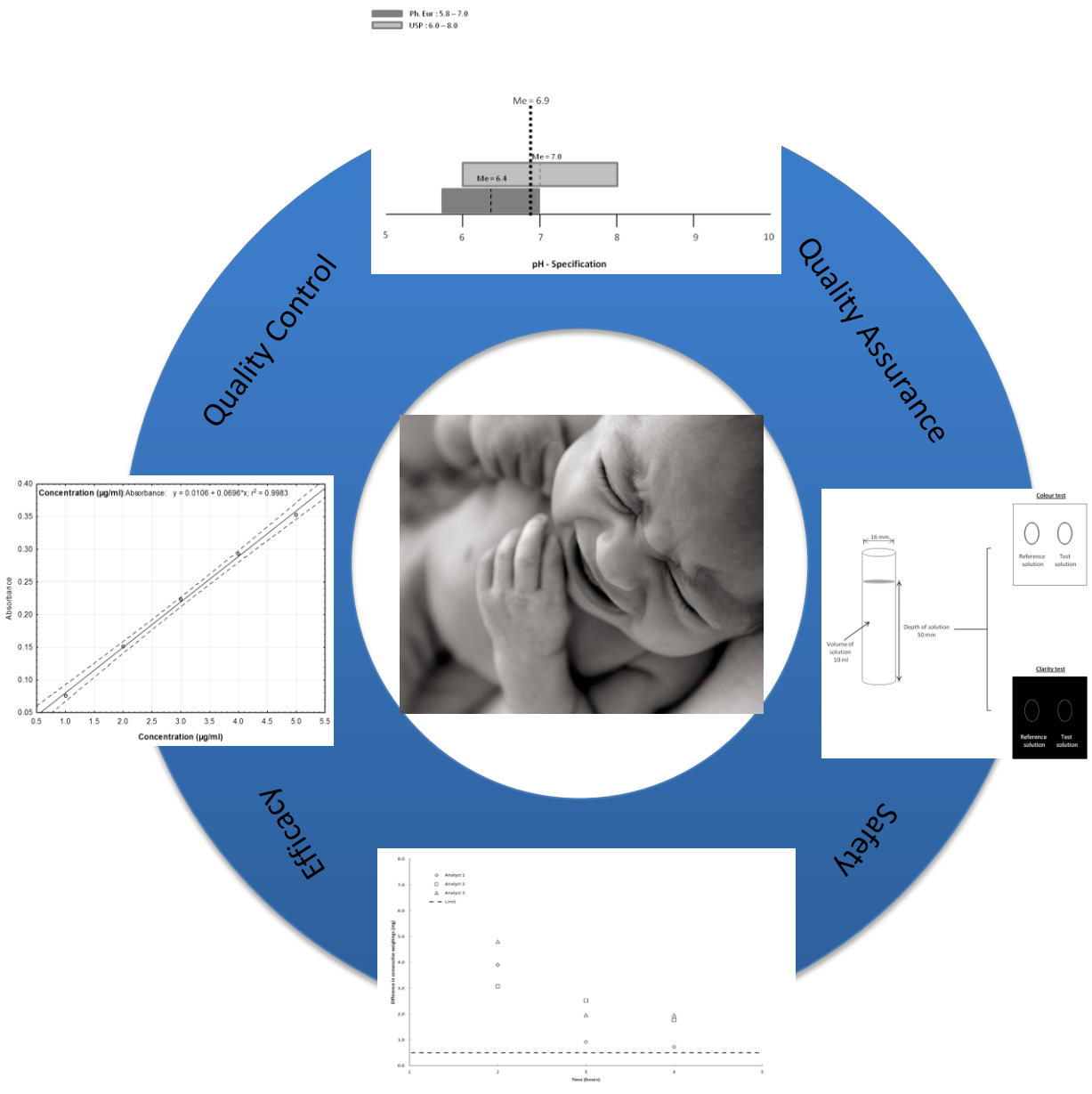
International pharmacopoeia monographs for zinc acetate and zinc gluconate active pharmaceutical ingredients used in the treatment of paediatric diarrhoea



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International pharmacopoeia monographs for zinc acetate and zinc gluconate active pharmaceutical ingredients used in the treatment of paediatric diarrhoea

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ABBREVIATIONS

%	Percentage
°C	Grades Celsius
µl	Microlitre
AAS	Atomic absorption spectrometry
Ag	Silver
Al	Aluminium
ANOVA	Analysis of variance
APIs	Active Pharmaceutical Ingredients
As	Arsenic
AsR	Arsenic reagent
AsTS	Arsenic test solution
Bi	Bismuth
BP	British Pharmacopoeia
Cas No.	Chemical abstracts service number
Cd	Cadmium
CENQAM	Centre for Quality Assurance of Medicines
Chem	Chemical reactions
C-Ion	Counter Ion
Cl	Chloride
ClTS	Chloride test solution
cm	Centimetres
Co.	Company

Cu	Copper
DA-EC	Diffuse adherent *E. coli*
DL	Detection limit
DLC	Detection limit criterion
EDTA	Ethylenediaminetetra –acetic acid
EH-EC	Enterohaemorrhagic *E. coli*
EI-EC	Enteroinvasive *E. coli*
EMA	European Medicines Agency
EML	Essential Medicines List
etc.	Etcetera
ET-EC	Enterotoxigenic *E. coli*
F_{crit}	Critical F value
Fe	Iron
FPP	Final Pharmaceutical Product
g	Grams
GLP	Good Laboratory Practises
GmbH & Co.KG	German: Limited partnership with a limited liability company as general partner
GMP	Good Manufacturing Practises
Hg	Mercury
HPLC	High Performance Liquid Chromatography
i.e.	That is
ICRS	International chemical reference substances
Inc.	Incorporated

INN	International Nonpropriety Name
Interm.	Intermediate
IPIs	Inactive Pharmaceutical ingredients
IR	Infra red
IUPAC	International Union of Pure and Applied Chemistry
IV	Intravenous
kg	Kilograms
KGaA	German: Limited partnership on shares
l	Litre
LA-EC	Localised adherent *E. coli*
LT	Heat labile
Ltd	Limited
M	Molar
me	Median
mEq	Milliequivalents
mg	Milligrams
ml	Millilitres
mm	Millimetres
mmol	Millimoles
mOsm	Milliosmoles
N/A	Not applicable
NaOH	Sodium hydroxide
NMT	Not more than
NRC	National Research Council

OHG	German: General partnership
ORS	Oral rehydration salts
Pb	Lead
PbS	Lead sulfide
PbTS	Lead test solution
Ph. Eur.	European Pharmacopoeia
Ph. Int.	*International Pharmacopoeia*
ppm	Parts per million
PQ	Prequalification
Pty	Propriety limited company
QC	Quality Control
R	Reagent
r^2	Correlation coefficient
RIIP	Research Institute for Industrial Pharmacy
RSD	Relative standard deviation
S	Sulfide
Sb	Antimony
SD	Standard deviation
Sn	Tin
SOR	Specific optical rotation
ST	Heat stable
TLC	Thin Layer Chromatography
TS	Test solution
UNICEF	The United Nations Children’s Fund

USA	United States of America
USP	United States Pharmacopoeia
UV	Ultra violet
V	Volume
VS	Volumetric solution
WHO	World Health Orangization

ABSTRACT

Acute diarrhoea is one of the largest health challenges globally, causing millions of child deaths every year.

A continued effort is made by the WHO, in collaboration with other institutions, to successfully combat diarrhoea. A new formulation for ORS (with a reduced osmolarity), in combination with zinc supplementation, was proposed to reduce the severity and duration of diarrhoea (WHO, 2006:1).

Appropriate zinc supplementation for the treatment of diarrhoea includes: zinc sulfate, zinc acetate dihydrate and zinc gluconate. With no monographs available in *The Ph. Int.* for zinc acetate dihydrate and zinc gluconate APIs, the development thereof has become a priority to the WHO.

During this study, suitable methods according to *The Ph. Int.* for the quality control testing of zinc acetate dihydrate and zinc gluconate APIs were investigated and proposed.

The following monograph requirements were proposed for zinc acetate dihydrate API:

- Identification of zinc by means of a precipitation reaction of zinc hydroxide and zinc sulfide,
- Identification of acetate by means of a precipitation reaction of ferric acetate,
- Clarity and colour of a 0.05 g/ml solution,
- pH value of a 0.05 g/ml solution,
- Assay by means of a complexometric titration with disodium EDTA,
- Impurities / Limit tests:
    - reducing substances by means of a reduction reaction with potassium permanganate,
    - chlorides by means of a precipitation reaction with silver nitrate,
    - sulfates by means of a precipitation reaction of barium sulfate,
    - arsenic by means of reaction between arsine and bromide,

- aluminium, cadmium, copper, iron and lead by means of atomic absorption spectrometry.

The following monograph requirements were proposed for zinc gluconate API:

- Identification of zinc by means of a precipitation reaction of zinc ferrocyanide,
- Identification of gluconate by means of a thin layer chromatographic separation method,
- Clarity and colour of a 0.01 g/ml solution,
- pH value of a 0.01 g/ml solution,
- Water by means of the Karl Fischer method,
- Assay by means of a complexometric titration with disodium EDTA,
- Impurities / Limit tests:
  - reducing sugars by means of a reduction reaction with cupri-tartaric test solution,
  - chlorides by means of a precipitation reaction with silver nitrate,
  - sulfates by means of a precipitation reaction of barium sulfate,
  - heavy metals by means of a precipitation reaction of sulfides in acidic solutions,
  - cadmium by means of atomic absorption spectrometry, and
  - microbial testing if required by *The Ph. Int.*

The proposed methods were then validated or verified according to international standards. Once the methods were proven to be fit for purpose, they were assembled into the respective monographs for inclusion in *The Ph. Int.*

The newly developed monographs were then evaluated by determining the compliance of commercially available zinc acetate dihydrate and zinc gluconate to the proposed specifications.

The study contributes to the WHO, pharmaceutical industry and medicines regulatory authorities by making these two monographs globally available, thus providing a quality gauge to ensure the availability of zinc acetate dihydrate and zinc gluconate APIs of pharmaceutical acceptable quality.

UITTREKSEL

Wêreldwyd is akute diarree, wat jaarliks die dood van miljoene kinders veroorsaak, een van die grootste gesondheidsuitdagings.

Daar word deur die Wêreldgesondheidsorganisasie (WGO), in samewerking met ander instansies, gepoog om diarree suksesvol te bekamp. ‘n Verbeterde formulering vir orale rehidrasie soute (met verlaagde osmolariteit), in kombinasie met sinkaanvulings, is reeds voorgestel om die graad en duur van diarree te beperk.

Toepaslike sinkaanvulling vir die behandeling van diarree sluit in: sinksulfaat, sinkasetaatdihidraat en sinkglukonaat. Die ontwikkeling van monografieë vir sinkasetaatdihidraat en sinkglukonaat aktiewe farmaseutiese bestanddele (AFB), het vir die WGO ’n prioriteit geword. Geen monografieë hiervoor is tans opgeneem in Die Internasionale Farmakopie nie.

Gedurende hierdie studie is geskikte metodes, volgens die vereistes van Die Internasionale Farmakopie, vir die kwaliteitsbeheer van sinkasetaatdihidraat en sinkglukonaat AFB ondersoek en voorgestel.

Die volgende monograafvereistes is voorgestel vir sinkasetaatdihidraat AFB:

- Identifikasie van sink deur middel van presipitasie van sinkhidroksied en sinksulfied,
- Identifikasie van asetaat deur middel van presipitasie van ysterasetaat,
- Helderheid en kleur van ’n 0.05 g/ml oplossing,
- pH waarde van ’n 0.05 g/ml oplossing,
- Inhoud bepaling deur middel van ‘n kompleksometriese titrasie met EDTA,
- Onsuiwerhede / Limiettoetse:
    - reduserende middels deur middel van ’n reduksiereaksie met kaliumpermanganaat,
    - chloriede deur middel van presipitaatvorming (reaksie met silwernitraat),
    - sulfate deur middel van presipitasie van bariumsulfaat,
    - arseen deur middel van ’n reaksie tussen arsien en bromied,

- aluminium, kadmium, koper, yster en lood deur middel van atoomabsorpsiespektrometrie.

Die volgende monograafvereistes is voorgestel vir sinkglukonaat AFB:

- Identifikasie van sink deur middel van presipitasie van sinkferrosianied,
- Identifikasie van glukonaat deur middel van ’n dunlaag chromatografiese metode,
- Helderheid en kleur van ’n 0.01 g/ml oplossing,
- pH waarde van ’n 0.01 g/ml oplossing,
- Water met die Karl Fischer metode
- Inhoud bepaling deur middel van ‘n kompleksometriese titrasie met EDTA,
- Onsuiwerhede / Limiettoetse:
    - reduserende suikers deur middel van ’n reduksiereaksie met kopertartraat toetsoplossing,
    - chloriede deur middel van presipitaatvorming (reaksie met silwernitraat),
    - sulfate deur middel van presipitasie van bariumsulfaat,
    - swaarmetale deur middel van die presipitasie van sulfiede in suuroplossings, en
    - kadmium deur middel van atoomabsorpsiespektrometrie.

Die voorgestelde metodes is daarna gevalideer of geverifieer volgens internasionale riglyne. Na bewys van die metodes se geskiktheid vir gebruik, is dit saamgestel in die onderskeie monografieë om ingesluit te word in Die Internasionale Farmakopie.

Die nuut ontwikkelde monografieë is daarna geëvalueer deur die toetsing van kommersieel beskikbare sinkasetaatdihidraat en sinkglukonaat om vas te stel of dit aan die voorgestelde spesifikasies voldoen.

Die studie lewer ’n bydra tot die farmaseutiese industrie en medisynebeheerrade deur die twee monografieë wêreldwyd beskikbaar te stel, en daardeur ’n kwaliteitstandaard te stel om die beskikbaarheid van sinkasetaatdihidraat en sinkglukonaat van farmaseutiese aanvaarbare gehalte te verseker.

AIMS AND OBJECTIVES

Nearly one in every five child deaths (approximately 1.5 million a year) is due to diarrhoea, which kills more children than AIDS, malaria and measles combined (Wardlow *et al.*, 2010:870).

The United Nations (UN) Millennium Development Goal 4 is to reduce the mortality rate of children under the age of five years by two thirds, between 1990 and 2015 (United Nations, 2010). For this target to be achieved, the management and treatment of diarrhoea need to be critically considered.

The WHO acknowledges the contribution that diarrhoea makes to the mortality rates and initiated a programme where a new formulation for oral rehydration salts (ORS), with a reduced osmolarity, and added zinc (by means of zinc sulfate, zinc acetate dihydrate or zinc gluconate) have been proposed to reduce the severity and duration of diarrhoea (WHO, 2006:1).

No monographs are currently available in *The Ph. Int.* for either zinc acetate dihydrate active pharmaceutical ingredient (API), or zinc gluconate API, to ensure the quality and safety thereof. The development of these monographs has therefore become a priority to the WHO.

The following study objectives were therefore set and pursued:

- Conduct a literature review of the pathogenesis, complications and treatment of diarrhoea (Chapter 1);
- Conduct a literature review of the pharmaceutical and pharmacological properties of zinc acetate dihydrate and zinc gluconate (Chapter 2);
- Investigate the process of monograph development, and the validation thereof to ensure its fitness for purpose (Chapter 3);
- Develop or propose suitable methods for the quality control testing of zinc acetate dihydrate API for possible inclusion in a monograph, according to the requirements of *The Ph. Int.* (Chapters 4 - 11);
- Validate the applicable methods in the zinc acetate dihydrate API monograph according to international standards (Chapters 4 – 11);

- Develop or propose suitable methods for the quality control testing of zinc gluconate API for possible inclusion in a monograph, according to the requirements of *The Ph. Int.* (Chapters 4 - 11);
- Validate the applicable methods in the zinc gluconate API monograph according to international standards (Chapters 4 – 11);
- Evaluate the compliance of commercially available zinc acetate dihydrate and zinc gluconate with the newly developed monographs.

The study will contribute to the WHO, pharmaceutical industry and medicines regulatory authorities with regards to the following:

- Assist the WHO in assuring that suitable methods and specifications are available for the quality control of zinc acetate dihydrate API and zinc gluconate API which are to be published in *The Ph. Int.*
- The publication of zinc acetate dihydrate API and zinc gluconate API monographs in *The Ph. Int.* will ensure that a quality gauge is available free of charge for use by manufacturers and quality control laboratories.
- The monographs developed would be used by the pharmaceutical industry to determine and ensure the quality of zinc acetate dihydrate API and zinc gluconate API, prior to the release thereof on the market.

The availability of safe and effective zinc salts will contribute to a reduction in the severity and duration of diarrhoea in children which may ultimately contribute to a reduction in the mortality rate of children.

"*There is no tragedy in life like the death of a child. Things never get back to the way they were.*" – Dwight D. Eisenhower

(Source: http://brainyquote.com)

CHAPTER 1

DIARRHOEA: PATHOGENESIS, COMPLICATIONS AND TREATMENT

1.1 Introduction

Worldwide diarrhoea is, second only to pneumonia, the leading cause of death in children younger than 5 years (Wardlow *et al.*, 2010:870). An estimated 1.87 million children below the age of 5 years died from diarrhoea in 2003 (WHO, 2005:3) and about 1.3 million children in 2008 (Global Health Council, 2010). Diarrhoea can be described as the passing of three or more unusually loose, watery stools in a 24 hour period (NDDIC, 2011). It is most common in children between 6 months and 2 years of age (WHO, 2005:4; WHO *et al.*, 2005:1). Acute diarrhoea is caused by infection of the bowel, has a rapid onset and may continue for several days. Persistent diarrhoea starts in a similar manner but lasts for 14 days or more. Diarrhoea has a high mortality rate due to complications such as dehydration and malnutrition (WHO *et al.*, 2005:1). Four clinical presentations of diarrhoea can be recognised (Table 1.1) where the basic underlying pathology and altered physiology are reflected (WHO, 2005:4).

Table 1.1 Clinical presentations of diarrhoea (WHO, 2005:4)

Type	Description and associated risks
Acute watery diarrhoea (including cholera)	Lasts several hours or days with the main danger being dehydration. If feeding is not continued, weight loss may also occur.
Acute bloody diarrhoea (also known as dysentery)	The main dangers include damage of the intestinal mucosa, sepsis and malnutrition. Dehydration may also occur.
Persistent diarrhoea	Lasts 14 days or longer. Dehydration may occur, but the main danger is malnutrition and serious non-intestinal infection.
Diarrhoea with severe malnutrition (marasmus or kwashiorkor)	Main dangers are severe systemic infection, dehydration, heart failure and vitamin and mineral deficiency.

1.2 Pathogenesis

The majority of infections are due to viruses, bacteria and protozoa, which are most commonly transmitted by the faecal-oral route through water, food and person-to-

person transmission (Kelly, 2011:201). During the past three decades numerous new microbial causes of diarrhoea have been identified. The most important acute diarrhoea-causing pathogens, the frequency of occurrence (incidence) as well as the pathogenesis are summarised in Table 1.2. In infants and young children a Rotavirus infection is the most common cause of acute diarrhoea and *Shigella* is the most common cause of bloody diarrhoea (Kelly, 2011:201; WHO, 2005:3,17).

Table 1.2 Acute diarrhoea-causing pathogens, incidence and pathogenesis in infants and young children (WHO, 2005:29)

Pathogen	Incidence	Pathogenesis
Viruses		
Rotavirus	Rotavirus is responsible for 15 - 25 % of diarrhoea episodes in children aged 6 - 24 months visiting treatment facilities, but for only 5 - 10 % of cases in the same age group in the community. Prevalence is worldwide and spread is by faecal/oral transmission or possibly by airborne droplets. Peak incidence of diseases is cold or dry seasons.	Rotavirus causes patchy damage to the epithelium of the small intestine, resulting in the blunting of the villi. There is some reduction in the activity of lactase and other dissacharidases, resulting in reduced absorption of carbohydrates, but this is usually of no clinical significance. The intestinal morphology and absorptive capacity return to normal within 2 - 3 weeks.
Bacteria		
Escherichia coli	*E. coli* causes up to one quarter of all cases of diarrhoea in developing countries. Transmission usually occurs through contaminated food (especially weaning foods) and water.	
a) Enterotoxigenic *E. coli* (ET-EC)	ET-EC is the major cause of acute watery diarrhoea in children and adults in developing countries, especially during the warm, wet season.	Two important virulent factors of ET-EC are: (1) colonisation factors that allow ET-EC to adhere to enterocytes of the small bowel, and (2) enterotoxins. ET-EC produces heat labile (LT) and / or heat stable (ST) enterotoxins that cause secretion of fluid and electrolytes, resulting in watery diarrhoea. ET-EC does not destroy the brush border or invade the mucosa.
b) Localised adherent *E. coli* (LA-EC)	In some urban areas, up to 30 % of acute diarrhoea cases in young infants are attributed to LA-EC. Many infections are acquired in hospital nurseries.	LA-EC is detected by patchy adherence to the HeLA cells or by specific gene probes. Entero-adherence and production of a potent cytotoxin are important mechanisms for causing diarrhoea.
c) Diffuse adherent *E. coli* (DA-EC)	DA-EC is widespread and appears to cause a small percentage of episodes of acute diarrhoea in young children.	DA-EC is detected by typical diffuse adherence to HeLa cells.

Table 1.2 Acute diarrhoea-causing pathogens, incidence and pathogenesis in infants and young children (WHO, 2005:29) (Continued)

Pathogen	Incidence	Pathogenesis
d) Enteroinvasive *E. coli* (EI-EC)	EI-EC is uncommon in developing countries. It causes sporadic food-borne outbreaks that affect children and adults. Symptoms of illness are similar to those of shigellosis.	EI-EC is similar to Shigella, both biochemically and serologically. Like Shigella, EI-EC penetrates and multiplies within the colonic epithelial cells.
e) Enterohaemorrhagic *E. coli (*EH-EC)	EH-EC is found in Europe and in parts of North and South America, where outbreaks may be caused by undercooked meat. Recent outbreaks in Southern Africa were traced to river water contaminated by cattle carcasses.	EH-EC produces a shigalike toxin that may be responsible for oedema and diffuse bleeding in the colon, as well as the haemolytic-uraemic syndrome that sometimes develops in children.
Shigella	Shigella causes 10 - 15 % of acute diarrhoea cases in children under 5 years and is the most common cause of bloody diarrhoea in children. Since the infectious dose is low (10 to 100 organisms), spread occurs by person-to-person contact. Food-borne and water-borne transmissions also occur. Peak incidence is in warmer seasons.	Shigella invades and multiplies within colonic epithelial cells, causing cell death and mucosal ulcers. Shigella occasionally invades the bloodstream. The virulence factors include: a smooth lipopolysaccharide cell wall antigen, antigens that promote cell invasion, and shiga toxin which is cytotoxic, neurotoxic and perhaps also causes watery diarrhoea.
Campylobacter jejuni	*C. jejuni* causes 5 - 15 % of diarrhoea cases in infants worldwide, but because it is also found in many without diarrhoea, the true proportion of cases due to *C. jejuni* is not known. In developing countries most children acquire immunity during the first year of life; the pathogen is frequently found in stools of healthy older children. Spread is by chickens and other animals.	*C. jejuni* probably induces diarrhoea by invasion of the ileum and the large intestine. Two types of toxins are produced: a cytotoxin and a heat-labile enterotoxin.
Vibrio cholerae O1 and O139	Cholera is endemic in many countries of Africa, Asia and Latin America, where epidemics often occur annually, usually during the hot, wet season. In such areas cholera occurs most often in children 2 - 9 years of age, and many cases are severe. In newly-affected areas, adults are also affected. Both contaminated water and food can transmit cholera; person-to-person spread is much less common.	*V. cholerae* adheres to and multiplies on the mucosa of the small intestine where it produces an enterotoxin which causes the diarrhoea. Cholera toxin is closely related to the heat-labile toxin (LT) of ET-EC.
Salmonella (non-typhoid)	*Salmonella* causes 1 - 5 % of gastroenteritis cases in most developing countries. Infection usually results from ingestion of contaminated animal products.	*Salmonella* invades the ileal epithelium. An enterotoxin causes watery diarrhoea. When mucosal damage occurs, diarrhoea may be bloody. Bacteraemia may occur and can lead to localised infection in other tissues, such as bone and meninges.

Table 1.2 Acute diarrhoea-causing pathogens, incidence and pathogenesis in infants and young children (WHO, 2005:29) (Continued)

Pathogen	Incidence	Pathogenesis
Protozoa		
Giardia duodenalis	*G. duodenalis* has a worldwide distribution, the prevalence of infection in young children approaching 100 % in some areas. Children aged 1 - 5 years are most commonly infected.	*G. duodenalis* infects the small bowel; the pathogenic mechanism is unclear. Flattening of the intestinal epithelium is seen in severe cases. Giardia infections are foodborne, waterborne, or spread by faecal-oral route; the latter occurs particularly in children living in crowded circumstances or attending day-care centres.
Entamoeba histolytica	Prevalence rates of *E. histolytica* infection vary widely but its distribution is worldwide. The incidence of disease increases with age, being highest in adult males.	*E. histolytica* invades the mucosa of the large intestine, where it is thought to elaborate neurohumoral substances that cause intestinal secretion and damage, resulting in an inflammatory type of diarrhoea.
Cryptosporidium	In developing countries, cryptosporidia may account for 5 - 15 % of childhood diarrhoea cases. *Cryptosporidia* are transmitted by the faecal-oral route.	Cryptosporidia attach to the microvillous surface of enterocytes and produce mucosal damage, which causes malabsorption and fluid secretion.

1.3 Complications and associated symptoms of diarrhoea

As previously stated, most diarrhoeal deaths are caused by dehydration and malnutrition. The four main complications and associated symptoms of diarrhoea are: dehydration, malnutrition, fever and convulsions.

1.3.1 Dehydration

According to the World Health Organization (WHO) dehydration occurs during diarrhoea when the loss of water and electrolytes through liquid stool, vomit, sweat and urine is not replaced adequately, leading to a deficit of water and electrolytes (WHO, 2005:4). The volume of fluid lost through the stools in one day can vary from 5 millilitres (ml) / kilogram (kg) (near normal) to 200 ml/kg, or more (WHO, 2005:4). The loss of electrolytes also varies, with sodium, potassium and chloride losses similar in range. In young children with severe dehydration the total body electrolyte deficit is usually about 70 to 110 millimoles (mmoles) / litre (l) of water deficit. The degree of dehydration is categorised according to symptoms and signs that reflect

the amount of fluid lost (Table 1.3) (WHO, 2005:4). Figure 1.1 and 1.2 depict the symptoms of a dehydrated infant and skin with decreased turgor respectively.

Table 1.3 Degree of dehydration according to signs and symptoms (WHO, 2005:4)

Degree of dehydration	Signs / Symptoms
Early stages	No signs or symptoms.
Increased dehydration	Thirst, restless or irritable behaviour, decreased skin turgor, sunken eyes, and sunken fontanel (in infants).
Severe dehydration	Evidence of hypovolaemic shock: diminished consciousness, lack of urine output, cool moist extremities, a rapid and feeble pulse, low / undetectable blood pressure and peripheral cyanosis.

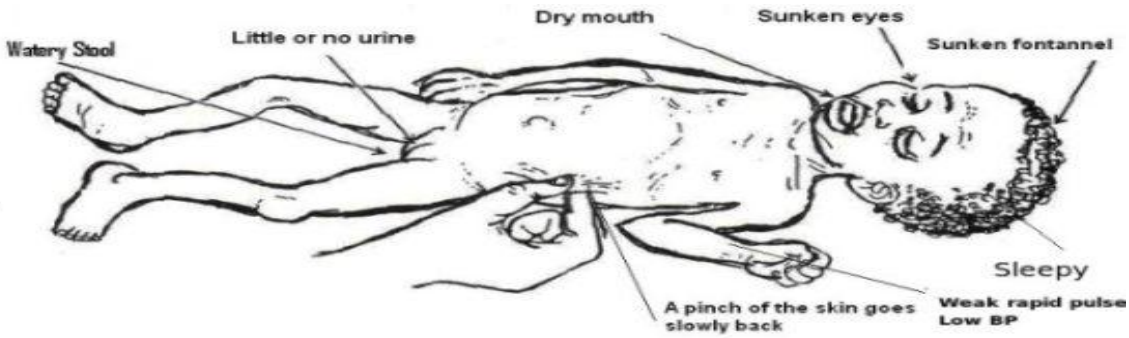


Figure 1.1 Symptoms of a dehydrated infant (Source: http://www.ashwini.org /asknk/index.php? option=com_content&view=article&id=83&Itemid=96).

Figure 1.2 Illustration of decreased skin turgor associated with dehydration (Source: http://www.nlm.nih.gov/medlineplus/ency/imagepages/17223.htm).

1.3.2 Malnutrition

In reality diarrhoea is as much a nutritional disease as a fluid and electrolyte loss disease. The morbidity impact of enteric pathogens is related to their ability to directly impair intestinal absorption, as well as their ability to cause diarrhoea, both of which impair nutritional status (Petri *et al*., 2008:1277). Children that die from diarrhoea are usually severely malnourished. The decrease of food intake and nutrient absorption, and increased nutrient requirement during diarrhoea join to cause failure to grow and weight loss (WHO, 2005:5). Any pre-existing malnutrition is worsened. In turn, the malnutrition contributes to more severe, prolonged and possibly more frequent diarrhoea, creating a vicious cycle (Petri *et al*., 2008:1277; WHO, 2005:5). Petri *et al*. (2008:1277) stresses the importance of a healthy intestinal tract in the first few formative years, because the predominant brain and synapse development in humans occurs in the first 2 years after birth. Thus to assure the optimal growth and development of the body, brain and neuronal synapses, absorption of key nutrients during the first few years is critical. This is demonstrated by the growth shortfalls that have been attributed to early childhood diarrhoea, as well as long-lasting and profound effects observed on fitness, cognition and schooling (Petri *et al*., 2008:1277).

1.3.3 Fever

Young children with diarrhoea may have fever due to severe dehydration, but the fever might also be caused by the infection, e.g. pneumonia, bacteraemia, urinary tract infection or otitis media (WHO, 2005:24).

1.3.4 Convulsions

Children with diarrhoea and a history of convulsions during the illness may be suffering from febrile convulsion, hypoglycaemia, hypernatraemia or hyponatraemia (WHO, 2005:25). Febrile convulsion usually occurs in infants when their body temperature exceeds 40 centigrade / Celsius (°C) or rises rapidly. Hypoglycaemia occasionally occurs in children with diarrhoea due to inadequate gluconeogenesis. Hypernatraemia occurs in children with diarrhoea, especially when given drinks that are hypertonic owing to its excessive content of sugar, whereas hyponatraemia

occurs when the diarrhoea sufferer only drinks water with little or no salt (WHO, 2005:16).

1.4 Treatment of diarrhoea

Although the mortality rate of diarrhoea has decreased drastically since 1978 from 4.5 million to 1.3 million annual deaths of children under the age of 5, acute diarrhoea remains a leading cause of childhood deaths (WHO *et al.*, 2006:1) (Figure 1.3). This has lead to revised recommendations from the WHO and The United Nations Children's Fund (UNICEF) for the treatment of diarrhoea in order to reduce the number of diarrhoea-related deaths. The revision is based on data published which illustrated a significant reduction in the severity and duration of diarrhoea when:

i. the osmolarity of the oral rehydration salts (ORS) is reduced, and
ii. zinc supplementation is provided to children with diarrhoea (WHO *et al.*, 2006:1).

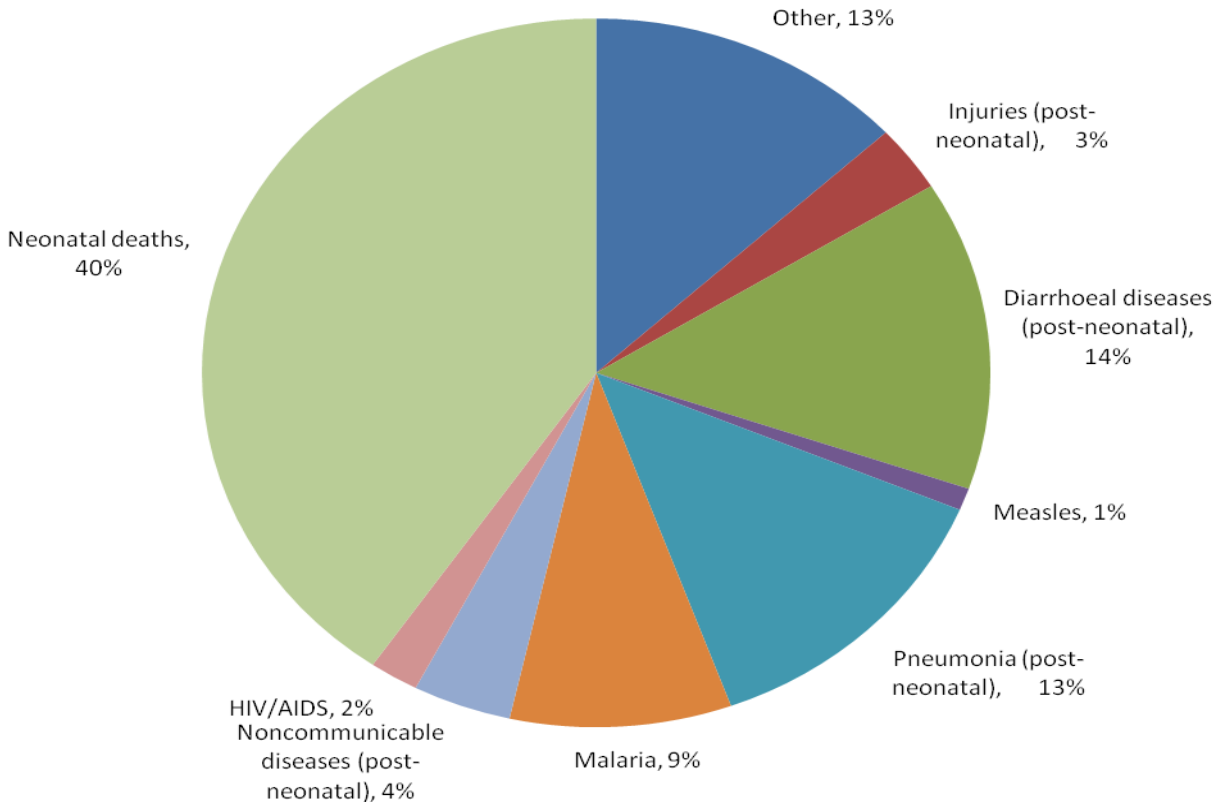


Figure 1.3 Main causes of death among children younger than five years of age globally (prepared based on information published in: WHO, 2011f).

1.4.1 Improved oral rehydration salts formulation

The original ORS formulation that has been recommended for the past 25 years contained 90 milliequivalents (mEq)/l of sodium with a 311 milliosmoles (mOsm)/l total osmolarity (WHO *et al.*, 2006:3). Although this formulation was safe and effective for the prevention and treatment of dehydration, it did not seem to reduce stool output nor seemed to have any other clinical benefits. For this reason numerous studies have been dedicated to the reformulation of ORS.

One approach was to avoid possible adverse effects of hypertonicity on net fluid absorption by reducing the osmolarity of the ORS solution. This was achieved by reducing the glucose and salt concentration of the solution (WHO *et al.*, 2006:3). According to the WHO *et al.* (2006:4), studies showed a 33 % reduction in the need for additional intravenous (IV) rehydration therapy in children given a reduced osmolarity solution: sodium concentration 75 mEq/l; glucose concentration 75 mmol/l; total osmolarity 245 mOsm/l. A combination of studies showed that lowered osmolarity solutions (between 210 and 268 mOsm/l, with sodium concentrations of 50 - 75 mEq/l) reduced stool output by about 20 % and the vomiting incidence by about 30 %. The use of 245 mOsm/l ORS solutions appeared to be as safe and at least as effective as standard ORS for use in children with cholera, and is as safe and as effective as the standard ORS solutions in treatment of adults with cholera (WHO *et al.*, 2006:4). The WHO and UNICEF recommended the improved ORS formulation summarised in Table 1.4.

Table 1.4 WHO and UNICEF recommended ORS formulation (WHO *et al.*, 2006:4)

Reduced osmolarity ORS	**Grams (g)/l**	**Reduced osmolarity ORS**	**mmol/l**
Sodium chloride	2.6	Sodium	75
Glucose, anhydrous	13.5	Chloride	65
Potassium chloride	1.5	Glucose, anhydrous	75
Trisodium citrate, dihydrate	2.9	Potassium	20
		Citrate	10
		Total osmolarity	**245**

The specifications for the improved solution after preparation are summarised in Table 1.5 (WHO *et al.*, 2006:4).

Table 1.5 WHO and UNICEF specifications for the new low osmolarity ORS solutions (WHO *et al.*, 2006:4)

Substance	Criteria
Glucose	At least equal to sodium and NMT 111 mmol/l
Sodium	60 – 90 mEq/l
Potassium	15 – 25 mEq/l
Citrate	8 – 12 mmol/l
Chloride	50 – 80 mEq/l
Total substance concentration	**200 – 310 mmol/l**

NMT = not more than

1.4.2 Zinc supplementation

The WHO *et al.* (2006:25) reported that zinc supplementation reduces the duration and severity of persistent and acute diarrhoea. These conclusions are based on review articles which indicated that zinc supplements had a positive effect on the treatment of acute diarrhoea (The Zinc Investigators & Collaborative Group, 1999:695 and 2000:1521). A summary of these studies and their results is presented in Table 1.6.

Table 1.6 Summary of the beneficial effects of zinc supplementation as treatment for acute diarrhoea (WHO *et al.*, 2006:24)

Beneficial effect	Number of studies with beneficial effects	Number of studies with statistical significance	% Reduction
Reduction of duration of the episode	11	8	25 %
Reduced proportion of the episode lasting more than 7 days	5	1	25 %
Reduction in stool volume	8	5	30 %

Further studies also showed that children with persistent diarrhoea, receiving zinc supplements, had a 24 % lower probability of continuing diarrhoea and a 42 % lower rate of treatment failure when compared to control groups (WHO *et al.*, 2006:24). In continuous trials, children receiving zinc supplements had 18 % lower diarrhoeal incidences and 25 % reduced prevalence than the control group. Short-course trials showed similar results to continuous trials with 11 % reduction in diarrhoeal incidence and 34 % reduction of prevalence. In the prevention of bloody diarrhoea, a number of studies have shown a positive impact on the prevalence of dysentery in

the month following zinc supplementations, whether given in a short course or continuous (WHO *et al.*, 2006:25).

1.4.3 Recommended treatment plans and other complimentary treatment

In order to develop and implement an appropriate treatment plan the patient (usually children under the age of 5 years) should be assessed for dehydration, bloody diarrhoea, persistent diarrhoea, malnutrition and serious non-intestinal infections (WHO, 2005:6). The degree of dehydration will determine the treatment plan (Figure 1.4). Treatment Plan A can be followed at home to prevent dehydration and malnutrition if no signs of dehydration are visible. Treatment Plan B is followed when some dehydration is visible (Table 1.7) and Treatment Plan C (Table 1.8) is to treat severe dehydration urgently (WHO, 2005:1).

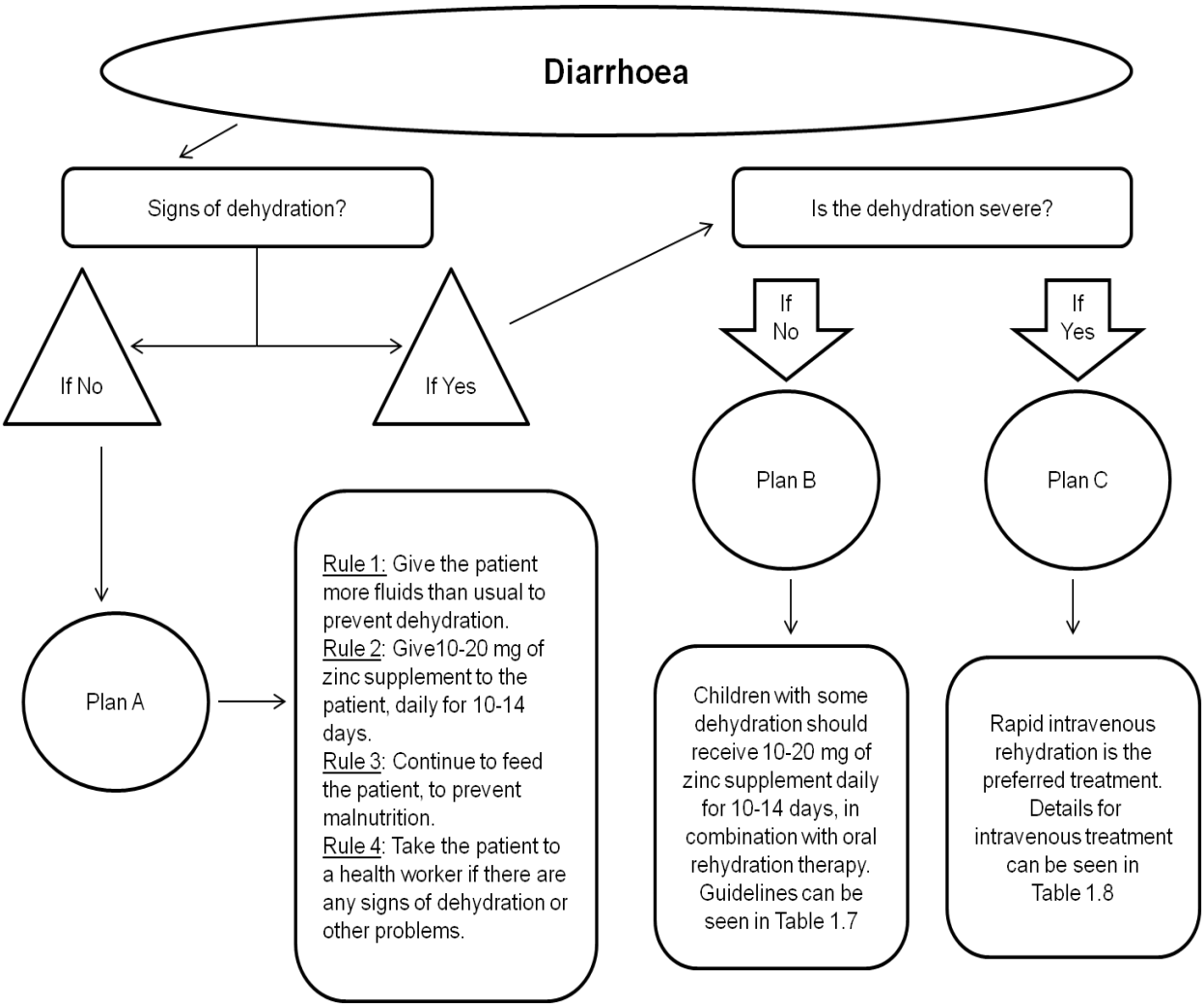


Figure 1.4 Treatment plan for diarrhoea (schematic presentation prepared based on information published in: WHO, 2005:7).

Table 1.7 Guidelines for treating children and adults with some dehydration (WHO, 2005:12)

Approximate amount of ORS solution to give in the first four hours						
Age[a]	**Less than 4 months**	**4 - 11 months**	**12 - 23 months**	**2 - 4 years**	**5 - 14 years**	**15 years and older**
Weight (in kg)	Less than 5	5 - 7.9	8 - 10.9	11 - 15.9	16 - 29.9	30 or more
Amount ORS (in ml)	200 - 400	400 - 600	600 - 800	800 - 1200	1200 - 2200	2200 - 4000

[a]Use the patient's age only when you do not know the weight. The approximate amount of ORS required (in ml) can also be calculated by multiplying the patient's weight in kg by 75.

- If the patient wants more ORS than shown, give more.
- Encourage the mother to continue breastfeeding her child.
- For infants under 6 months who are not breastfed, if using the old WHO ORS solution containing 90 mmol/l of sodium, also give 100 - 200 ml clean water during this period. However, if using the new reduced (low) osmolarity ORS solution containing 75 mmol/l of sodium, it is not necessary.

NOTE: During the initial stages of therapy, while still dehydrated, adults can consume up to 750 ml per hour, if necessary, and children up to 20 ml per kg body weight per hour.

Table 1.8 Guidelines for intravenous treatment of children and adults with severe dehydration (WHO, 2005:14)

Start intravenous fluids immediately. If the patient can drink, give ORS by mouth until the drip is set up. Give 100 ml/kg Ringer's Lactate Solution[a] divided as follows:

Age	**First give 30 ml/kg in:**	**Then give 70 ml/kg in:**
Infants under 12 months	1 hour[b]	5 hours
Older	30 minutes[b]	2.5 hours

- Reassess the patient every 1 – 2 hours. If hydration is not improving, give the intravenous drip more rapidly.
- After six hours (infants) or three hours (older patients), evaluate the patient and choose the appropriate Treatment Plan (A, B or C) to continue treatment.

[a] If Ringer's Lactate Solution is not available, normal saline may by used.
[b] Repeat once if radial pulse is still very weak or not detectable.

Other complementary treatment and anti-microbials should not be given routinely to children with diarrhoea, except in cases of dysentery diarrhoea, suspected cases of cholera with severe dehydration and laboratory proven, symptomatic infection with *Giardia duodenalis* (WHO, 2005:25). Dysentery may be treated with ciprofloxacin for three days, or for five days with another oral antimicrobial to which most *Shigella* in the area are sensitive (WHO, 2005:17). Agents that are thought of as "anti-diarrhoeals" are dangerous and have no practical benefit. Anti-diarrhoeal products include adsorbents (that is {i.e}. kaolin, attapulgite, smectite, activated charcoal, cholestyramine), anti-motility (i.e. loperamide hydrochloride, diphenoxylate with

atropine, tincture of opium, camphorated tincture of opium, paregoric, codeine), and bismuth subsalicylate. Anti-motility agents are not recommended for children and infants because of respiratory depression concerns (Kelly, 2011:204). The abovementioned active pharmaceutical ingredients (APIs) on their own or in combination should never be indicated for the treatment of acute diarrhoea (WHO, 2005:25). Anti-emetics, steroids and purgatives should also be avoided (WHO, 2005:26). There is however one “anti-diarrhoeal” that has been developed that can be safely used in children (Chacón, 2010:298; Salazar-Lindo *et al.*, 2000:466). It is an enkephalinase inhibitor, racecadotril, which has a pro-absorptive activity (Salazar-Lindo *et al.*, 2000:463). This is an effective agent for reducing stool weight and bowel frequency (Salazar-Lindo *et al.*, 2000:465).

1.5 Conclusion

Diarrhoea is a current and real time problem in our modern day society, especially in malnourished populations. The United Nations (UN) Millennium Development Goal 4 is to reduce by two thirds, between 1990 and 2015, the under five mortality rate (United Nations, 2010). For this goal to be reached, diarrhoea as major contributor to the mortality rate needs to be eradicated.

The majority of infections are caused by various viruses, bacteria and protozoa, with the main complications of diarrhoea being dehydration, malnutrition, fever and convulsions. The WHO, in collaboration with institutions like UNICEF, John Hopkins Bloomberg School of Public Health and USAID, has invested time and resources to combat diarrhoea by the recommendation of a new ORS in combination with zinc supplementations. Various articles have been published to show the positive effects of zinc supplementation on diarrhoea. These positive effects include: a reduction in the prevalence of dysentery; lower diarrhoeal incidences; lower probability of continuing diarrhoea; reduction in the duration of the diarrhoeal episode; and reduction in stool volume and vomiting.

1.6 Aim of this study

Based on the information presented in this chapter it is clear that zinc plays an integral part in the treatment of diarrhoea. Primary health care needs should be

satisfied by essential medicines (WHO *et al.*, 2007:11). For this reason zinc sulfate products were included in the WHO's Essential Medicines List (EML) for Adults (WHO, 2011c:25) and the list for Children (WHO, 2011d:22). Zinc sulfate is also listed in the Prequalification Programme (a United Nations Programme managed by WHO) that aims to make quality priority medicines available for the benefit of those in need (WHO, 2011b). Zinc is therefore considered to be the essential moiety with the desired therapeutic effect, where any of the water soluble zinc salts may be used in the management of diarrhoea. The EML recommends a daily dose of 10 milligrams (mg) to 20 mg elemental zinc for the treatment of diarrhoea (WHO *et al.*, 2007:11).

The zinc salts commonly used for zinc supplementation for the treatment of diarrhoea include: zinc sulfate, zinc acetate dihydrate and zinc gluconate (WHO *et al.*, 2006:32). To ensure that these salts are of pharmaceutical acceptable quality, reliable test methods and strict specifications need to be set. Monographs provide a quality gauge to evaluate the quality of medicines. With no monographs available in *The International Pharmacopoeia (Ph. Int*.) for zinc acetate dihydrate and zinc gluconate APIs, this study aimed to contribute to the combating of diarrhoea by developing these quality standards.

Chapter 2 provides an overview of the pharmaceutical and pharmacological properties of zinc acetate dihydrate and zinc gluconate, which were considered during the development of the monographs.

CHAPTER 2

ZINC SALTS: PHARMACEUTICAL AND PHARMACOLOGICAL BACKGROUND

2.1 Introduction

Zinc is a naturally occurring mineral and a vital micro-nutrient in humans (Cerner Multum Incorporated {Inc.}, 2010). Lately, zinc supplements have been used in the management of acute and persistent diarrhoea to reduce both the severity and duration thereof (WHO *et al*., 2006). The zinc salts of choice include zinc sulfate, zinc acetate dihydrate and zinc gluconate, which are all water-soluble. All three zinc salts are considered equally effective in diarrhoea management, although zinc sulfate was the most widely used essentially due to the cost effectiveness thereof (WHO *et al*., 2006; WHO *et al*., 2007). Due to less gastrointestinal side effects, zinc acetate dihydrate is the therapeutically preferred salt (Brewer, 2001:106; Trepanier, 2008; Wintergerst *et al.*, 2006:90) above zinc gluconate and zinc sulfate; where zinc gluconate is better tolerated than zinc sulfate (Meynadier, 2000:269).

The aim of this chapter was to focus on the pharmaceutical and pharmacological properties of the acetate and gluconate salts. Zinc sulfate was included in this chapter for the sake of completeness.

2.2 Description of zinc salts

2.2.1 Formulae and molecular weight

The molecular weight, together with the empirical formula, for each of the zinc salts can be seen in Table 2.1.

Table 2.1 Empirical formulae and molecular weights of zinc salts

Zinc salt	Empirical formula	Molecular weight
Zinc acetate	$C_4H_6O_4Zn \cdot 2H_2O$ (dihydrate) $C_4H_6O_4Zn$ (anhydrous)	219.49 g/mol 183.48 g/mol
Zinc gluconate	$C_{12}H_{22}O_{14}Zn$	455.69 g/mol
Zinc sulfate	$ZnSO_4 \cdot H_2O$ (monohydrate)	179.46 g/mol

The structural formulae of the zinc salts are represented in Figure 2.1.

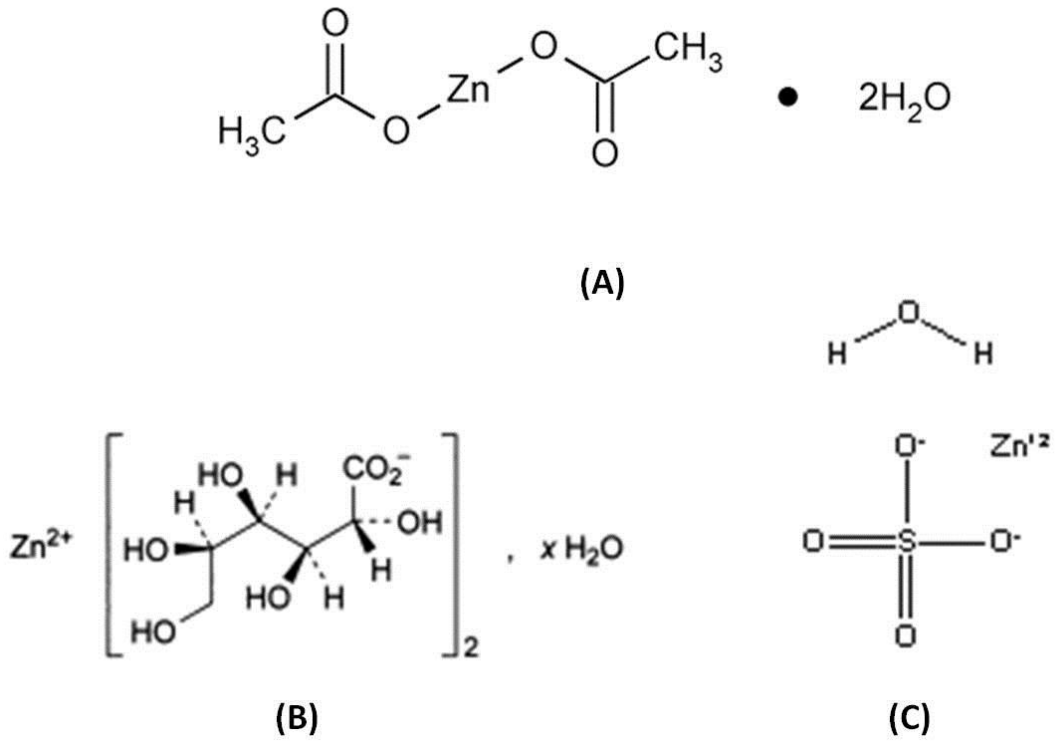


Figure 2.1 Structural formula of (A) zinc acetate dihydrate (USP, 2011); (B) zinc gluconate (BP, 2011); and (C) zinc sulfate monohydrate (Source: http://img.guidechem.com/casimg/7446-19-7.gif7).

2.2.2 Nomenclature

2.2.2.1 Chemical names

Zinc acetate dihydrate, zinc gluconate and zinc sulfate (Sweetman, 2002:1398).

2.2.2.2 Nonproprietary names

Zinc acetate dihydrate, zinc gluconate and zinc sulfate (Sweetman, 2002:1398).

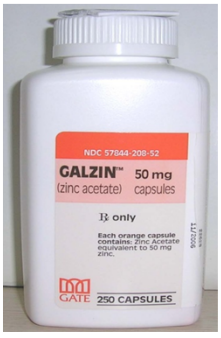
2.2.2.3 Proprietary names

Zinc acetate dihydrate products available: Zincris Syrup® (Cipla), Galzin® (Teva Pharmaceuticals), ProstAvan® (Melaleuca Inc.) and Wilzin® (Orphan Pharma) (Cerner Multum Inc., 2010).

Zinc gluconate oral solution (Sanjing Pharm) and Cold-EEZE® (ProPhase Labs) contain zinc gluconate (Cerner Multum Inc., 2010). The following zinc gluconate products are available in South Africa: Zinc Tablets® (Vital Health Foods) and Zinc Tablets® (Natrodale) (Rossiter & Blockman, 2010:96).

Zinc sulfate products available: ZinCfant ® (Nutriset), Eye-Sed® (Scherer Laboratories Inc.), Verazinc® (Forest Pharmaceuticals), Zinc-220® (ALTO® Pharmaceuticals), Zinca-Pak® (Solo Pak Medical Products Inc.), Mar-Zinc® (Marlop Pharmaceuticals Inc.), Rivasol® (Riva Laboratories), Orazinc®, Orazinc 110®, Orazinc 220® (Mericon Industries Inc.), Micro Zn® (Sandoz Inc.), Zinc CR® (Vitaline Corporation), and Zincate® (Paddock Laboratories Inc.) (Cerner Multum Inc., 2010).

Examples of final pharmaceutical products (FPP) which contain zinc acetate dihydrate, zinc gluconate and zinc sulfate are shown in Figure 2.2.



(A)

Source:http://www.kodc.or.kr/drugimage/galzin.jpg

(B)

Source:http://www.overstockdrugstore.com/product_images/p/091108100143.jpg

(C)

Source:http://www.nutriset.fr/assets/images/visuelsProduits/ZincfantPriveENG.jpg

Figure 2.2 Examples of FPP of (A) zinc acetate dihydrate, (B) zinc gluconate and (C) zinc sulfate.

2.2.3 Odour, colour and appearance

Zinc acetate dihydrate presents as white or colourless crystals (or flakes) which have a faint acetous odour and an astringent metallic taste (Hayes & Martin, 1994:8).

Zinc gluconate is a white to off-white, hygroscopic, crystalline powder (USPDI, 1998:IV/544).

Zinc sulfate is efflorescent crystals or a white crystalline powder that is colourless, odourless and transparent with a sharp metallic taste (Hayes & Martin, 1994:8).

2.3 Pharmaceutics

The mentioned zinc salts can be presented in one or more of the following preparations: eye drops, lozenges, mints, chewing gum, oral solutions, syrups, capsules, (dispersible) tablets and injections.

2.3.1 Oral administration of zinc salts

2.3.1.1 Liquid dosage forms

ZinCfant® (Nutriset) is a dispersible zinc sulfate tablet available in both 10 mg and 20 mg tablets for the treatment of acute diarrhoea in infants and young children. It is also used in the treatment of the micro-nutrient deficiency diseases according to WHO and UNICEF criteria (Nutriset, 2006).

Zincris Syrup® (Cipla) contains zinc acetate dihydrate equivalent to 20 mg elemental zinc in each 5 ml for an array of applications, including acute and persistent diarrhoea, respiratory tract infections, common cold, Wilson`s disease and as an immunity booster (Cipla, 2009).

Zinc gluconate oral solution (Sanjing Pharm) is available in 10 ml vials which contain zinc gluconate for the promotion of healthy skin, growth development and body immunity (Sanjing Pharmaceutical Co., Ltd., 2010).

2.3.1.2 Solid dosage forms

Wilzin® (Orphan Pharma) hard capsules are available in two strengths, i.e. 25 mg and 50 mg of elemental zinc as zinc acetate dihydrate for the treatment of Wilson's disease (Anon, 2004).

Galzin® (Teva Pharmaceuticals) capsules are available in 25 mg and 50 mg of elemental zinc as zinc acetate for treatment of Wilson's disease (Medicine Net., Inc., 2010).

ProstAvan® (Melaleuca Inc.) contains zinc acetate dihydrate equivalent to 15 mg elemental zinc as zinc supplement to promote prostate health and is available in tablets, soft gel capsules and extended release tablets (Drugs.com, 2010b).

Orazinc 220® (Mericon Industries Inc.), Zinc CR® (Vitaline Corporation), Mar-Zinc® (Marlop Pharmaceuticals Inc.), Zinc-220® (ALTO® Pharmaceuticals) and Verazinc® (Forest Pharmaceuticals) are all capsule products containing 220 mg zinc sulfate equivalent to 50 mg zinc to treat and to prevent zinc deficiency (Drugs.com, 2010c).

Orazinc® tablets and Orazinc 110® capsules (Mericon Industries Inc.) contain 110 mg zinc sulfate equivalent to 25 mg elemental zinc (Drugs.com, 2010c).

Cold-EEZE® (ProPhase Labs) products are cherry flavoured lozenges containing 104 mg zinc gluconate trihydrate, to reduce the duration and severity of common colds (Drugs.com, 2010a).

ZOX® mints and TheraBreath Plus® chewing gum (TheraBreath) contain zinc gluconate as breath freshener and plaque inhibitor (Katz, 2012).

2.3.2 Nasal administration of zinc salts

Cold-Eeze® (ProPhase Labs) and Zicam® (Matrixx Initiatives) are nasal sprays designed for the treatment of common colds. However, these products were discontinued due to the risk of anosmia, a potentially permanent loss of smell (Eby & Halcomb, 2006:37).

2.3.3 Ophthalmic administration of zinc salts

Eye-Sed® (Scherer Laboratories Inc.) is a 0.25 % solution of zinc sulfate for the relief of mild eye irritation (Drugs.com, 2010).

2.3.4 Parenteral administration of zinc salts

Zinca-Pak® (Solo Pak Medical Products Inc.) injections are available in two strengths, i.e. 1 mg/ml (as sulfate as 4.39 mg heptahydrate or 2.46 mg anhydrous) or 5 mg/ml (as 21.95 mg sulfate) solutions for IV administration (Drugs.com, 2010).

2.4 Pharmacodynamics

2.4.1 Mechanism of action

Zinc plays an important immunological and metabolic role in the human body.

2.4.1.1 Effects on immunity

Zinc salts improve immunity and are proposed to modulate host resistance to various infections. Some studies suggest that zinc may reduce the duration of cold symptoms, possibly due to the reduction of inflammatory cytokines (Cipla, 2009).

2.4.1.2 Effects on the gastrointestinal tract

Zinc improves the transport of electrolytes and water across the intestinal mucosa. In contrast, zinc deficiency can impair the intestinal brush border, increase secretion in response to bacterial enterotoxins and lead to perturbations in the intestinal permeability (Cipla, 2009).

A study by Altaf *et al.* (2002:29) showed that there was an influence on the intestinal villi when rats, with induced diarrhoea, were treated with oral rehydration salts (ORS) with added zinc supplements. An expansion of the intercellular space, an indication of vigorous absorption, was observed in rats who received ORS with added zinc. The intercellular space of rats, who received ORS without zinc, did not show any significant expansion (Figure 2.3).

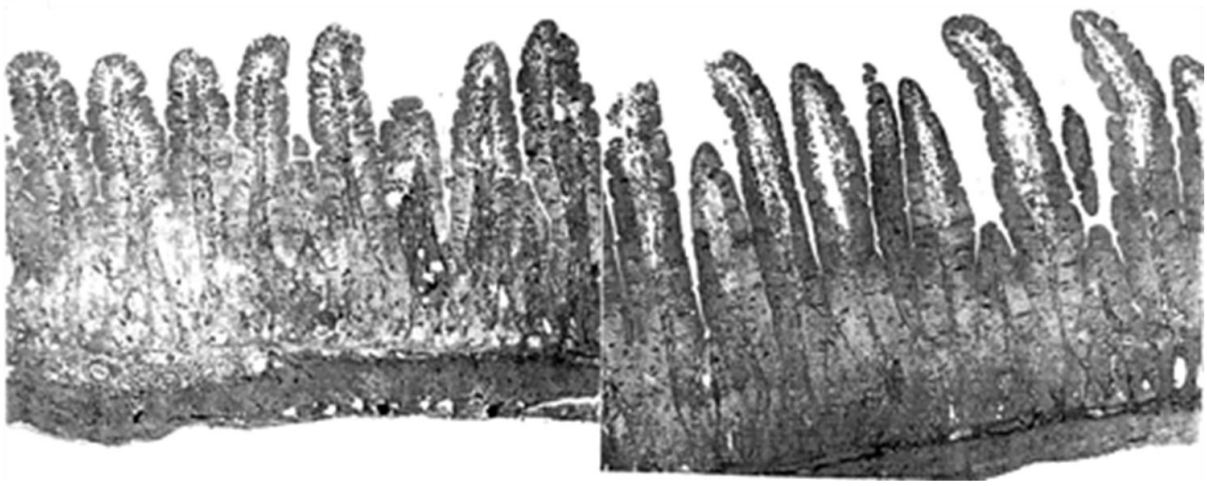


Figure 2.3 Intestinal villi from rats after two days in recovery from cathartic-induced diarrhoea with ORS (a) without zinc or (b) with zinc (Altaf *et al.,* 2002:30).

2.4.1.3 Effects on cellular metabolism

Zinc plays a fundamental role in cellular metabolism (Cipla, 2009) by acting as an integral part of several enzymes required for protein and carbohydrate metabolism, wound healing, normal growth maintenance, skin hydration, taste-, and smell senses (Cerner Multum Inc., 2010).

2.4.1.4 Effects in Wilson's disease

Wilson's disease is a rare hereditary disease caused by a defect in the body's ability to metabolise copper that results in an accumulation of copper deposits in main organs such as the brain, liver, and kidneys (The Learning Company Inc., 1997). The active moiety in zinc supplements is the zinc cation. These cations reduce and maintain systemic copper by competing on the luminal side of the intestinal epithelium for absorption area and induce the synthesis of metallothionein (Anon, 2004:12). Metallothionein binds metals, including copper, to form a non-toxic complex which is excreted in the stool (Cipla, 2009).

2.4.2 Indications

Zinc salts are indicated for the treatment of acute and persistent diarrhoea, respiratory tract infections, common colds, Wilson's disease and zinc deficiencies (Cipla, 2009). Recommended dosage and administration intervals of zinc

supplements are given in Table 2.2. Dosages are expressed as elemental zinc. Zinc supplementations are recommended between meals, at least one hour before or two to three hours after meals. In case gastric intolerance occurs, zinc can be taken with food, preferable protein (meat) (Anon, 2004:2; Cipla, 2009).

Table 2.2 Recommended dosage and administration intervals of elemental zinc (Anon, 2004:2; Cerner Multum Inc., 2010; Cipla, 2009)

Indication	Dosage	Administration intervals
Acute diarrhoea		
Children below 6 months	10 mg	Daily for 10 - 14 days
Children above 6 months	20 mg	Daily for 10 - 14 days
Respiratory tract infections		
Children below 6 months	10 mg	Daily for 10 - 14 days
Children above 6 months	20 mg	Daily for 10 - 14 days
Wilson's disease		
Adults	50 mg	Three times daily (maximum dose of 50 mg 5 times daily.
Children from 1 - 6 years	25 mg	Twice daily
Children from 6 - 16 years	25 mg	Three times daily
Adolescents (16 years/body weight above 57 kg)	50 mg	Three times daily
Pregnant females	25 mg	Three times daily
Sickle cell anaemia	10 - 15 mg	Daily
Acrodermatitis enteropathica	1 - 2 mg/kg body weight	Daily
Ophthalmic	1 - 2 drops	Four times daily
Dietary allowances		
Children up to 3 years	5 - 10 mg	Daily
Children 4 - 6 years	10 mg	Daily
Children 7 - 10 years	10 mg	Daily
Adolescent and adult males	15 mg	Daily
Adolescent and adult females	12 mg	Daily
Pregnant females	15 mg	Daily
Breast-feeding females	16 - 19 mg	Daily

2.4.3 Toxicity and side effects

After oral ingestion of a high dose of zinc salts, the following side effects may occur, namely abdominal pain, dyspepsia, nausea, vomiting, diarrhoea, gastric irritation, gastritis, fever and respiratory distress (Troy *et al.,* 2005:1717). Chronic large zinc doses may depress immune function and resulting copper deficiency may cause hypochromic anaemia (Cipla, 2009; Troy *et al.,* 2005:1717).

Hypotension, jaundice, pulmonary oedema, corrosion and inflammation of the mucous membranes of the mouth and stomach, indigestion, unusual tiredness or weakness, ulceration of the stomach followed by perforation, are all symptoms that also have been reported (Cerner Multum Inc, 2010; Cipla, 2009).

2.4.4 Contraindications

Contraindications include hypersensitivity to zinc salts or any component of the zinc containing supplement (Anon, 2004:3).

2.4.5 Precautions

2.4.5.1 Drug interactions

Zinc absorption may be reduced by penicillamine, phosphorus-containing preparations, iron-supplements and tetracyclines. Zinc supplements may reduce the absorption of copper and fluoroquinolones (Sweetman, 2002:1398).

2.4.5.2 Renal impairment

Caution should be exercised in patients with renal impairment to avoid zinc accumulation. Patient monitoring is advised (Cipla, 2009).

2.4.5.3 Lactation

Zinc-induced copper deficiency in the breast-fed infant may occur and zinc is excreted in human breast milk. Therefore high zinc concentrations, such as found in treatment of Wilson's disease, should be avoided during lactation (Anon, 2004:4).

2.5 Pharmacokinetics

2.5.1 Solubility

The solubility of zinc salts in water and in alcohol, according to the British Pharmacopoeia's (BP) definition of solubility, is summarised in Table 2.3 (BP, 2011).

Table 2.3 Solubility of zinc salts (BP, 2011)

Zinc salt	Solubility* in water	Solubility* in alcohol
Zinc acetate dihydrate	Freely soluble (in 1 to 10 ml)	Soluble 96 % ethanol (in 1 to 30 ml)
Zinc gluconate	Soluble (in 1 to 30 ml)	Practically insoluble (in more than 10 000 ml)
Zinc sulfate	Very soluble (in less than 1 ml)	Practically insoluble (in more than 10 000 ml)

*Solubility = gram per number of millilitre of solvent

2.5.2 Absorption

Zinc is poorly absorbed, with a few dietary factors influencing the absorption. The zinc intake plays a role in absorption, where increasing amounts of zinc will decrease the fractional % zinc absorption (Lönnerdal, 2000:1378S). Protein quantity and quality in a meal is positively correlated to zinc absorption (Lönnerdal, 2000:1379S). Iron and agents, such as hydrogen gas, which increase gastric pH, may decrease zinc absorption (Cipla, 2009). According to Lönnerdal (2000:1381S) toxic levels of cadmium inhibit zinc absorption. When zinc molecules form a complex with low-molecular-weight ligands or chelators (for example histidine), and that complex is absorbed, it is likely that the net effect of zinc absorption will be positive (Lönnerdal, 2000:1381S). Zinc bioavailability can also be markedly decreased by phytic acid, particularly in the presence of large amounts of calcium (Hayes & Martin, 1994:11; Sweetman, 2002:1398; Troy *et al.,* 2005:1717).

Absorption mainly occurs in the duodenum and jejunum of the small intestines. Bioavailability vary between sources, but ranges from 20 % - 30 % (Cerner Multum Inc., 2010; Sweetman, 2002:1398).

2.5.3 Distribution

Zinc is widely distributed throughout the body, where skin, bone, liver, skeletal muscle and prostatic fluids have the highest zinc concentrations (Hayes & Martin, 1994:11; Sweetman, 2002:1398; Troy *et al.,* 2005:1717). In the blood, approximately 80 % of absorbed zinc is distributed to erythrocytes, with the about 98 % of the remaining zinc in leukocytes bound to plasma proteins (Table 2.4).

Although zinc binds to all plasma proteins, it binds most loosely to albumin (Troy *et al.,* 2005:1717).

Zinc occurs in the body in two different combinations. Firstly, zinc binds loosely to a protein to form a metal-protein complex, which acts as its carrier and transport mechanism. Secondly, as a metalloenzyme in which zinc is an integral part of an important enzyme system, such as carbonic anhydrase for CO_2 regulation (Hayes & Martin, 1994:12).

Table 2.4 Zinc binding proteins (Anon, 2004:6; Cipla, 2009; Hayes & Martin, 1994:11)

Plasma protein	Percentage binding
Albumin	55 %
Alpha 2- macroglobun / transferrin	30 % - 40 %
Retinol-binding protein	1 % - 2 %
Amino acids	1 %

2.5.4 Elimination half-life

Zinc is not metabolised. The elimination half-life of zinc from the body is 5 to 16 months and plasma elimination half-life is around 1 hour after the administration of a 45 mg dose (Anon, 2004:6; Hayes & Martin, 1994:12). The kidneys have little or no role in the regulation of zinc content in the body. Up to 80 % of dietary zinc is excreted through the intestines (faeces), with relatively little (15 % - 25 %) from urine and sweat (Anon, 2004:6; Cipla, 2009; Troy *et al.,* 2005:1717).

2.6 Conclusion

Zinc plays an important immological and metabolic role in the human body. Zinc has an array of applications and is available in a range of preparations. Water-soluble zinc salts have been used to treat respiratory tract infections, common colds, Wilson's disease, bad breath and mild eye irritations; as well as for the promotion of growth development, body immunity, healthy skin and plaque inhibition. For the purpose of this study the primary indication of these water-soluble zinc salts is the treatment of acute and persistent diarrhoea, to reduce both the severity and duration thereof. This can be attributed to the effect of zinc on the gastrointestinal tract where it improves the transport of electrolytes and water across the intestinal mucosa.

As mentioned in Chapter 1, the elemental zinc provided for the treatment of diarrhoea should be of adequate quality, and monographs provide this quality gauge to evaluate the quality of medicines. Chapter 3 will focus on the importance of monographs and the process to develop these quality standards.

CHAPTER 3

MONOGRAPHS OF *THE INTERNATIONAL PHARMACOPOEIA*

3.1 Introduction

Monographs play a critical role in the quality control testing (QC-testing) of APIs and pharmaceutical dosage forms to ensure the quality, safety and efficacy of the product for patient use. The access to validated and peer-reviewed monographs is of essence to the pharmaceutical industry (BP, 2011; *Ph. Int*., 2011; USP, 2011).

The aim of this chapter was to investigate the process of monograph development and the validation thereof to ensure its fitness for purpose.

3.2 Definition

A monograph is defined by The Learning Company Inc. (1997) as a scholarly piece of writing or essay or book on a very specific, often limited subject, drafted by experts in that specific field.

The National Research Council (NRC) of Canada (2010) defines a monograph as follows: "*A monograph is a specialised scientific book. As learned treatises on clearly defined topics, which may be intra-, inter-, or cross-disciplinary, monographs generally are written by specialists for the benefit of other specialists. Although usually regarded as a component of the review literature of science, monographs are works that demand the highest standards of scholarship. Their preparation calls for exceptional breadth and depth of knowledge on the part of their authors, who, inter alia, must be able to collect, collate, analyse, integrate, and synthesise all relevant contributions to the archival literature of the scientific and engineering journals and to add original material as required. The value of monographs lies in the coherence and comprehensiveness of the information and knowledge they contain, which is important to the specialised researchers to whom they are directed and, therefore, to the advancement of science and engineering generally. Most monographic manuscripts are critically reviewed and tightly edited. The resulting books can be expected to have a reasonably long shelf life*" (NRC, 2010).

3.3 Importance

Worldwide sales of counterfeit medicines were estimated to be able to top 75 billion US dollars in 2010, a 90 % rise in five years, according to an estimate published by the Centre for Medicine in the Public Interest in the United States of America (USA) (as quoted by a Bulletin of the WHO, 2010a). The WHO describes substandard medicines as "*pharmaceutical products that do not meet their quality standards and specifications. Each pharmaceutical product that a manufacturer produces has to comply with quality assurance standards and specifications, at release and throughout its shelf-life, according to the requirements of the territory of use. Normally, these standards and specifications are reviewed, assessed and approved by the applicable national or regional medicines regulatory authority before the product is authorised for marketing*" (WHO, 2010b:3).

Substandard medicines are ineffective and put lives at risk. An outright lack of therapeutic benefits may cause death, especially in infants, and insufficient active ingredients lead to API resistance. Not only do substandard medicines undermine confidence in health-care systems, health professionals, pharmaceutical manufacturers and distributors, it also places a financial burden on patients and governments (Bate & Boateng, 2007:2). This highlights the need for strict regulatory standards such as monographs to ensure quality medicine.

The availability of monographs in *The Ph. Int.* provides a quality platform for medicines regulatory authorities to combat counterfeit products. *The Ph. Int.* comprises a collection of recommended procedures (also known as monographs) for analysis and specifications for the determination of pharmaceutical substances, excipients and dosage forms that is intended to serve as source material for reference or adaptation by any WHO Member State wishing to establish pharmaceutical requirements. The pharmacopoeia, or any part of it, has legal status whenever a national or regional authority expressly introduces it into appropriate legislation (WHO, 2009:299).

Pharmacopoeial specifications represent only one element of the quality assurance of medicines. APIs and final dosage forms should be manufactured according to the current requirements of Good Manufacturing Practices (GMP) (WHO, 2011e).

It should be understood that a distinction exists between pharmacopoeial standards and manufacturers' release specifications (*Ph. Int.,* 2011):

- Pharmacopoeial standards are publicly-available compliance specifications which provide a means for an independent check of the quality of a product at any time during its shelf-life by a QC laboratory.
- The manufacturers' release specifications should be compatible with pharmacopoeial specifications; however they may differ in several respects. The compliance specifications of the manufacturer may need to be more exacting than corresponding pharmacopoeial requirements.

The Ph. Int. also states that: "*the degree of protection provided by pharmacopoeial standards will depend not only on their technical content but also to a great extent on how they are used. The specified tolerances and limits allow for the inherent variations that occur during production and packaging, as well as for subsequent degradation within normal handling and storage conditions and for any acceptable variance of analytical results*" (*Ph. Int*., 2011).

3.4 Development of monographs for publication in *The International Pharmacopoeia*

The Ph. Int. develops monographs in collaboration with WHO collaborating centres. The Research Institute for Industrial Pharmacy (RIIP®), incorporating the Centre for Quality Assurance of Medicines (CENQAM®), is a WHO collaborating centre for the quality assurance of medicine. The following steps are stipulated by the WHO (WHO, 2011a) for the development of new monographs (for a schematic diagram see Figure 3.1):

Step 1: Identification of specific pharmaceutical products for which QC specifications need to be developed, confirmation by all WHO parties concerned (including Department of Essential Medicines and Pharmaceutical Policies (EMP), specific disease programmes and the Prequalification Programme).

Step 2: Provision of the contact details from manufacturers of the above products in collaboration with all parties concerned.

Step 3: Contact manufacturers for provision of QC specifications and samples.

Step 4: Identify and contact QC laboratories for collaboration in the project (a number of laboratories depending on how many pharmaceutical products have been identified in step 1). Contract for laboratory work.

Step 5: Prepare the contract for drafting the specifications and undertaking the necessary laboratory work.

Step 6: Search for information on QC specifications available in the public domain.

Step 7: Laboratory testing, development and validation of QC Specifications.

Step 8: Support WHO host organisation for International Chemical Reference Substances (ICRS) (Council of Europe) in the establishment of ICRS.

Step 9: Contact collaborating manufacturers for the availability of the respective substances to establish ICRS, as necessary.

Step 10: Follow the consultative process, mailing of draft specifications to Expert Panel and specialists, including providing drafts on the website.

Step 11: Discussion of comments with contract laboratories, WHO Collaborating Centres and additional laboratory testing to verify and/or validate specifications and the ICRS host organisation.

Step 12: Consultation to discuss the comments and test results received as feedback.

Step 13: Recirculation for comments.

Step 14: As step 10.

Step 15: Present the drafts to the WHO Expert Committee on Specifications for Pharmaceutical Preparations for possible formal adoption. If not adopted repeat steps 11 to 13 as often as necessary. If adopted proceed to step 16.

Step 16: Incorporate all changes agreed during the discussion leading to adoption together with any editorial points.

Step 17: Where necessary, also take account of any further comments that may still be received during comment deadlines for recirculated texts (Step 12 and beyond) falling shortly after the meeting.

Step 18: In all cases, confirm the amended text by correspondence with the relevant experts and/or contract laboratory before making it available on the WHO Medicines website.

Step 19: Make “final texts” available on the Medicines website to provide users such as prequalification (PQ) assessors and manufacturers with the approved specifications in advance of the next publication date.

Step 20: Include in *The Ph. Int.*

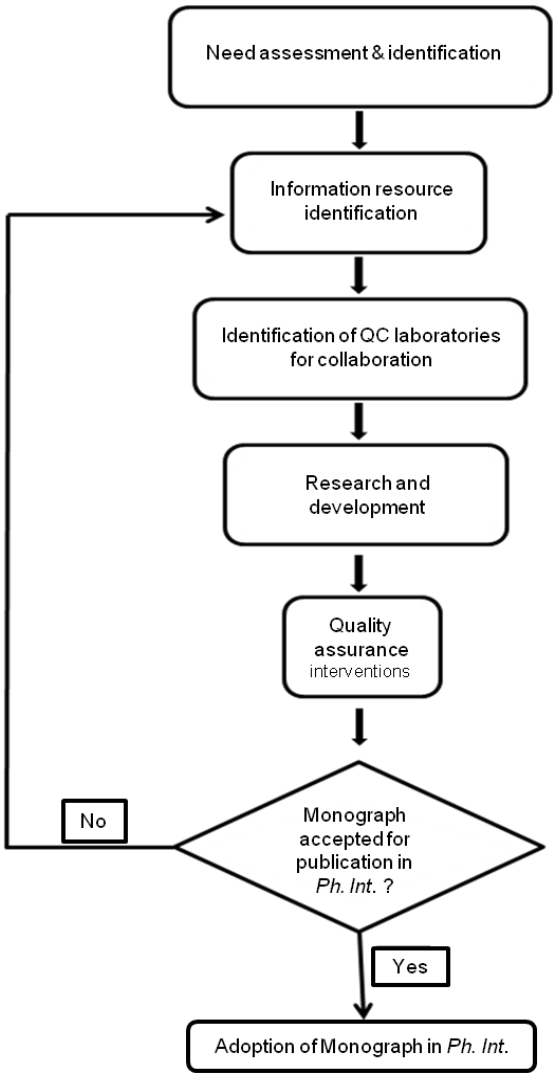


Figure 3.1 Schematic diagram of the steps followed in the development of new monographs (WHO, 2011a).

3.5 Structure of monographs for APIs to be published in *The International Pharmacopoeia*

The basic structure of an API monograph to be published in *The Ph. Int.* is shown in Figure 3.2 (*Ph. Int*., 2011), which ensures the uniformity and standard of the monographs.

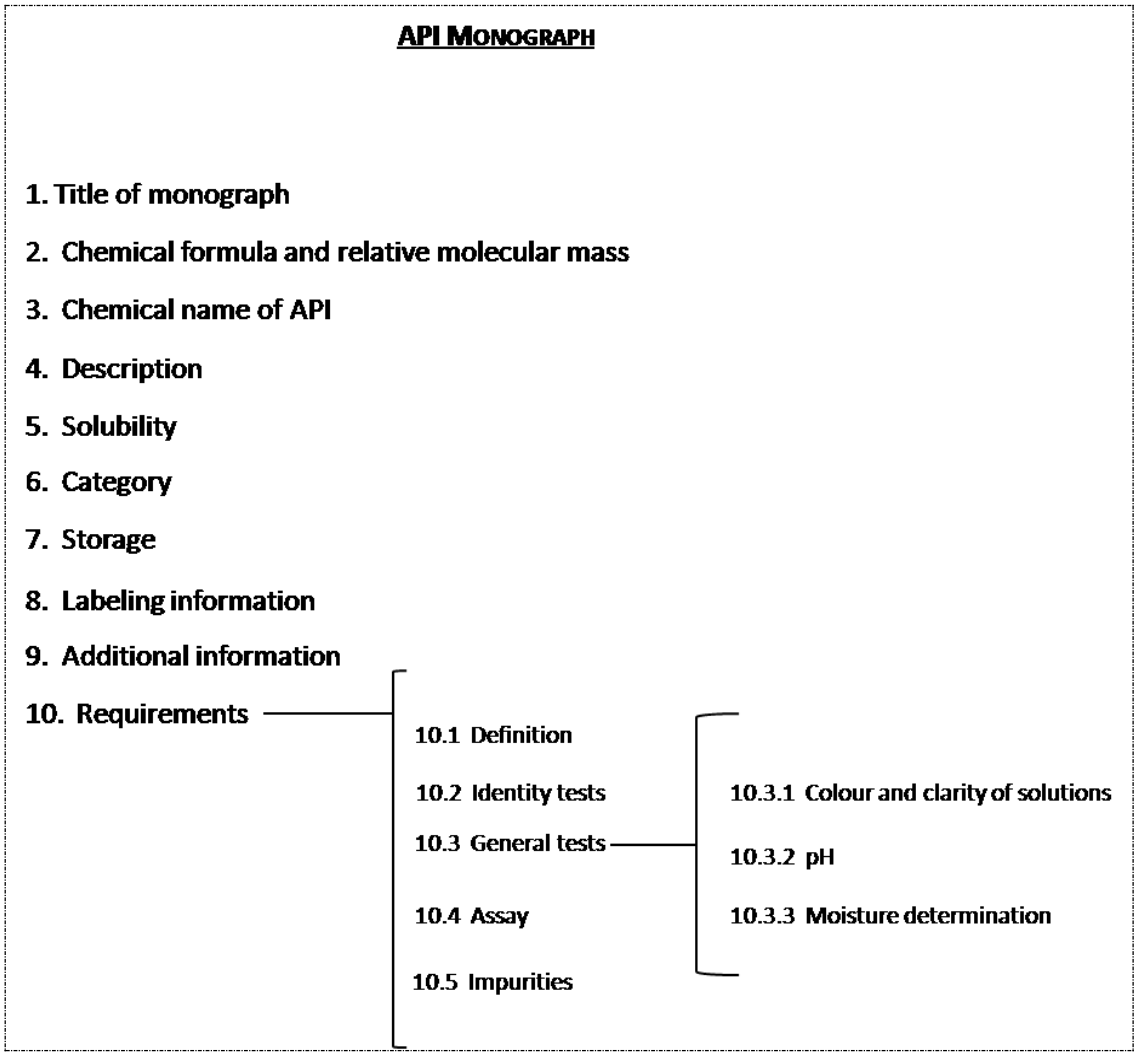


Figure 3.2 The basic structure of an API monograph to be published in *The Ph. Int.* (*Ph. Int*., 2011).

3.5.1 Title of monograph

The title of the monograph should indicate that the monograph is intended for a specific API. The name of the API in the monograph must be given in Latin and English. The Latin name should be a singular form of the recommended or proposed International Nonproprietary Name (INN) (*Ph. Int.*, 2011).

The Ph. Int. specifies that for salts the name of the acid component should be placed in the nominative case (either second declension neuter or third declension masculine) and the other component in the genitive case (e.g. Zinci sulfas). On the other hand, for compounds that are not derived from true acids, both components of the title are placed in the nominative case, treating the main component as a neuter substantive and using an adjectival form of the complementary component in agreement with this substantive (e.g. Cloxacillinum with "natricus" as the adjectival form of Natrium, thus Cloxacillinum natricum) (*Ph. Int.,* 2011).

3.5.2 Chemical formula and relative molecular mass

The empirical chemical formula and the relative molecular mass should be reported for APIs whose chemical composition is known. A graphic formula should be reported for organic substances (*Ph. Int.*, 2011).

3.5.3 Chemical name of active pharmaceutical ingredient

The chemical name of the API should be given in accordance with the International Union of Pure and Applied Chemistry (IUPAC) system. More than one systematic name may be given in the event when equally acceptable alternative names may be construed from the IUPAC system. *The Ph. Int.* also requires that the registry number established by the Chemical Abstracts Service of the American Chemical Society (CAS No.) should be reported for the API to assist with the identification of the API (*Ph. Int.,* 2011).

3.5.4 Description

In this section a general description of the appearance of the API should be provided. *The Ph. Int.* clearly states that the information provided under this heading is not to be interpreted in a strict sense and should not necessarily be regarded as an analytical requirement (*Ph. Int.,* 2011).

3.5.5 Solubility

The approximate solubility of the API should be reported in this section. According to *The Ph. Int.* (2011) the approximate solubility of the API should be evaluated at

20 °C and should then be classified according to the approximate solubility classification system tabulated in Table 3.1.

Table 3.1 Approximate solubility classification system of *The Ph. Int.* (2011)

Solubility*	Class
Less than 1 part	Very soluble
From 1 to 10 parts	Freely soluble
From 10 to 30 parts	Soluble
From 30 to 100 parts	Sparingly soluble
From 100 to 1 000 parts	Slightly soluble
From 1 000 to 10 000 parts	Very slightly soluble
More than 10 000 parts	Practically insoluble

* The expression "part" describes the number of ml of solvent represented by the stated number of parts in which 1 g of solid is soluble.

3.5.6 Category

The category is indicative of the principal pharmacological action and therapeutic use or, for excipients, the main pharmaceutical use. *The Ph. Int.* warns that users should not assume that the substance has no other action or use than that reported in the monograph (*Ph. Int.,* 2011).

3.5.7 Storage

In this section information should be provided with regards to the storage conditions of the API to ensure the integrity thereof. Storage conditions should be discussed with specific reference to the type of container to be used, photosensitivity of the API and the recommended temperatures for storage of the API (*Ph. Int.,* 2011).

3.5.7.1 Containers

The container in which the API is stored should protect the API and ensure the integrity thereof. It is critical that the API should not react in any way, physically or chemically, with the container and by so doing altering its quality. The container should also protect the API from interacting with the immediate environment such as moisture sorption, oxidation due to exposure to oxygen, photolysis due to direct ultraviolet (UV) exposure, etcetera (etc.).

The Ph. Int. utilises the permeability terms tabulated in Table 3.2 to describe the required containers which should be used for the storage of APIs (*Ph. Int.,* 2011).

Table 3.2 Permeability terms used to describe the required containers which should be used for the storage of APIs (*Ph. Int.,* 2011)

Term	Requirements
Well-closed containers	The contents must be protected from loss and extraneous matter.
Tightly closed containers	Protection from extraneous matter, loss, efflorescence, deliquescence and evaporation of content must be ensured. Containers to be opened and closed must be designed to close airtight with every reclosure.
Hermetically closed containers	Content must be impervious to air or any other gas; as well as protected from extraneous matter and loss.
Tamper-evident container	A device must be fitted that clearly reveals whether the container has ever been opened.

3.5.7.2 Protection from light

Light sensitive APIs should be sheltered from light utilising one or more of the following two approaches:

- Storage in a light-resistant container that either has special coating applied to the container (Figure 3.3 (A)), or is made with material with the inherent property to protect from the effects of light (Figure 3.3 (B)).
- Storage in a container which is coated with a suitable light-resistant (opaque) covering. Alternatively, the container can be stored in a dark place (*Ph. Int.,* 2011).

(A) (B)

Figure 3.3 Example of (A) a container with a special coating and (B) a container which is made of high density polyethylene which can protect APIs from light exposure.

3.5.7.3 Temperature

Storage temperatures should be stated in the monograph if a temperature other than room temperature (15 to 25 °C, depending on climatic conditions, up to 30 °C) is required (*Ph. Int.,* 2011).

3.5.8 Labelling information

The relevant authority should stipulate whether any specific labelling statements should appear on the container, the packaging or a leaflet accompanying the package. Labelling information is rarely provided for APIs but could be added if deemed necessary (*Ph. Int.,* 2011).

3.5.9 Additional information

This information includes any characteristics of the API such as polymorphism, hygroscopicity, stability, precautions, melting point/behaviour, etc. (*Ph. Int.,* 2011). Attention may be drawn to particular hazards in certain monographs by means of a warning statement. *The Ph. Int.* (2011) clearly states that the absence of such a statement is not to be considered to mean that no hazard exists.

3.5.10 Requirements

Requirements or specifications are defined by the European Medicines Agency (EMA) as a list of tests, references to analytical procedures, and appropriate acceptance criteria, which are numerical limits, ranges, or other criteria for the tests described (EMA, 2000:3). The set of criteria is established to which the API should conform to be considered acceptable for its intended use. "Conformance to specifications" means that the API, when tested according to the listed analytical procedures, will meet all the listed acceptance criteria (EMA, 2000:3). A list of general requirements specified by *The Ph. Int.* can be seen in Figure 3.4.

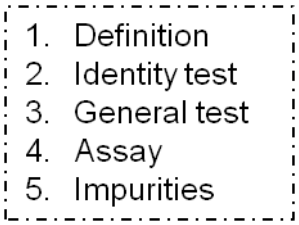


Figure 3.4 A list of monograph tests and requirements according to *The Ph. Int.* (2011).

When an API complies with all the requirements set out in *The Ph. Int.*, it can be stated that is it of pharmacopoeial quality.

3.5.10.1 Definition

The statement or definition constitutes the instructions or quantitative requirements with which the material must comply (*Ph. Int.,* 2011). For example, zinc sulfate's definition is: "*Zinc sulfate monohydrate contains not less than 99.0% and not more than 101.0% of $ZnSO_4,H_2O$. Zinc sulfate heptahydrate contains not less than 99.0% and not more than 104.0% of $ZnSO_4,7H_2O$"* (*Ph. Int.*, 2011). The definition is thus an expansion of the purity requirements of the API.

3.5.10.2 Identity tests

It is important to establish the identity of the API and to discriminate between compounds of closely related structure which are likely to be present (EMA, 2000:9). Wherever possible, infrared (IR) spectrum characteristics are used as the principal test of identification, due to the uniqueness of a well developed fingerprint region (Silverstein *et al.*, 1981:81). If the new API is a salt, identification testing should be specific for the individual ions. According to the EMA (2000:9) an identification test that is specific for the salt itself should suffice.

The Ph. Int. recommends the use of the following identity tests:

- IR,
- Thin-layer chromatography (TLC),
- High-performance liquid chromatography (HPLC),
- UV,
- Specific optical rotation (SOR),

- Chemical reactions (Chem), and
- Counter-ion (C-ion).

The Ph. Int. further proposes that there should preferable be a subsidiary choice where such tests are included for APIs. Choice between the following should be allowed (as appropriate):

- IR and other tests, or
- IR + SOR and other tests + SOR

Whenever applicable a test for C-ion should be included, and if a choice is given, include both options (*Ph. Int.*, 2011).

3.5.10.3 General tests

3.5.10.3.1 Clarity and colour of solution

A black background is used to determine the clarity of the solution. The light source must be such that opalescence standard TS2 (as described in *The Ph. Int.,* 2011 section 1.11 Colour of liquids) can be readily distinguished from water (*Ph. Int.,* 2011). A solution is considered clear if its opalescence is not more pronounced than that of opalescence standard TS2 (*Ph. Int.,* 2011). *The Ph. Int.* stipulates that a colourless solution should be not more intensely coloured than any of the standard colour solutions Bn0, Yw0, Gn0, or Rd0 (as described in *The Ph. Int.,* 2011 section 1.11 Colour of liquids).

3.5.10.3.2 pH

The pH of an API in solution is a chemical property which may be utilised as a supplementary identification test. pH testing may be utilised to distinguish between:

- Free base and salt of an API: for example – amikacin versus amikacin sulfate (*Ph. Int.,* 2011).
- Different salts of the same API: for example – quinine sulphate versus quinine bisulfate (*Ph. Int.,* 2011).

3.5.10.3.3 Moisture determination

According to the EMA, water content is important in cases where the API is known to be hygroscopic or degraded by moisture or when the drug substance is known to be a stoichiometric hydrate (EMA, 2000:11). The data on the effects of hydration or moisture adsorption can be used to justify the acceptance criteria. A detection procedure that is specific for water (e.g., Karl Fischer titration) is preferred; however in some cases, a LOD procedure may be considered adequate (ICH, 1999:10).

3.5.10.4 Assay

It is important to determine the purity (content) in a specific, stability-indicating manner (EMA, 2000:9). Unless otherwise indicated, assay testing is usually carried out at ambient temperature (between 15 and 25 °C, or up to 30 °C in some climatic zones) (*Ph. Int.,* 2011). All glassware used should be of suitable quality, and unless specified otherwise, all solutions are prepared using 'Purified water' / water reagent (R) (distilled or demineralised) (*Ph. Int.,* 2011). The specification for the assay test is expressed in the definition section (3.5.10.1) of the monograph.

3.5.10.5 Impurities

Impurities must be tested to ensure that they are below the specified limits. The impurity concentration is usually given as a percentage or in parts per million (ppm) by weight (*Ph. Int.,* 2011). According to *The Ph. Int.* (2011) a list of all known and potential impurities may be given for information at the end of the monograph, if it has been shown to be controlled by the tests in a monograph.

3.6 Method validation and verification

To conform to the requirements as set out in the ICH guideline Q2 R1 (2005:1) methods used for pharmaceutical analysis must be sufficiently accurate, specific, sensitive and precise (Ermer & Miller, 2005:V). Formal validation studies are performed for a newly developed method, or when the validation of an existing method needs to be completed according to ICH guidelines. Method verification or transfer verification needs to be performed to illustrate the suitability (fitness for use) of a method under actual conditions in the individual laboratory.

The current GMP regulations (21 CFR 211.194(a) (2)) states that the "suitability of all testing methods used shall be verified under actual conditions of use" (FDA, 2011).

Previously validated methods (i.e. established methods) can be obtained from manufactures, monographs or scientific journals. Where validated methods are available, transfer validation / verification applies. Transfer validation tests determine whether the method may be successfully employed at the intended facility at the specified conditions. It is assumed that a certain amount of validation is already available for the method retrieved from the monograph, so no or only a few performance parameters need to be considered during the transfer verification process (Ermer & Miller, 2005:302). Table 3.3 serves as a guideline when selecting the typical validation / verification parameters which should be evaluated for compendial methods.

Table 3.3 The typical validation / verification parameters which should be evaluated for compendial methods (Pappa, 2006:11)

API	Final product
Identification	
Specificity	Specificity
Impurities	
Specificity Limit of detection	Specificity Limit of detection
Assay	
Precision Specificity Limit of quantitation	Precision Specificity Limit of quantitation

It is recognised that different test procedures require unique validation schemes or approaches. Validation requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. According to the ICH guideline (ICH, 2005:3) on the Validation of analytical procedures: Text and methodology Q2 (R1), Table 3.4 serves as a guideline when selecting the typical validation parameters which should be considered. These parameters will now be defined, according to ICH Q2 (R1) (2005:1-13), as they were applied during the monograph development process.

Table 3.4 The typical validation parameters which should be considered according to the ICH Expert working group (2005:3)

Type of analytical procedure	**Identification**	**Testing for impurities**		**Assay** - **Dissolution (measurement only)** - **Content/potency**
Characteristics		Quantitative	Limit	
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm. Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

Interm. = Intermediate

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

(1) In cases where reproducibility has been performed, intermediate precision is not needed.

(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(3) May be needed is some cases.

3.6.1 Specificity

Specificity can be defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. (ICH, 2005:4).

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). According to the ICH guideline (2005:4) on the Validation of analytical procedures: Text and methodology Q2 (R1), this definition has the following implications:

- Identification: to ensure the identity of an analyte.
- Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

- Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample (ICH, 2005:4).

3.6.2 Accuracy

The closeness of agreement between the value found and which is accepted either as a conventional true value or an accepted reference value is described as accuracy or trueness (ICH, 2005:4).

3.6.3 Precision

Precision can be considered at three levels: repeatability, intermediate precision and reproducibility (ICH, 2005:4). It expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample or sample solutions under the prescribed conditions. Precision may be expressed as the variance, standard deviation or coefficient of variation of a series of measurements (ICH, 2005:4).

3.6.3.1 Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision (ICH, 2005:5).

3.6.3.2 Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. (ICH, 2005:5).

3.6.3.3 Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardisation of methodology) (ICH, 2005:5).

3.6.4 Detection limit

The detection limit is the lowest amount of analyte in a sample which can be detected, though not necessarily quantified as an exact value (ICH, 2005:5).

3.6.5 Quantitation limit

The quantitation limit is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. It is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products (ICH, 2005:5).

3.6.6 Linearity

The linearity is the ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample (ICH, 2005:5).

3.6.7 Range

The range of an analytical procedure is the interval between, and including, the upper and lower concentration (amounts) of analyte where the sample has a suitable level of precision, accuracy and linearity (ICH, 2005:5).

3.6.8 Robustness

Robustness is the capacity of an analytical procedure to remain unaffected by small, but deliberate variations in method parameters and serves as an indication of its reliability during normal usage (ICH, 2005:5).

3.7 Conclusion

Monographs play a critical role in QC-testing of APIs and pharmaceutical dosage forms to ensure the quality, safety and efficacy thereof. This chapter highlighted the steps and general requirements for the development of monographs for publication in *The Ph. Int.* The information presented in this chapter was used as a platform in

the following chapters for the development of zinc acetate dihydrate and zinc gluconate API monographs, starting in Chapter 4 with identification tests.

CHAPTER 4

IDENTIFICATION TESTS

4.1 Introduction

As mentioned in section 3.5.10.2 (Chapter 3), the purpose of identification tests in a monograph is to confirm the identity of the API being investigated. The ICH guideline "Specifications: Test procedures and acceptance criteria for new drug substances and new drug products: chemical substances – Q6A" states that identification testing should be specific and able to discriminate between compounds of closely related structure (ICH, 1999:6). There are a number of ways to confirm the true identity of an API, namely: determination of physical constants, chromatographic tests and chemical tests (Kar, 2005:10). A schematic presentation of the approaches for identification of APIs can be seen in Figure 4.1.

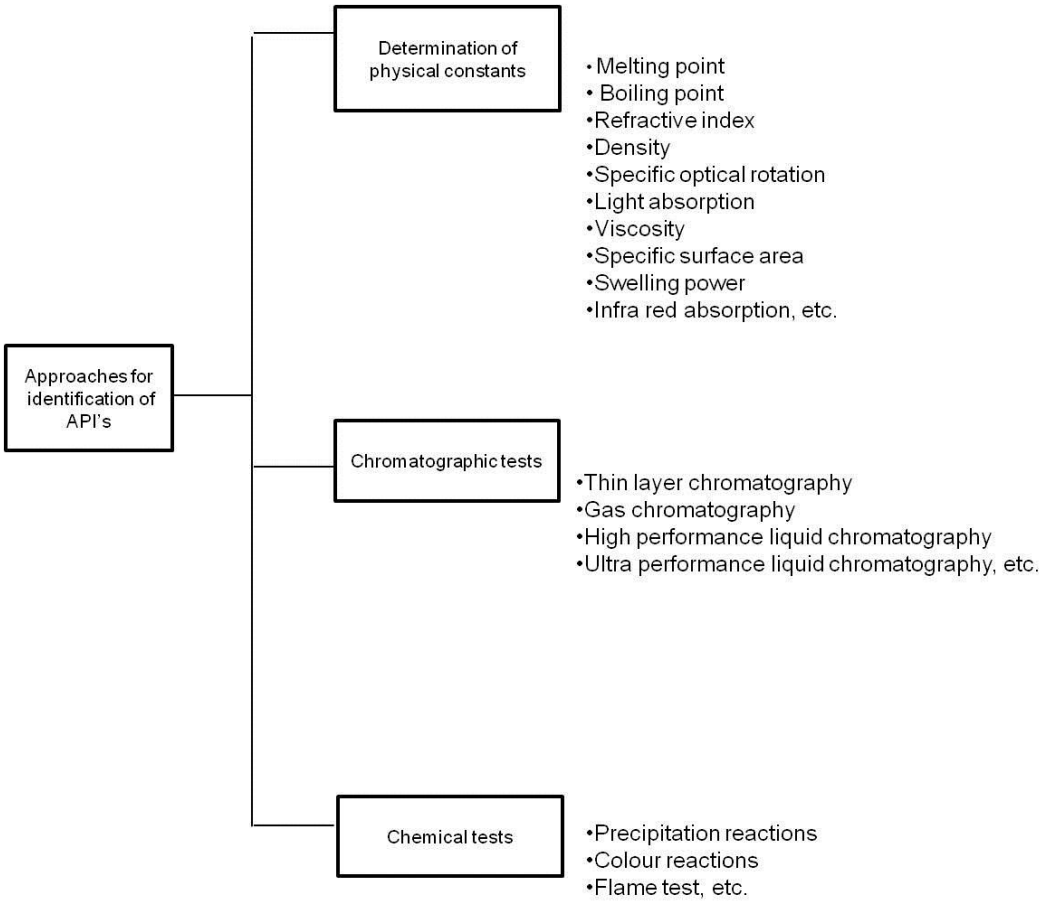


Figure 4.1 A schematic presentation of the approaches for identification of APIs (constructed based on information presented by Kar, 2005:10-15).

Both zinc salts investigated in this study consist of a metal cation and an ionic salt. The ICH guideline "Specifications: Test procedures and acceptance criteria for new drug substances and new drug products: chemical substances – Q6A" states that in the instance where the API is a salt, an identification test for the individual ions itself should suffice (ICH, 1999:6). Based on the identification tests recommended by *The Ph. Int.* section 3.5.10.2 (Chapter 3) and the chemical properties of the zinc salts, the following approaches were considered for identification tests:

a) Identification of the zinc cation: - by means of (i) chemical tests: flame test or (ii) a precipitation test.
b) Identification of acetate: - by means of a chemical test: precipitation test.
c) Identification of gluconate: - by means of a chromatographic test: TLC.

The aim of this chapter was to establish identification methods for zinc, acetate and gluconate to be included in *The Ph. Int.* monographs.

4.2 General identification test for zinc in zinc salts by means of a flame test

Zinc has an electron configuration of $[Ar]3d^{10}4s^2$ and is a member of group 12 of the periodic table. It is a moderately reactive metal and strong reducing agent. Zinc burns in air with a bright bluish-green flame, giving off zinc oxide fumes (Hogan *et al.*, 2010).

4.2.1 Materials and equipment

A list of the materials used in the identification test for zinc in zinc salts by means of a flame test is tabulated in Table 4.1. Table 4.2 lists the equipment used for this test.

Table 4.1 Materials used in the identification test for zinc in zinc salts by means of a flame test

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate*	A892302 020	Merck KGaA	Germany
Zinc gluconate*	K38073079 025	Merck KGaA	Germany
Hydrochloric acid	1034315	Merck Chemicals (Pty) Ltd.	South Africa

KGaA = German: Limited partnership on shares; Pty = Propriety limited company; Ltd. = Limited

* Only one manufacturer was able to provide samples for testing (refer to Chapter 12 - section 12.6)

Table 4.2 Equipment used in the identification test for zinc in zinc salts by means of a flame test

Equipment	Supplier	Country of origin
BernzOmatic PBLAN bunsen burner	CADAC	South Africa
Platinum wire	Industrial Analytical	South Africa

4.2.2 Procedure

Prepare a platinum wire by cleaning it repeatedly with hydrochloric acid (~250 g/l) TS to remove any traces of possible contaminants. Introduce the zinc salts on the platinum wire to a hot, non-luminous blue flame and observe the colour of the flame that results. Take care to repeat the cleansing of the platinum wire between samples. The flame should be a bluish-green colour.

4.2.3 Results

Zinc acetate dihydrate burned with a bluish-green flame. The zinc gluconate seemed to melt when introduced to the flame and had a characteristic smell of caramelised sugar (Table 4.3).

Table 4.3 Photographs of the zinc salts during the zinc identification test by means of a flame test

Hot, non-luminous blue flame	Zinc acetate dihydrate	Zinc gluconate
		

4.2.4 Discussion

Zinc acetate dihydrate API burned with a bluish-green flame which is a positive test for zinc. The zinc gluconate API seemed to melt when introduced to the flame, which may be attributed to the presence of the gluconate sugar.

4.2.5 Conclusion

The flame test may be utilised for the identification of zinc in zinc acetate dihydrate API, but is considered not suitable for zinc gluconate API.

4.3 General identification test for zinc in zinc salts by means of precipitation tests

Zinc reacts readily with acids, alkalis and other non-metals to yield water soluble or insoluble salts. This chemical property of zinc is commonly employed in identification tests for zinc (Hogan *et al.*, 2010). The European Pharmacopoeia (Ph. Eur.) (Ph. Eur., 2011) and BP (BP, 2011) have quantitative reactions for the identification of zinc in zinc acetate dihydrate and zinc gluconate APIs, which were adopted for the identification of zinc in the respective APIs for inclusion in *The Ph. Int.* monographs.

The specificity of these methods will be discussed in sections 4.3.2.3 and 4.3.3.3.

4.3.1 Materials and equipment

A list of all the materials used in the identification test for zinc acetate dihydrate API is tabulated in Table 4.4; and for zinc gluconate tabulated in Table 4.5. A Sartorius ED623S+ balance (IMP, South Africa) was used during these tests.

Table 4.4 Materials used in the identification test for zinc in zinc acetate dihydrate

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Sodium hydroxide	ME9M51063	Merck Chemicals (Pty) Ltd.	South Africa
Ammonium chloride	K36849224657	Merck KGaA	Germany
Sodium sulfide	A0308455	Across Organics	Belgium
Water R	N/A	RIIP®/CENQAM®	South Africa

N/A = Not Applicable

Table 4.5 Materials used in the identification test for zinc in zinc gluconate

Material	Batch number	Manufacturing company	Country of origin
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Potassium hexacyanoferrate (II) trihydrate	A0046684906	Merck KGaA	Germany
Hydrochloric acid	1034315	Merck Chemicals (Pty) Ltd.	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

4.3.2 Identification test for zinc in zinc acetate dihydrate API

4.3.2.1 Procedure

Dissolve 0.1 g zinc acetate dihydrate in 5 ml of water R and add 0.2 ml of sodium hydroxide (~400 g/l) TS. A white precipitate is formed. Add a further 2 ml of sodium hydroxide (~400 g/l) TS. The precipitate dissolves. Add 10 ml of ammonium chloride (~100 g/l) TS. The solution remains clear. Add 0.1 ml of sodium sulfide TS. A flocculent white precipitate is formed.

4.3.2.2 Results

Initially the zinc solution was a clear solution. When 0.2 ml of sodium hydroxide (~400 g/l) TS was added to the test solution, a white precipitate formed. A further 2 ml of sodium hydroxide (~400 g/l) TS was then added to the test solution and the precipitate dissolved (Figure 4.2).

Ten millilitres of ammonium chloride (~100 g/l) TS was then added to the test solution, and the solution remained clear. When 0.1 ml of sodium sulfide TS was added to the test solution a flocculent white precipitate formed (Figure 4.2).

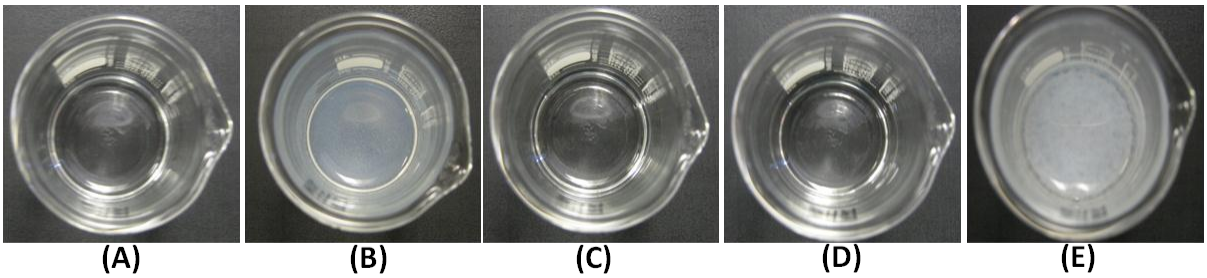


Figure 4.2 Photographs of the test solution during the various stages for the identification of zinc in zinc acetate dihydrate; (A) Initial clear test solution; (B) Precipitation with addition of 0.2 ml sodium hydroxide (~400 g/l) TS; (C) Precipitate dissolved upon addition of 2 ml sodium hydroxide (~400 g/l) TS; (D) Clear solution upon addition of ammonium chloride (~100 g/l TS); and (E) Flocculent white precipitate formed upon addition of sodium sulfide TS.

4.3.2.3 Discussion

Brown (2012) recommends the following basic hydroxide-ammonia precipitation reaction to identify zinc: Add to the zinc solution 0.4 ml of sodium hydroxide (~40 g/l) TS, a white precipitate should form. Drop-wise add ammonia (~260 g/l) TS to the solutions until the precipitate disappears.

The abovementioned method is not recommended due the lack of specificity thereof. All of the following metals namely, Be^{2+}, Pb^{2+}, Al^{3+}, Sn^{2+}, Sn^{4+} and Sb^{3+} form a white precipitate with sodium hydroxide that redissolves in excess sodium hydroxide (Pederson, 2006:89).

However, the method used by the Ph. Eur. / BP (and is also recommended for this test) could be considered specific: In the first step the 0.2 ml sodium hydroxide (~400 g/l) TS is added to the 5 ml test solution. A white gelatinous precipitate of zinc hydroxide is formed:

$$Zn^{2+}_{(aq)} + 2OH^-_{(aq)} \rightarrow Zn(OH)_2 \downarrow$$

Zinc hydroxide is amphoteric, and will readily dissolve with a further addition of 2 ml of sodium hydroxide (~400 g/l) TS due to the formation of the zinc hydro complex:

$$Zn(OH)_2\downarrow + 2OH^- \rightarrow [Zn(OH)_4]^{2-}$$

Pederson (2006:89) illustrated that up to this point in the experiment the test would give a positive reaction with Be^{2+}, Pb^{2+}, Al^{3+}, Sn^{2+}, Sn^{4+} and Sb^{3+}, since all these metals produce a white precipitate that redissolves in excess sodium hydroxide.

In the next step 10 ml of ammonium chloride (~100 g/l) TS is added to the test solution which has to remain clear due to the conversion of the zinc hydro complex into a soluble ammonia complex, the tetramminezincate(II) ion:

$$[Zn(OH)_4]^{2-} + 4NH_3 \rightarrow [Zn(NH_3)_4]^{2-} + 4OH^-$$

If the observation in the first two steps was caused by Be^{2+} or Al^{3+}, the addition of ammonium chloride would cause it to reprecipitate as hydroxides due to a lowering of the OH^- concentration (Pederson, 2006:89).

During the last step 0.1 ml of sodium sulfide TS is added to the solution which caused the formation of a white flocculent precipitate of zinc sulfide:

$$[Zn(NH_3)_4]^{2-} + S^{2-} \rightarrow ZnS \downarrow + 4NH_3$$

Pederson (2006:89) explained that a relatively high degree of selectivity is hereby achieved as the sulfide precipitates of Pb^{2+}, Sn^{2+}, Sn^{4+} and Sb^{3+} are black, brown, yellow and orange-red respectively. This gives a clearly negative result for Zn^{2+}.

Based on the abovementioned discussion and the fact that the Ph. Eur. / BP utilises this method, it could be considered adequately specific to identify zinc in zinc acetate dihydrate API, and no formal method validation is required.

4.3.2.4 Conclusion

The method proposed by Brown (2012) is not recommended for the identification of zinc in zinc acetate dihydrate API (or zinc gluconate API) due to the lack of specificity thereof.

The appearance and disappearance of the white precipitates during the various steps described in sections 4.3.2.2 and 4.3.2.3 is a positive and selective identification for zinc.

The precipitation test described in section 4.3.2.1 may thus be utilised for the identification of zinc in zinc acetate dihydrate API, and is therefore recommended for inclusion in *The Ph. Int.* monograph.

4.3.3 Identification test for zinc in zinc gluconate API

4.3.3.1 Procedure

Dissolve 0.1 g zinc gluconate in 5 ml of water R. Add 0.5 ml of potassium ferrocyanide (~53 g/l) TS. A white precipitate is formed that does not dissolve upon the addition of 5 ml of hydrochloric acid (~330 g/l) TS.

For the abovementioned method the following needs to be incorporated into the "*Reagents, test solutions and volumetric solutions*" section of *The Ph. Int.:*

Potassium ferrocyanide (~53 g/l) TS.

A solution of potassium ferrocyanide R containing about 53 g of $K_4Fe(CN)_6$ per litre.

4.3.3.2 Results

Initially the zinc solution was a clear solution. When 0.5 ml of potassium ferrocyanide (~53 g/l) TS was added to the test solution, a white precipitate formed. The white precipitate did not dissolve upon addition of 5 ml of hydrochloric acid (~330 g/l) TS (Figure 4.3).

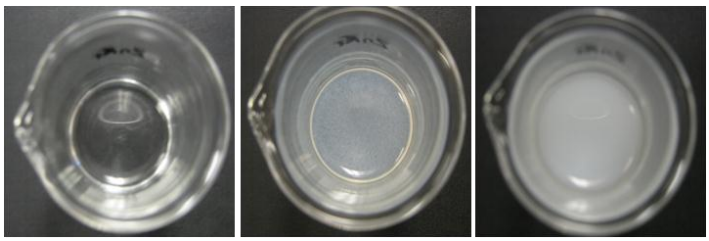


Figure 4.3 Photographs of the test solution during the various stages of the identification of zinc in zinc gluconate; (A) Initial clear test solution; (B) Precipitation with addition of 0.5 ml of potassium ferrocyanide (~53 g/l) TS; and (C) Precipitate remained upon addition of 5 ml of hydrochloric acid (~330 g/l) TS.

4.3.3.3 Discussion

The zinc ion (Zn^{2+}) reacted with the potassium ferrocyanide [$Fe(CN)_6$] to form zinc ferrocyanide which yielded the white precipitate illustrated in Figure 4.3. This reaction is described by the following chemical equation:

$$2Zn^{2+}\,(aq) + K_4[Fe(CN)_6] \rightarrow Zn_2[Fe(CN)_6] \downarrow + 4K^{+}\,(aq)$$

The ferrocyanides of zinc and copper are very insoluble in hydrochloric acid (Hibbard, 1934:424), yielding a white zinc ferrocyanide precipitate (and a brownish-red copper ferrocyanide). The presence of the white precipitate, after the addition of the hydrochloric acid, is thus a positive identification for zinc.

The sensitivity and specificity of the potassium ferrocyanide test for the qualitative identification of zinc is discussed by several authors (Bennett & McKee, 1928:475; Mehlig, 1927:722). Based on the aforementioned and the fact that the Ph. Eur. / BP utilises this method, it could be considered adequately specific to identify zinc in zinc gluconate API, and no formal method validation is required.

4.3.3.4 Conclusion

The appearance of a white precipitate with the addition of 0.5 ml of potassium ferrocyanide (~53 g/l) TS in the test solution, that does not dissolve upon the addition of 5 ml of hydrochloric acid (~330 g/l) TS, is a positive test for zinc.

The precipitation test described in section 4.3.3.1 may thus be utilised for the identification of zinc in zinc gluconate API, and is therefore recommended for inclusion in *The Ph. Int.* monographs.

4.4 General identification test for acetate in zinc acetate dihydrate API by means of a precipitation test

The Ph. Eur. / BP monograph for zinc acetate dihydrate API specifies an olfactory test (evolution of acetic acid vapours when heated with oxalic acid R) for the identification of acetate (BP, 2011; Ph. Eur., 2011). This method is not recommended. Although there is limited danger associated with some of the foulest

odours, in some situations the olfactory detection is not recommended due to safety and reliability concerns (Joyce *et al.,* 2010:21-22).

The United States Pharmacopoeia (USP) (USP, 2011) specifies a precipitation reaction that consists of two parts. Part I is a lanthanum nitrate test, which produces a blue precipitate. Part II is the basic ferric chloride test which produces a red precipitate. In a publication by Joyce *et al.* (2010:23) it was illustrated that the lanthanum nitrate test, as well as the basic ferric chloride test were sufficiently specific to identify acetate in zinc acetate dihydrate independently.

To avoid exposure to toxic (and expensive) lanthanum nitrate and iodine (used in the lanthanum nitrate test) it is recommended that only the basic ferric chloride test is to be included in the zinc acetate dihydrate API monograph for *The Ph. Int*.

Based on the abovementioned discussion the ferric chloride method could be considered adequately specific to identify acetate, and no formal method validation is required.

4.4.1 Materials and equipment

A list of all the materials used in the identification test for acetate in zinc acetate dihydrate API can be seen in Table 4.6. A Sartorius ED623S+ balance (IMP, South Africa) was used during this test.

Table 4.6 Materials used in identification test for acetate in zinc acetate dihydrate

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Ferric chloride hexahydrate	1032580	Merck Chemicals (Pty) Ltd.	South Africa
Hydrochloric acid	1034315	Merck Chemicals (Pty) Ltd.	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

4.4.2 Procedure

Dissolve 0.2 g zinc acetate dihydrate in 4 ml of water R and add 4 ml of ferric chloride (~65 g/l) TS. A red-brown colour is formed. Boil the solution; a red-brown

precipitate is produced. Add drop wise sufficient hydrochloric acid (~250 g/l) TS to dissolve the precipitate; a yellow colour appears.

4.4.3 Results

Initially the test solution (0.05 g/ml) was clear. A red-brown colour formed when 4 ml of the ferric chloride (~65 g/l) TS was added to 4 ml of acetate solution (0.05 g/ml). A red-brown precipitate formed when this solution was boiled. When hydrochloric acid (~250 g/l) TS was added to the solution the precipitate disappeared and the solution turned yellow. A faint acetic acid odour was also detected (Table 4.7).

Table 4.7 Photographs of the zinc acetate dihydrate solution during the various stages of the acetate identification test

Initial test solution (0.05 g/ml)	Test solution with the addition of ferric chloride (~65 g/l) TS	Red-brown precipitate formed when boiled	Red-brown precipitate dissolved with the addition of hydrochloric acid (~250 g/l) TS and the solution turned yellow
			

4.4.4 Discussion

The iron ion (Fe^{3+}) reacted with the acetate ion ($CH_3CO_2^-$) to form ferric acetate which yielded the red-brown colour illustrated in Table 4.7. The ferric acetate precipitated when crystallisation was induced through boiling. This reaction is described by the following chemical equation:

$$2FeCl_{3\,(aq)} + 3Zn(C_2H_3O_2)_{2\,(aq)} \rightarrow 3ZnCl_{2\,(aq)} + 2Fe(C_2H_3O_2)_3 \downarrow$$

Once hydrochloric acid (~330 g/l) TS was introduced to the ferric acetate, iron(III)chloride ($FeCl_3$) and acetic acid (CH_3COOH) formed, which was detected by the disappearance of the precipitate. The yellow colour which appeared is characteristic of iron(III)chloride (O'Neil *et al.,* 2006:687). During the reaction an

acetic acid odour was detected. The formation of the iron(III)chloride and the acetic acid is illustrated in the following chemical equation:

$$Fe(C_2H_3O_2)_{3\,(s)} + 3HCl \rightarrow FeCl_{3\,(aq)} + 3CH_3COOH_{\,(aq)}$$

4.4.5 Conclusion

The proposed basic ferric chloride test is suitable for the identification of acetate in zinc acetate dihydrate API and is therefore recommended for inclusion in *The Ph. Int.* monograph.

4.5 General identification test for gluconate in zinc gluconate by means of thin layer chromatography

The Ph. Eur. / BP and USP monograph for zinc gluconate specify a TLC method for the identification of gluconate (BP, 2011; Ph. Eur., 2011; USP, 2011). The suitability of this method was evaluated for possible adoption in *The Ph. Int*.

4.5.1 Introduction to thin layer chromatography

TLC is a chromatographic separation technique in which a mobile phase passes over a stationary phase that consists of a thin layer (sorbent) applied to a solid support (for example a glass plate, glass fibre, terephthalate films or aluminium foil) (Hahn-Deinstrop, 2007:15). An example of a typical chromatogram is illustrated in Figure 4.4.

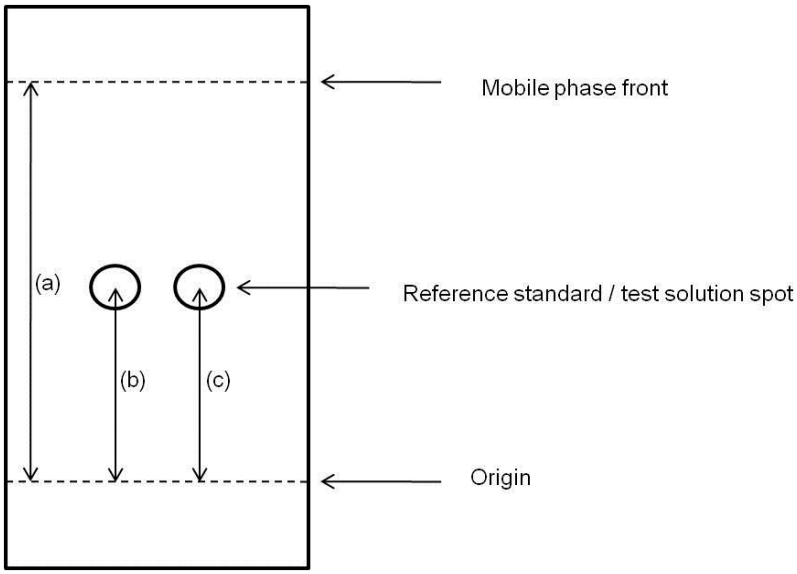


Figure 4.4 Basic components of a chromatogram to demonstrate the origin, reference standard / sample spot and the mobile phase front; a) distance from origin to mobile phase front, b) distance from origin to centre of reference standard solution spot, and c) distance from origin to centre of test solution spot.

The substance under investigation (test solution) is applied at the start line (origin) and, as the mobile phase progresses by capillary action, it allows the mixture of substances (if it is a mixture) to separate into its components (Figure 4.5).

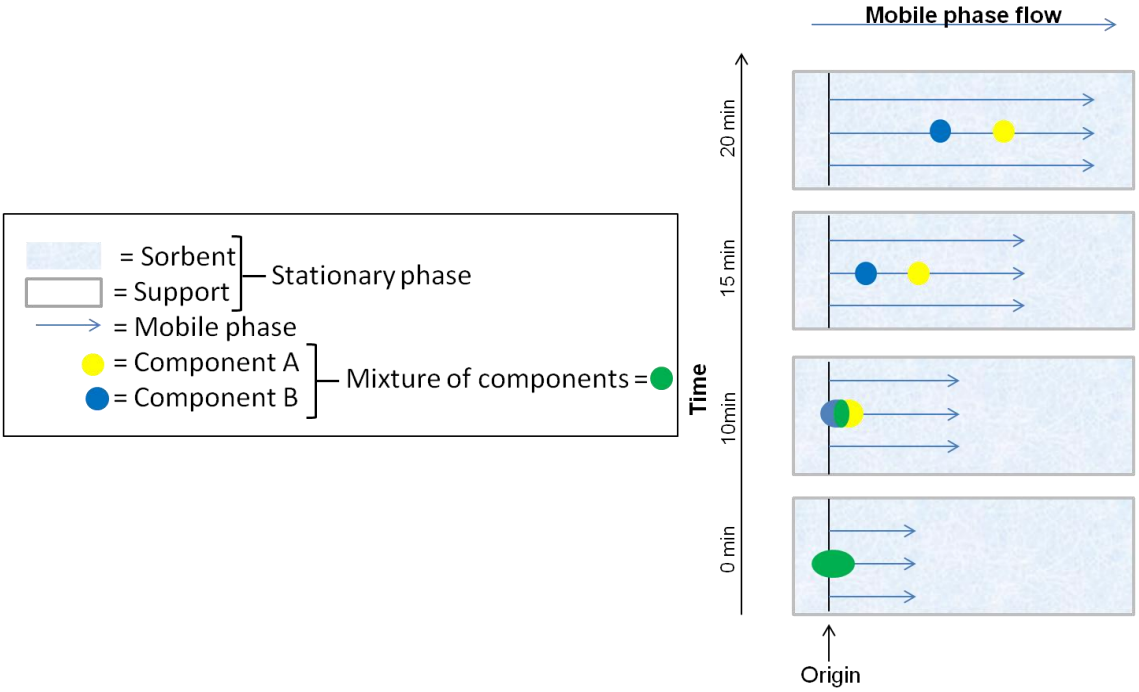


Figure 4.5 Schematic presentation of the separation of a mixture of components A and B by a moving mobile phase while being absorbed on the stationary phase.

As the mobile phase moves over the surface of the stationary phase it transports the analyte past sorbent particles of the stationary phase. The analyte molecules are only free to move with the mobile phase if they are not bound to the surface of the sorbent particles. The ability of an analyte to bind to the surface of the sorbent particles in the presence of mobile phase can be viewed as the sum of two competitive interactions: the mobile phase may compete for interaction with the stationary phase or compete for interaction with the analyte (Yves Rubin research group, 2011:2).

For example: the polar groups in the mobile phase may compete with the analyte for binding sites on the surface of the sorbent particles, for example silica gel. If a highly polar mobile phase is used, it might interact more strongly with the surface of the silica gel and reduce available sites on the stationary phase to bind with the analyte. The analyte will thus move quickly past the stationary phase (Yves Rubin research group, 2011:2).

Similarly, polar groups in the mobile phase can interact strongly with polar functionality in the analyte and prevent interaction of the analyte with the surface of the sorbent particles (i.e. silica gel) which leads to the rapid movement of the analyte past the stationary phase (Yves Rubin research group, 2011:2).

There are various sorbents available for the various applications, of which silica gel is probably the most commonly used (Hahn-Deinstrop, 2007:22). The empirical formula for silica is SiO_2; where the oxygen atoms on the surface of the silica gel particles are bound to protons (Scott, 1980:49). These hydroxyl groups render the surface of the gel particle highly polar (Figure 4.6). To achieve separation, the test solution must have a relatively equal affinity for the mobile phase and the packing material (silica gel).

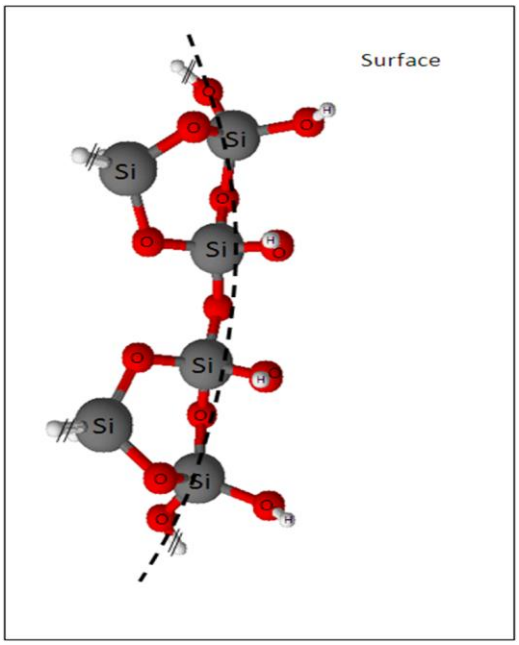


Figure 4.6 Schematic presentation of the silica gel particle surface illustrating the hydroxyl groups responsible for the polar property of silica gel plates.

Several different types of stationary phases are available, but the most common for TLC is alumina and silica. These are listed according to polarity in Figure 4.7.

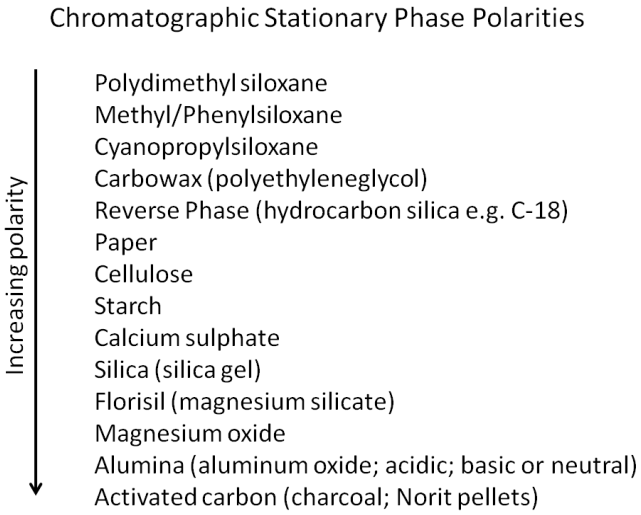


Figure 4.7 Common stationary phases listed by increasing polarity (Source:http://courses.chem.psu.edu/chem36/Experiments/PDF's_for_techniques/TLC.pdf).

As mentioned previously, the more polar compounds will adhere more strongly to the polar stationary phase, for example silica or alumina. The elution order for some functional groups from silica or alumina is listed in Figure 4.8.

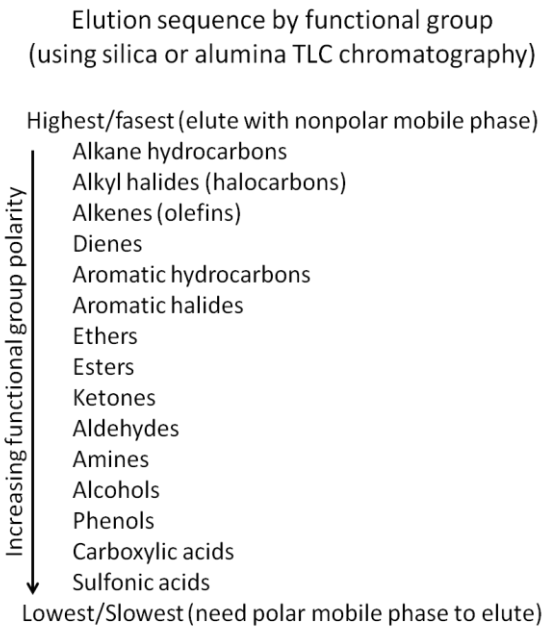


Figure 4.8 The elution order for some functional groups from silica or alumina (Source:http://courses.chem.psu.edu/chem36/Experiments/PDF's_for_techniques/TLC.pdf).

The mobile phase polarity can also be adjusted to change the chromatographic separation. Figure 4.9 lists some common mobile phases according to increasing polarity.

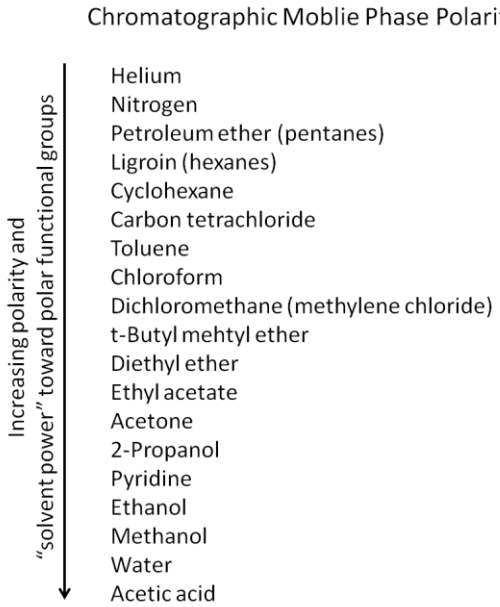


Figure 4.9 Common mobile phases according to increasing polarity (Source: http://courses.chem.psu.edu/chem36/Experiments/PDF's_for_techniques/TLC.pdf).

Some of the applications of TLC include (but not limited to):

- Determination of the number of components in a mixture;
- Verification of a substance's identity;
- Monitoring of the progress of a reaction (especially during synthesis of APIs);
- Determination of appropriate conditions for column chromatography, and
- Analysis of the fractions obtained from column chromatography (Hahn-Deinstrop, 2007:22).

4.5.2 Evaluation of the suitability of the Ph. Eur. / BP / USP thin layer chromatographic method for the identification of gluconate in zinc gluconate

The Ph. Eur. / BP and USP monograph for zinc gluconate API specify the following TLC method for the identification of gluconate:
"Test solution: Dissolve 20 mg of the substance to be examined in 1 ml of water R.

Reference solution: Dissolve 20 mg of calcium gluconate CRS in 1 ml of water R, heating if necessary in a water-bath at 60 °C.

Plate: TLC silica gel plate R (5 - 40 µm) [or TLC silica gel plate R (2 - 10 µm)].

Mobile phase: concentrated ammonia R, ethyl acetate R, water R, ethanol (96 %) R (10:10:30:50 volume (V)/V/V/V).

Application: 1 microlitre (µl).

Development: Over 3/4 of the plate.

Drying: At 100 - 105 °C for 20 minutes, then allow to cool to room temperature.

Detection: Spray with a solution containing 25 g/l of ammonium molybdate R and 10 g/l of cerium sulfate R in dilute sulphuric acid R, and heat at 100 - 105 °C for about 10 minutes.

Results: The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution" (BP, 2011; Ph. Eur., 2011; USP, 2011).

The abovementioned method was executed. Additional positive and negative control solutions (prepared in the same concentrations as the test solution) were prepared to evaluate the specificity of the method. Potassium gluconate was used as a positive control and zinc acetate dihydrate was used as a negative control. The materials used are tabulated in Table 4.8.

Table 4.8 Materials used in the gluconate identification test

Material	Batch number	Manufacturing company	Country of origin
Ammonia solution (25 %)	1035758	Merck Chemicals Pty. Ltd.	South Africa
Ethyl acetate	SAAR2235020LC	Merck Chemicals Pty. Ltd.	South Africa
Ethanol	1036688	Merck Chemicals Pty. Ltd.	South Africa
Sulphuric Acid	1035329	Merck Chemicals Pty. Ltd.	South Africa
Cerium (IV) sulfate	13590-82-4	Sigma-Aldrich Co.	USA
Ammonium molybdate	SAAR1123420EM	Merck Chemicals Pty. Ltd.	South Africa
Calcium gluconate anhydrous (USP)	292-28-5	Sigma-Aldrich Co.	Germany
Potassium gluconate	031 M0141 V	Sigma-Aldrich Co.	USA
Zinc sulfate heptahydrate	1034607	Merck Chemicals Pty. Ltd.	South Africa
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
DC-Fertigfolien ALUGRAM® SIL G (Pre-coated aluminium sheets, Silica gel 60, layer 0.20 mm)	904099	Macherey-Nagel GmbH & Co.KG	Germany
DC-Fertigplatten SIL G-25 (Pre-coated glass TLC-plates, SIL-G-25, layer 0.25 mm)	905128	Macherey-Nagel GmbH & Co.KG	Germany
Water R	N/A	RIIP®/CENQAM®	South Africa

Co. = Company; GmbH & Co. KG = German: Limited partnership with a limited liability company as general partner

When the method was executed, poor chromatography was obtained. The reference solution, test solution and positive control solution revealed severe streaking. Figure 4.10 shows a photograph of the chromatogram with the streaking indicated by the dashed ovals.

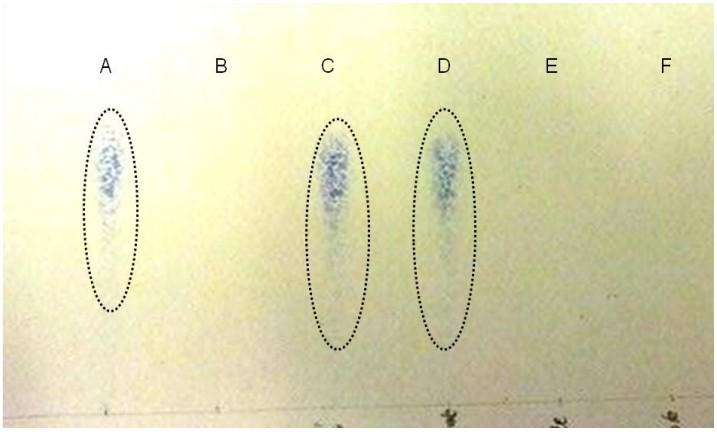


Figure 4.10 Photograph of the TLC chromatogram for identification of gluconate. A) Reference solution (calcium gluconate), B) Solvent (water R), C) Positive control solution (potassium gluconate 20 mg/ml), D) Test solution (zinc gluconate 20 mg/ml), E) Negative control solution 1 (zinc sulfate 20 mg/ml), F) Negative control solution 2 (zinc acetate dihydrate 20 mg/ml).

Possible reasons for the streaking included: (i) the sample being overloaded, and / or (ii) the high affinity of functional groups for silica gel, such as the carboxylic acid (-COOH) of gluconate.

To overcome possible sample overloading the concentration of the test solution and reference solutions was reduced from 20 mg/ml to 10 mg/ml. The streaking was slightly reduced, but unfortunately desired spots were still not obtained (Figure 4.11).

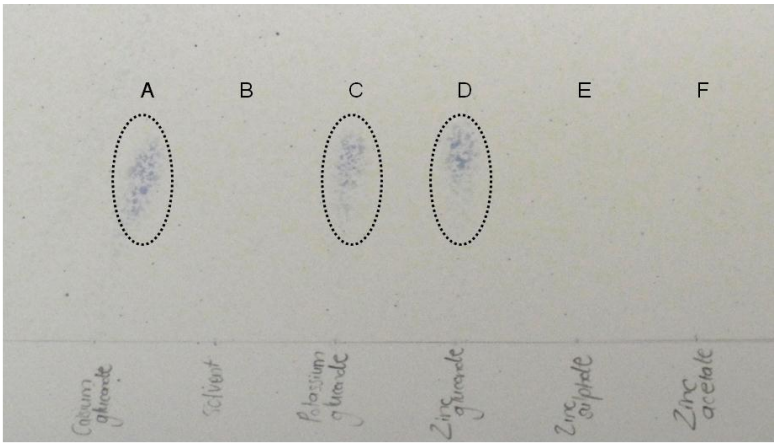


Figure 4.11 Photograph of the TLC chromatogram with 10 mg/ml concentrations: A) Reference solution (calcium gluconate 10 mg/ml), B) Solvent (water R), C) Positive control solution (potassium gluconate 10 mg/ml), D) Test solution (zinc gluconate 10 mg/ml), E) Negative control solution 1 (zinc sulfate 10 mg/ml), F) Negative control solution 2 (zinc acetate dihydrate 10 mg/ml).

In an attempt to reduce the high affinity of functional groups for silica gel two possibilities were considered. Firstly, the addition of ammonium hydroxide or acetic acid to the solvent to decrease the affinity for the silica gel. Secondly, changes to the mobile phase composition to increase the affinity for the mobile phase. The first possibility was not found viable due to the fact that the original method already contained ammonium hydroxide, and the stringent smell of ammonium hydroxide and acetic acid was to be avoided.

To evaluate the effect of changes in the polarity of the mobile phase on the chromatography, the following approach was followed: three new mobile phases were prepared using combinations of the solvents presented in Figure 4.12 to obtain 3 mobile phases with different polarities. They were:

Mobile phase A: Ethyl acetate R, ethanol (96 %) R (50:50 V/V).

Mobile phase B: Water R, ethanol (96 %) R (50:50 V/V).

Mobile phase C: Ethyl acetate R, water R, ethanol (96 %) R (50:25:25 V/V/V).

The TLC method of the Ph. Eur. / BP was executed once again, but the abovementioned three mobile phases were used and only the zinc gluconate test solution was applied to the chromatogram. The chromatograms obtained are illustrated in Figure 4.12.

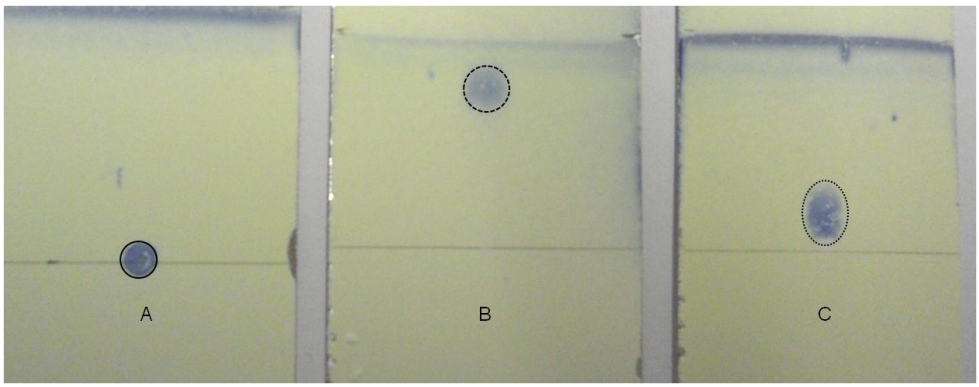


Figure 4.12 Photograph of the three TLC chromatograms studied for the influence of different composite mobile phases: A) ethyl acetate R, ethanol (96 %) R (50:50 V/V), B) water R, ethanol (96 %) R (50:50 V/V), and C) ethyl acetate R, water R, ethanol (96 %) R (50:25:25 V/V/V).

The order of the relative polarity (or “solvent power”) of the different mobile phases is: Mobile phase B > Mobile phase C > Mobile phase A.

Based on the chromatography obtained in Figures 4.10 and 4.12, the following mobile phase was proposed: Ethyl acetate R, water R, ethanol (96 %) R (10:50:40 V/V/V).

The Ph. Eur. / BP TLC method was adjusted as follows and executed:

Prepare the mobile phase by mixing 10 volumes of ethyl acetate R, 50 volumes of water R and 40 volumes of ethanol (~750 g/l) TS.

Prepare the following solutions:

A) Water R as solvent.
B) Reference solution 1 by dissolving about 20 mg calcium gluconate R in 1 ml of water R.
C) Reference solution 2 by dissolving about 10 mg calcium gluconate R in 1 ml of water R.
D) Positive control solution 1 by dissolving about 20 mg potassium gluconate R in 1 ml of water R.
E) Positive control solution 2 by dissolving about 10 mg potassium gluconate R in 1 ml of water R.
F) Test solution 1 by dissolving about 20 mg of zinc gluconate in 1 ml of water R.
G) Test solution 2 by dissolving about 10 mg of zinc gluconate in 1 ml of water R.
H) Negative control solution 1 by dissolving about 20 mg zinc acetate dihydrate R in 1 ml of water R.
I) Negative control solution 2 by dissolving about 20 mg zinc sulfate R in 1 ml of water R.

Prepare the spray solution by transferring 2.5 g of ammonium molybdate and 1.0 g cerium sulfate into a 100 ml volumetric flask, dilute and make up to volume with sulphuric acid (~100 g/l) TS.

Saturate the developing chamber with the mobile phase for at least an hour, using suitable filter paper to line the chamber. Prepare a TLC plate, with silica gel R5 as the coating substance, by lightly drawing an application line with a pencil at least 1.5 centimetres (cm) from the bottom of the plate. Also make small spots with the pencil for the application areas for the test solutions and the reference solutions

prepared. Take care to make the first spot at least 2.0 cm from the side of the plate, with at least 1.5 cm between the spots.

Apply separately to the plate 1 µl of solutions (A) – (I). Allow the spots to dry in a current of cool air. Place the dried TLC plate in the saturated chamber to develop. Allow the mobile phase to run at least three-quarters of the plate's height (usually 10 - 15 cm). The distance of the mobile phase should be marked, as soon as the plate is removed from the chamber. After removing the plate from the chromatographic chamber, dry the plate for 20 minutes at 105 °C. Spray the plate with the spray solution (Ammonium molybdate / cerium sulfate / sulfuric acid TS) and heat the plate at 105 °C for 10 minutes. Examine the chromatogram in daylight.

For the abovementioned method the following needs to be incorporated into the "*Reagents, test solutions and volumetric solutions*" section of *The Ph. Int.:*

Ammonium molybdate / cerium sulfate / sulfuric acid TS.
Procedure. Dissolve 2,5 g ammonium molybdate R and 1,0 g cerium sulfate R in sulfuric acid (~100 g/l) to produce 100 ml.

Calcium gluconate R.
$C_{12}H_{22}CaO_{14},H_2O$.
A commercially available reagent of suitable grade.

Cerium sulfate R.
CeO_8S_2.
A commercially available reagent of suitable grade.

The positions of the spots were determined by calculating the retardation factor (R_f). The R_f values were calculated with the following equation (Hahn-Deinstrop, 2007:4):

$$Rf = \frac{\text{Distance from origin to centre of spot}}{\text{Distance from origin to mobile phase front}}$$

4.5.3. Results

A photograph of the chromatogram with the newly proposed identification method can be seen in Figure 4.13, with the calculated R_f values tabulated in Table 4.9.

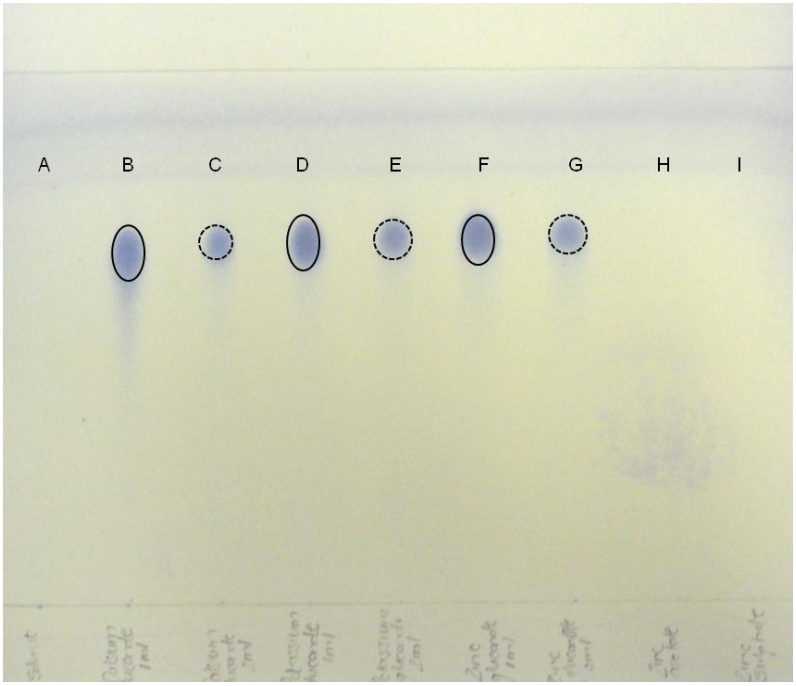


Figure 4.13 Photograph of the TLC chromatogram obtained with the newly proposed identification method: A) Solvent (water R), B) Reference solution (calcium gluconate 20 mg/ml), C) Reference solution (calcium gluconate 10 mg/ml), D) Positive control solution (potassium gluconate 20 mg/ml), E) Positive control solution (potassium gluconate 10 mg/ml), F) Test solution (zinc gluconate 20 mg/ml), G) Test solution (zinc gluconate 10 mg/ml), H) Negative control solution 1 (zinc acetate dihydrate 20 mg/ml), I) Negative control solution 2 (zinc sulfate 20 mg/ml).

Table 4.9 Calculated R_f values for the TLC chromatogram obtained with the new proposed mobile phase (see Figure 4.13)

Solutions	Position	Concentration (mg/ml)	Distance (mm)	R_f
Mobile phase	Front*	N/A	119	N/A
Solvent	**A**	0	No spot obtained	N/A
Reference	**B**	20	78	0.7
Reference	**C**	10	79	0.7
Positive control	**D**	20	80	0.7
Positive control	**E**	10	81	0.7
Test	**F**	20	82	0.7
Test	**G**	10	81	0.7
Negative control	**H**	20	No spot obtained	N/A
Negative control	**I**	20	No spot obtained	N/A

* Mobile phase front = distance from origin to mobile phase front

4.5.4 Discussion of the method development experiment results

The spots of the 20 mg/ml reference solution, test solution and positive control solution revealed a degree of streaking, whereas the 10 mg/ml solutions produced round, well-distinguished spots (Figure 4.13). No spots were obtained with the solvent and the negative control solutions.

The test solution spots complied with the pharmacopoeial criteria: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution. The calculated R_f values were similar (Table 4.9), which confirmed the visual results.

Based on the results obtained the following changes (Table 4.10) are proposed to the Ph. Eur., BP and USP identification method. The validation of the recommended changes is discussed in section 4.5.5.

Table 4.10 Pharmacopoeial methods versus newly proposed method for identification of gluconate by means of TLC

Property	Ph. Eur. / BP/ USP method	Proposed method
Plate	TLC silica gel plate R	TLC silica gel plate R
Mobile phase composition	Concentrated ammonia R, ethyl acetate R, water R, ethanol (96 %) R (10:10:30:50 V/V/V/V).	Ethyl acetate R, water R, ethanol (96 %) R (10:50:40 V/V/V).
Concentration of solutions	20 mg/ml	10 mg/ml
Application volume	1 µl	1 µl
Detection	Spray with a solution containing 25 g/l of ammonium molybdate R and 10 g/l of cerium sulfate R in dilute sulphuric acid R, and heat at 100 - 105 °C for about 10 minutes.	Spray with a solution containing 25 g/l of ammonium molybdate R and 10 g/l of cerium sulfate R in dilute sulphuric acid R, and heat at 100 - 105 °C for about 10 minutes.
Results	The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.	The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

4.5.5 Validation report of the gluconate identification test

A method validation was performed to verify the validity and suitability of the amended method. The validation parameter (according to Table 3.4 – Chapter 3) which should be considered for an identification test is specificity. However, for this validation study intermediate precision and robustness were also evaluated.

4.5.5.1 Specificity

By using positive and negative control solutions, the discriminating power of an identification test can be demonstrated (ICH, 2005:7). A positive control solution is defined as a solution that contains the substance (in this case gluconate) that needs to be identified, for example potassium gluconate R. The *positive control substance* does not necessarily have to contain the same cation (Zn^{2+}) as the substance under investigation. A *negative control solution* is defined as a solution that does not contain the substance to be identified (in this case gluconate). A *reference solution* contains the substance which is to be identified (i.e. calcium gluconate R). A *test solution* refers to a solution which is prepared from the substance under investigation (i.e. zinc gluconate).

4.5.5.1.1 Procedure

Prepare the mobile phase by mixing 10 volumes of ethyl acetate R, 50 volumes of water R and 40 volumes of ethanol (~750 g/l) TS.

Prepare the following solutions:

A) Water R as solvent.
B) Reference solution 1 by dissolving about 20 mg of calcium gluconate R in 1 ml of water R.
C) Reference solution 2 by dissolving about 10 mg of calcium gluconate R in 1 ml of water R.
D) Positive control solution 1 by dissolving about 20 mg potassium gluconate R in 1 ml of water R.
E) Positive control solution 2 by dissolving about 10 mg potassium gluconate R in 1 ml of water R.
F) Test solution 1 by dissolving about 20 mg zinc gluconate in 1 ml of water R.

G) Test solution 2 by dissolving about 10 mg zinc gluconate in 1 ml of water R.
H) Negative control solution 1 by dissolving about 20 mg zinc acetate dihydrate R in 1 ml of water R.
I) Negative control solution 2 by dissolving about 20 mg zinc sulfate R in 1 ml of water R.

Prepare the spray solution by transferring 2.5 g of ammonium molybdate and 1.0 g cerium sulfate into a 100 ml volumetric flask, dilute and make up to volume with sulphuric acid (~100 g/l) TS.

Saturate the developing chamber with the mobile phase for at least an hour, using suitable filter paper to line the chamber. Prepare a TLC plate, with silica gel R5 as the coating substance, by lightly drawing an application line with a pencil at least 1.5 cm from the bottom of the plate. Also make small spots with the pencil for the application areas for the test solutions and the reference solutions prepared. Take care to make the first spot at least 2.0 cm from the side of the plate, with at least 1.5 cm between the spots.

Apply separately to the plate 1 µl of solutions (A) – (I). Allow the spots to dry in a current of cool air. Place the dried TLC plate in the saturated chamber to develop. Allow the mobile phase to run at least three-quarters of the plate's height (usually 10 - 15 cm). The distance of the mobile phase should be marked, as soon as the plate is removed from the chamber. After removing the plate from the chromatographic chamber, dry the plate for 20 minutes at 105 °C. Spray the plate with the spray solution (ammonium molybdate (25 g/l) TS / cerium sulfate (10 g/l) / sulfuric acid (~100 g/l) TS) and heat the plate at 105 °C for 10 minutes. Examine the chromatogram in daylight.

Determine the R_f values (as described in section 4.5.2) for all spots detected on the chromatogram.

4.5.5.1.2 Acceptance criteria

To illustrate the specificity of this method only the spots obtained from the 10 mg/ml concentration solutions will be taken into consideration.

i. The principal spots obtained with solutions C, E & G correspond in position, appearance, and intensity.
ii. No spots should be obtained with solutions A, H & I.

4.5.5.1.3 Results and discussion

A photograph of the chromatogram obtained using the method for specificity can be seen in Figure 4.14. The R_f values of the spots obtained are tabulated in Table 4.11.

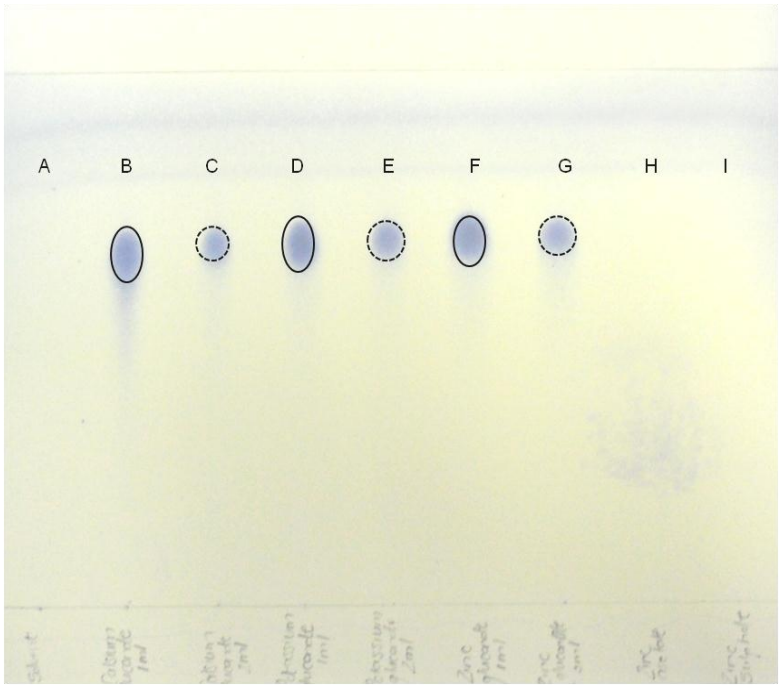


Figure 4.14 Photograph of the TLC chromatogram obtained with the newly proposed identification method: A) Solvent (water R), B) Reference solution (calcium gluconate 20 mg/ml), C) Reference solution (calcium gluconate 10 mg/ml), D) Positive control solution (potassium gluconate 20 mg/ml), E) Positive control solution (potassium gluconate 10 mg/ml), F) Test solution (zinc gluconate 20 mg/ml), G) Test solution (zinc gluconate 10 mg/ml), H) Negative control solution 1 (zinc acetate dihydrate 20 mg/ml), I) Negative control solution 2 (zinc sulfate 20 mg/ml).

The principal spots obtained with solution C, E & G corresponded in position (similar R_f values), appearance, and intensity. No spots were obtained with solutions A, H & I. It can thus be concluded that this method is specific for the identification of gluconate.

Table 4.11 Calculated R_f values of the spots obtained in the TLC chromatogram obtained with the newly proposed gluconate identification method (see Figure 4.14)

Solutions:	Position	Concentration (mg/ml)	Distance (mm)	R_f
Mobile phase	Front*	N/A	119	N/A
Solvent	**A**	0	No spot obtained	N/A
Reference	**B**	20	78	0.7
Reference	**C**	10	79	0.7
Positive control	**D**	20	80	0.7
Positive control	**E**	10	81	0.7
Test solution	**F**	20	82	0.7
Test solution	**G**	10	81	0.7
Negative control	**H**	20	No spot obtained	N/A
Negative control	**I**	20	No spot obtained	N/A

* Mobile phase front = distance from origin to mobile phase front

4.5.5.2 Intermediate precision

Intermediate precision expresses the closeness of agreement of results in the presence of within-laboratories variations (ICH, 2005:5). Intermediate precision was illustrated using three different analysts who prepared the reference solutions and the test solutions in two different concentrations (i.e. 10 mg/ml and 7 mg/ml).

4.5.5.2.1 Procedure

Prepare the mobile phase by mixing 10 volumes of ethyl acetate R, 50 volumes of water R and 40 volumes of ethanol (~750 g/l) TS.

Prepare the following solutions:

A) Reference solution 1 by dissolving about 10 mg of calcium gluconate R in 1 ml of water R.
B) Reference solution 2 by dissolving about 7 mg of calcium gluconate R in 1 ml of water R.
C) Test solution 1 by dissolving about 10 mg zinc gluconate in 1 ml of water R.
D) Test solution 2 by dissolving about 7 mg zinc gluconate in 1 ml of water R.

Prepare the spray solution by transferring 2.5 g of ammonium molybdate and 1.0 g cerium sulfate into a 100 ml volumetric flask, dilute and make up to volume with sulphuric acid (~100 g/l) TS.

Saturate the developing chamber with the mobile phase for at least an hour, using suitable filter paper to line the chamber. Prepare a TLC plate, with silica gel R5 as the coating substance, by lightly drawing an application line with a pencil at least 1.5 cm from the bottom of the plate. Also make small spots with the pencil for the application areas for the test solutions and the reference solutions prepared. Take care to make the first spot at least 2.0 cm from the side of the plate, with at least 1.5 cm between the spots.

Apply separately to the plate 1 µl of solutions (A) – (D). Allow the spots to dry in a current of cool air. Place the dried TLC plate in the saturated chamber to develop. Allow the mobile phase to run at least three-quarters of the plate's height (usually 10 - 15 cm). The distance of the mobile phase should be marked, as soon as the plate is removed from the chamber. After removing the plate from the chromatographic chamber, dry the plate for 20 minutes at 105 °C. Spray the plate with the spray solution (ammonium molybdate (25 g/l) TS / cerium sulfate (10 g/l) / sulfuric acid (~100 g/l) TS) and heat the plate at 105 °C for 10 minutes. Examine the chromatogram in daylight.

Determine the R_f and R_r values of all the spots obtained on the chromatograms. Seeing that R_f values can be greatly affected by experimental conditions in practice, the R_r value (relative retardation factor) is usually used by pharmacopoeias (*Ph. Int*., 2011). The R_r value is the ratio of the distance moved by the test solution and a reference standard solution (also known as R_{St}). The R_r values were calculated using the following equation (*Ph. Int*., 2011):

$$R_r = \frac{Distance\ from\ origin\ to\ centre\ of\ sample\ spot}{Distance\ from\ origin\ to\ centre\ of\ reference\ standard\ spot}$$

Perform a one-way analysis of variance (ANOVA) on the R_f and R_r values calculated, using a 95 % confidence interval. The one-way ANOVA is interpreted by comparing the F test statistic, where the F value is the ratio of the variance between and the variance within items, and the F_{crit} (critical F value) value is the largest value of F that is statistically significant (Bolton, 1997:272-273).

4.5.5.2.2 Acceptance criteria

i. The principal spot obtained with solution C corresponds in position, appearance, and intensity with that obtained with solution A.
ii. The principal spot obtained with solution D corresponds in position, appearance, and intensity with that obtained with solution B.
iii. The R_f values of the spots obtained from solutions A - D obtained by the three analysts should be comparable ($F < F_{crit}$).
iv. The R_r values of the spots obtained from solutions A - D obtained by the three analysts should be comparable ($F < F_{crit}$).

4.5.5.2.3 Results and discussion

A photograph of the chromatograms obtained using the method for intermediate precision is displayed in Figure 4.15. The calculated R_f values and one-way ANOVA results are tabulated in Table 4.12. The principal spot obtained with solution C corresponded in position, appearance, and intensity with that obtained with solution A by all three analysts. The principal spot obtained with solution D corresponded to that obtained with solution B by all three analysts.

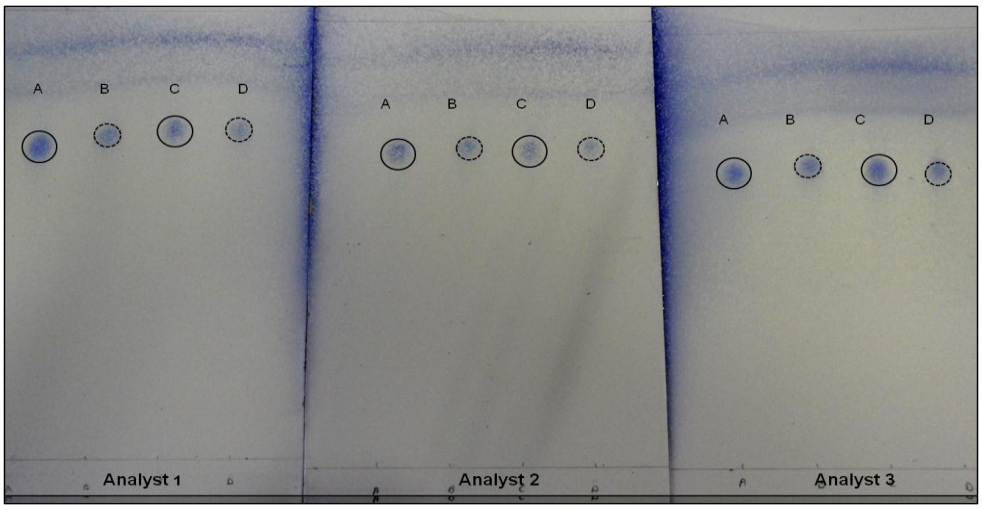


Figure 4.15 Photograph of the TLC chromatograms obtained by the three analysts using the method for intermediate precision. Description of the spots: A) Reference solution (calcium gluconate 10 mg/ml), B) Reference solution (calcium gluconate 7 mg/ml), C) Test solution (zinc gluconate 10 mg/ml), D) Test solution (zinc gluconate 7 mg/ml).

Table 4.12 Calculated R_f values of the spots obtained in the TLC chromatograms of the three analysts using the method for intermediate precision (see Figure 4.15), and a summary of the one-way ANOVA results obtained

Analyst	Solution/Spot	Distance (mm)	Mobile phase front* (mm)	R_f value
1	A	100	151	0.7
	B	103		0.7
	C	105		0.7
	D	104		0.7
2	A	101	150	0.7
	B	102		0.7
	C	100		0.7
	D	102		0.7
3	A	93	145	0.6
	B	100		0.7
	C	99		0.7
	D	99		0.7

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Analyst 1	4	2.73	0.683	0.00029167
Analyst 2	4	2.70	0.675	3.3333E-05
Analyst 3	4	2.69	0.673	0.00049167

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000216667	2	0.000108	0.39795918	0.682948703	4.256495
Within Groups	0.00245	9	0.000272			
Total	0.002666667	11				

* Mobile phase front = distance from origin to mobile phase front

The one-way ANOVA was used to compare the R_f values of the spots obtained from the four different solutions by the three analysts, where the null hypothesis states that there is no significant difference between the mean of the R_f values: H_0: $\mu_A = \mu_B = \mu_C$ (Bolton, 1997:273). The alternative hypothesis state that there is difference between any two of the mean R_f values: H_a: $\mu_a \neq \mu_b \neq \mu_c$. If the calculated F value is equal to or greater than F_{crit} at the specified confidence interval ($\alpha = 0.05$), the null hypotheses can be rejected (Table 4.13) (Bolton, 1997:273).

Table 4.13 Interpretation of the one-way ANOVA results with respect to F and F_{crit} (Bolton, 1997:273)

Test statistic results	Conclusion
$F < F_{crit}$	Fail to reject the null hypothesis. Accept H_0: $\mu A = \mu B = \mu C$ Thus there is no significant difference between the mean R_f values.
$F \geq F_{crit}$	Reject the null hypothesis. Accept H_a: $\mu_a \neq \mu_b \neq \mu_c$ Thus there is a significant difference between the mean R_f values.

The F value (0.4) calculated was smaller than F_{crit} (4.3), indicating that there is no significant difference between the mean R_f values of the spots obtained from solutions A - D by the three analysts.

The R_r values of the spots obtained from solutions A - D and a summary of the one-way ANOVA results by the three analysts are tabulated in Table 4.14. The F value (0.7) calculated was smaller than (9.6), indicating that there is no significant difference between the mean R_r values of the spots obtained from solutions A - D by the three analysts.

Table 4.14 Calculated R_r values of the spots obtained from solutions A - D on the TLC chromatograms by the three analysts using the method for intermediate precision (see Figure 4.15) and a summary of the one-way ANOVA results obtained

Analyst	Spots	Distance (mm)	R_r value
1	A	100	1.1
	C	105	
	B	103	1.0
	D	104	
2	A	101	1.0
	C	100	
	B	102	1.0
	D	102	
3	A	93	1.1
	C	99	
	B	100	1.0
	D	99	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Analyst 1	2	2.06	1.03	0.0008
Analyst 2	2	1.99	0.995	5E-05
Analyst 3	2	2.05	1.025	0.00245

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00143	2	0.00072	0.6515152	0.5821304	9.5520945
Within Groups	0.00330	3	0.00110			
Total	0.00473	5				

It can thus be concluded that this method complies with the required intermediate precision requirements.

4.5.5.3 Robustness

To investigate the robustness of the proposed TLC method minor changes / adjustments were made to the following parameters:

i) Concentration of the reference/test solutions: - the influence of a reduction in the concentration of the reference- and test solutions on the chromatography was investigated. The spots obtained from the 10 mg/ml reference- and test

solutions were compared to those obtained from the 7 mg/ml reference- and test solutions.

ii) Different TLC plate supports: - the specified solutions were applied to (i) a silica gel aluminium support plate; and (ii) silica gel glass support plate. The spots obtained from the aluminium support plate were compared to those obtained from the glass support plate.

The influence of changes to the composition of the mobile phase was not investigated due to the fact that the sensitivity of the method towards such changes was already discussed in section 4.5.2. The following procedure was executed by three individual analysts.

4.5.5.3.1 Procedure

Prepare the mobile phase by mixing 10 volumes of ethyl acetate R, 50 volumes of water R and 40 volumes of ethanol (~750 g/l) TS.

Prepare the following solutions:

A) Reference solution 1 by dissolving about 10 mg of calcium gluconate R in 1 ml of water R.
B) Reference solution 2 by dissolving about 7 mg of calcium gluconate R in 1 ml of water R.
C) Test solution 1 by dissolving about 10 mg zinc gluconate in 1 ml of water R.
D) Test solution 2 by dissolving about 7 mg zinc gluconate in 1 ml of water R.

Prepare the spray solution by transferring 2.5 g of ammonium molybdate and 1.0 g cerium sulfate into a 100 ml volumetric flask, dilute and make up to volume with sulphuric acid (~100 g/l) TS.

Saturate the developing chamber with the mobile phase for at least an hour, using suitable filter paper to line the chamber. Prepare two TLC plates, aluminium support and glass support plates, with silica gel R5 as the coating substance. Lightly draw an application line with a pencil at least 1.5 cm from the bottom of the plate. Also make small spots with the pencil for the application areas for the test solutions and reference solutions prepared. Take care to make the first spot at least 2.0 cm from the side of the plate, with at least 1.5 cm between the spots.

Apply separately to the two prepared plates 1 µl of solutions (A) - (D). Allow the spots to dry in a current of cool air. Place the dried TLC plate in the saturated chamber to develop. Allow the mobile phase to run at least three-quarters of the plate's height (usually 10 - 15 cm). The distance of the mobile phase should be marked, as soon as the plate is removed from the chamber. After removing the plate from the chromatographic chamber, dry the plate for 20 minutes at 105 °C. Spray the plate with the spray solution (ammonium molybdate (25 g/l) TS / cerium sulfate (10 g/l) / sulfuric acid (~100 g/l) TS) and heat the plate at 105 °C for 10 minutes. Examine the chromatogram in daylight.

Determine the R_f values (as described in section 4.5.2) of the spots obtained on the chromatograms.

Calculate the R_r value (as described in section 4.5.5.2) of spots A and C, and the R_r value of spots B and D.

Perform a one-way analysis of variance (ANOVA) on the R_r values of the 7 mg/ml versus the 10 mg/ml concentrations, using a 95 % confidence interval.

Also, perform a one-way analysis of variance (ANOVA) on the R_f values of all the spots obtained from the glass plate support versus all the spots obtained from the aluminium plate support (using a 95 % confidence interval).

4.5.5.3.2. Acceptance criteria

The TLC method to identify gluconate in zinc gluconate should remain unaffected by small, deliberate changes; where:

i. The principal spot obtained with solution C corresponds in position, appearance, and intensity with that obtained with solution A.
ii. The principal spot obtained with solution D corresponds in position, appearance, and intensity with that obtained with solution B.
iii. The R_r values of the spots obtained from solutions A and C should be comparable to the R_r values of the spots obtained from solutions B and D ($F < F_{crit}$).
iv. The R_f values of spots A - D obtained on the glass plate support and the aluminium plate support should be comparable ($F < F_{crit}$).

4.5.5.3.3 Results and discussion

The chromatograms obtained when the method was executed on the aluminium support plates can be seen in Figure 4.15; with the calculated R_f values of the spots obtained tabulated in Table 4.12.

The execution of the method on the glass support plates can be seen in Figure 4.16; with the calculated R_f values of the spots obtained tabulated in Table 4.15.

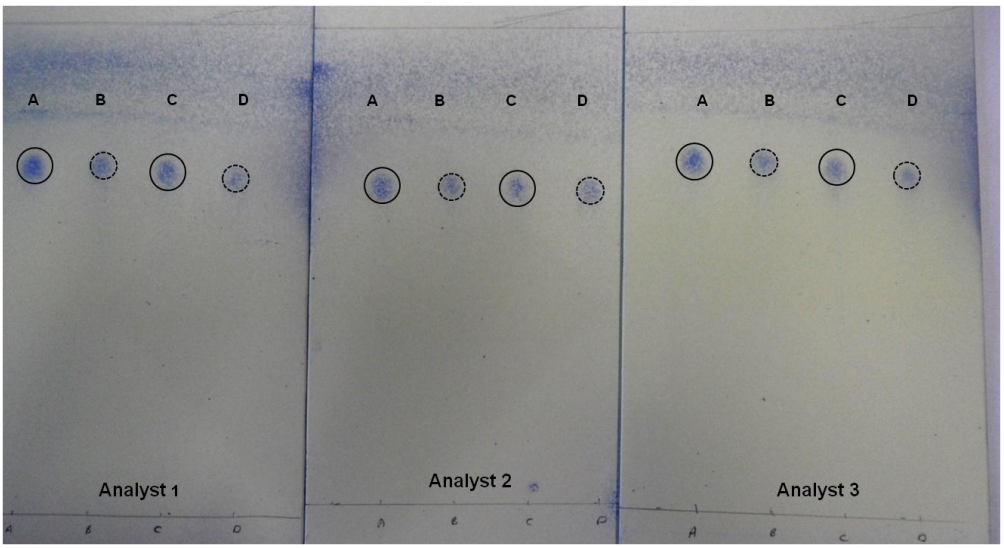


Figure 4.16 Photograph of the spots obtained on glass support TLC plates using the method for robustness: A) Reference solution (calcium gluconate 10 mg/ml), B) Reference solution (calcium gluconate 7 mg/ml), C) Test solution (zinc gluconate 10 mg/ml), D) Test solution (zinc gluconate 7 mg/ml).

Table 4.15 Calculated R_f values of the spots obtained on glass supported TLC chromatograms using the procedure for robustness (see Figure 4.16)

Analyst	Spot	Distance (mm)	Mobile phase front* (mm)	R_f value
1	A	95	137	0.7
	B	97		0.7
	C	95		0.7
	D	93		0.7
2	A	88	134	0.7
	B	88		0.7
	C	87		0.7
	D	87		0.7
3	A	96	132	0.7
	B	97		0.7
	C	95		0.7
	D	94		0.7

* Mobile phase front = distance from origin to mobile phase front

The effect of a lower solution concentration on the chromatography (for the aluminium and glass support plates) can be seen in Figures 4.15 and 4.16. The principal spot obtained with solution C corresponded in position, appearance, and intensity with that obtained with solution A. The principal spot obtained with solution D corresponded in position, appearance, and intensity with that obtained with solution B.

The comparison of the R_r values of the spots obtained with the two different concentrations (on both the glass and aluminium support plates) is summarised in Table 4.16. The results of the one-way ANOVA on the R_r values of spots A & C and B & D are summarised in Figure 4.17.

Table 4.16 Comparison of R_r values of the spots A & C and B & D obtained with the two different concentrations (10 mg/ml and 7 mg/ml)

Analyst	Plate support	R_r (Spots A&C)	R_r (Spots B&D)
1	Glass	1.0	1.0
	Aluminium	1.1	1.0
2	Glass	1.0	1.0
	Aluminium	1.0	1.0
3	Glass	1.0	1.0
	Aluminium	1.1	1.0

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Rr A&C	6	6.2	1.0333333	0.002666667
Rr B&D	6	6	1.0000000	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.003333333	1	0.0033333	2.5	0.144928	4.964603
Within Groups	0.013333333	10	0.0013333			
Total	0.016666667	11				

Figure 4.17 Results of the one-way ANOVA for R_r values of the spots A & C and B & D obtained with the two different concentrations (10 mg/ml and 7 mg/ml).

The F value (2.5) calculated is smaller than F_{crit} (5.0), which indicated that there was no significant difference between the R_r values of the spots obtained from the two different concentrations. This indicated that the results obtained from the proposed method remained unaffected when small changes are made to the concentration of the reference solution and the test solution.

The comparison of the R_f values of the spots obtained for both plate supports is summarised in Table 4.17. The influence of the different plate supports used was investigated by means of a one-way ANOVA on the R_f values of the spots obtained on the two different plates. These results can be seen in Figure 4.18.

Table 4.17 Comparison of R_f values of the spots obtained from solutions A - D on the glass and aluminium TLC plates by each analyst

Analyst	Solutions	R_f for Aluminium plate	R_f for Glass plate
1	A	0.7	0.7
	B	0.7	0.7
	C	0.7	0.7
	D	0.7	0.7
2	A	0.7	0.7
	B	0.7	0.7
	C	0.7	0.7
	D	0.7	0.7
3	A	0.6	0.7
	B	0.7	0.7
	C	0.7	0.7
	D	0.7	0.7

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Aluminum plate	12	8.12	0.6767	0.000242424
Glass plate	12	8.28	0.6900	0.000909091

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001066667	1	0.001066667	1.852631579	0.187258	4.300949
Within Groups	0.012666667	22	0.000575758			
Total	0.013733333	23				

Figure 4.18 Results of the one-way ANOVA results for the R_f values of the spots obtained on the two different TLC plates (glass and aluminium).

The F value (1.9) calculated is smaller than F_{crit} (4.3), which indicated that there was no significant difference between the R_f values of the spots obtained on the aluminium and glass TLC plates. This indicated that the results obtained from the proposed method remained unaffected when glass or aluminium was used as TLC plate support.

4.5.6 Conclusion

An amended TLC method was developed for the identification of gluconate in zinc gluconate API. The proposed method was validated with regards to the following criteria: specificity, intermediate precision and robustness. The acceptance criteria for each validation parameter were met, therefore the method can be considered to be successfully validated.

4.6 Chapter conclusion

The identification of zinc in zinc acetate dihydrate was successfully achieved using the identification test for zinc described in the Ph. Eur. / BP monographs, by means of a precipitation reaction of zinc hydroxide and zinc sulfide.

The identification of zinc in zinc gluconate was successfully achieved using the identification test for zinc described in the Ph. Eur. / BP monographs, by means of a precipitation reaction of zinc ferrocyanide (that is insoluble in hydrochloric acid).

The flame test could be used to identify zinc in zinc acetate dihydrate, but not for zinc gluconate, and will for this reason not be recommended as a general test for identification of zinc in the mentioned zinc salts.

Acetate was readily identified using the prescribed precipitation test method in section 4.4 (basic ferric chloride test); and the identification of gluconate by means of TLC can be successfully executed using the newly proposed method in section 4.5.

The following three chapters will focus on the general tests, as given in Figure 3.2 (Chapter 3), namely colour and clarity of solutions, pH and moisture determination.

CHAPTER 5

GENERAL TESTS: CLARITY AND COLOUR

5.1 Introduction

There are a number of miscellaneous characteristics included in official compendia to ascertain the purity, authenticity and identification of an API. Clarity and colour is included under these. When an API is dissolved at a known concentration (in a specified solvent) it gives rise to a clear solution that may be either clear or possess a definite colouration which may be characteristic to the specific API (Kar, 2005:14).

The aim of this chapter was to establish a clarity and colour method and a clarity and colour specification for zinc acetate dihydrate and zinc gluconate API solutions to be included in *The Ph. Int.* monographs.

The tests for clarity and colour of liquids are carried out by comparing test solutions with reference colour solutions specified in the monograph. The composition of the reference solutions is selected based on the hue and intensity of the colour of the test solutions corresponding to the limits permitted in the specifications (*Ph. Int*., 2011). The specifications for clarity and colour of zinc salt solutions should be clear and colourless due to the high solubility of zinc salts in water (Table 2.3) (BP, 2011).

According to *The Ph. Int.* (*Ph. Int*., 2011) a solution is considered colourless if it is not more intensely coloured than any of the standard colour solutions Bn0, Yw0, Gn0, or Rd0 and clear if its opalescence is not more pronounced than that of opalescence standard TS2. These reference solutions are prepared by preparing a stock colour standard solution using cobalt colour TS, copper colour TS, dichromate colour TS and iron colour TS. All four of the colour test solutions need to be standardised by means of titration with either sodium thiosulfate or disodium edetate. These, in turn, need to be standardised as well; the whole process is both time consuming and costly. The prevalence of preparation errors by an analyst should also not be excluded.

The Ph. Eur. states that a liquid is considered clear if its clarity is the same as that of purified water, or the solvent used, when examined visually using identical testtubes

of colourless, transparent, neutral glass with a flat base and internal diameter of 15 - 25 mm, with the depth of 40 mm. Alternatively, when purified water or solvent is not used, it is considered clear if its opalescence is not more pronounced than that of reference suspension I. The same is true for colour tests: a solution is colourless if it has the appearance of purified water or the solvent or is not more intensely coloured than reference solution B9 (Ph. Eur., 2011).

The benefits of using purified water as reference solution include: i) cost-effectiveness, ii) requires no additional sample preparation (thus more time-effective and excludes potential analyst errors). *The Ph. Int.* and the Ph. Eur. are in the process of harmonisation. Based on the mentioned reasons the Ph. Eur. / BP methods were used for the clarity and colour testing of zinc salt solutions.

5.2 The clarity and colour properties of zinc acetate dihydrate and zinc gluconate solutions

According to the Ph. Eur. / BP a 0.1 g/ml zinc acetate dihydrate solution and a 0.02 g/ml zinc gluconate solution should be used for the clarity and colour testing (Ph. Eur., 2011).

5.2.1 Materials and equipment

Information of materials used in the clarity and colour study of zinc acetate dihydrate and zinc gluconate solutions can be seen in Table 5.1. A Sartorius ED623S+ balance (IMP, SA) was used during this test.

Table 5.1 Information of materials used in the clarity and colour study of zinc salt solutions

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Water R	N/A	RIIP®/CENQAM®	South Africa

5.2.2 Procedure

Prepare the following zinc salt solutions:

A) Dissolve about 10 g of zinc acetate dihydrate in a 100 ml of water R (100 mg/ml).
B) Dissolve about 1 g of zinc gluconate in a 50 ml of water R (20 mg/ml).

Carry out the test in flat-bottomed tubes of transparent glass that are matched as closely as possible in internal diameter and in all other respects (tubes of about 16 millimetres {mm} internal diameter are suitable). Use 10 ml of the prepared test solution (zinc salt solution) and 10 ml of the standard solution (water R) for both the clarity and colour tests. The depth of liquid should be about 50 mm (Figure 5.1). View the test and reference solutions down the vertical axis of the tubes in diffused light against a specified background (*Ph. Int*., 2011). For colour testing a white background is used and for clarity testing a black background is specified (Figure 5.1).

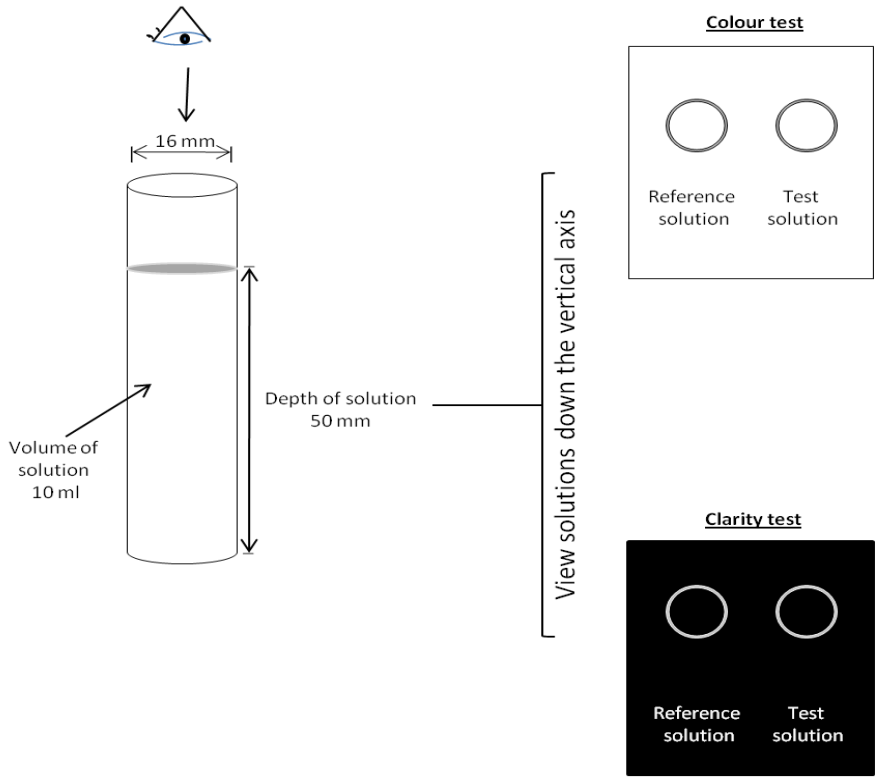
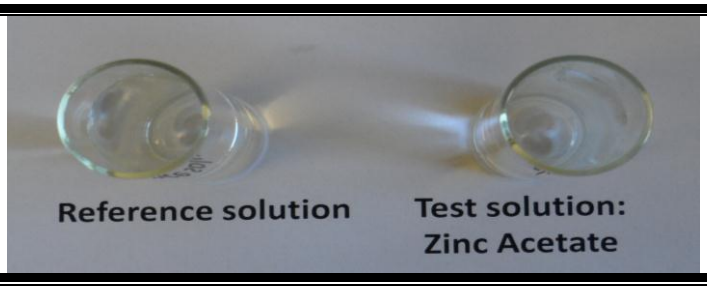
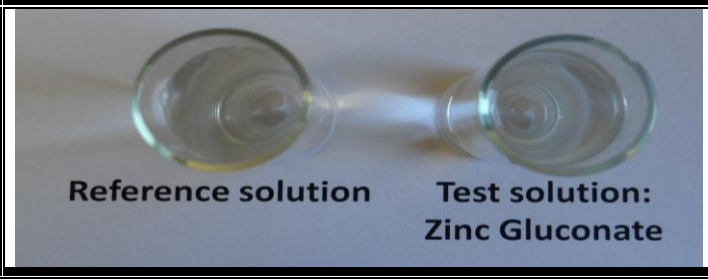


Figure 5.1 Schematic presentation of the experimental setup for clarity and colour test according to *The Ph. Int. (Ph. Int*., 2011).

5.2.3 Results

When the test solutions and colour reference solution were viewed down the vertical axis of the tubes in diffused light against a white background, they were observed to be visually similar (Table 5.2).

Table 5.2 Photographs of the test solutions and colour reference solution during the colour test as viewed down from the vertical axis

Zinc acetate dihydrate	
Zinc gluconate	

When the test solutions and clarity reference solution were viewed down the vertical axis of the tubes in diffused light against a black background they were observed to be visually similar (Table 5.3).

Table 5.3 Photographs of the zinc salt solutions and clarity reference solution during the clarity test as viewed down from the vertical axis

Zinc acetate dihydrate	
Zinc gluconate	

5.2.4 Discussion

The zinc acetate dihydrate solution and zinc gluconate solution were compared to water R, which served as both the clarity - and colour reference solutions. The zinc acetate dihydrate and the zinc gluconate solutions were observed to be visually identical to water R.

5.3 Conclusion

Water R proved to be a cost- and time-effective reference solution for clarity and colour determination of zinc acetate dihydrate and zinc gluconate salts, eliminating time consuming solution preparations.

The zinc acetate dihydrate and zinc gluconate solutions at the specified concentrations were found to be clear and colourless, complying with the set specification of *The Ph. Int*. The clarity and colour test will therefore be recommended to be included into *The Ph. Int.* monographs.

CHAPTER 6

GENERAL TESTS: pH

6.1 Introduction

The acidity or alkalinity of an aqueous solution is defined by the pH value of the solution. This characteristic can be used to distinguish between different solutions or between the salt forms of the same active moiety (*Ph. Int.*, 2011).

The determination of the pH value may be carried out by measuring the potential difference between electrodes immersed in standard and test solutions. The standard solutions used are assigned a definite pH value by convention *(Ph. Int*., 2011). The pH is calculated by means of the following equation:

$$pH\ (X) = pH\ (S) + \frac{E - Es}{2.3026\ RT/F}$$

Where: R denotes the gas constant, T the thermodynamic temperature (in K), and F the Faraday constant. Thus defined, the quantity pH is a dimensionless number.

A glass electrode has wide applicability in pH measurements due to its immediate response to rapid changes of hydrogen ion concentrations, even in poorly buffered solutions. Since the mechanism of this electrode involves no electron exchange it is the only electrode sensitive to hydrogen ions that is not disturbed by oxidising or reducing agents *(*Kotz & Treichel, 1999:817). As pH values are dependent on temperature, the measurements should be carried out at selected constant temperatures *(Ph. Int*., 2011).

The aim of this chapter was to establish a pH method and specification for zinc acetate dihydrate and zinc gluconate API solutions to be included in *The Ph. Int.* monographs.

The USP and the Ph. Eur. recommend a 0.05 g/ml zinc acetate dihydrate solution for determining the pH value, with the following pH specifications: USP = 6.0 - 8.0 and Ph. Eur. = 5.8 - 7.0 (Ph. Eur., 2011; USP, 2011). Figure 6.1 displays the pH value specifications for the Ph. Eur. and the USP.

A pH specification of 6.0 - 7.0 would ensure compliance to both the Ph. Eur. and USP monographs. Based on the fact that *The Ph. Int*. and the Ph. Eur. / BP are harmonising, a pH specification (for the 0.05 g/ml zinc acetate dihydrate solution) of 5.8 - 7.0 is recommended.

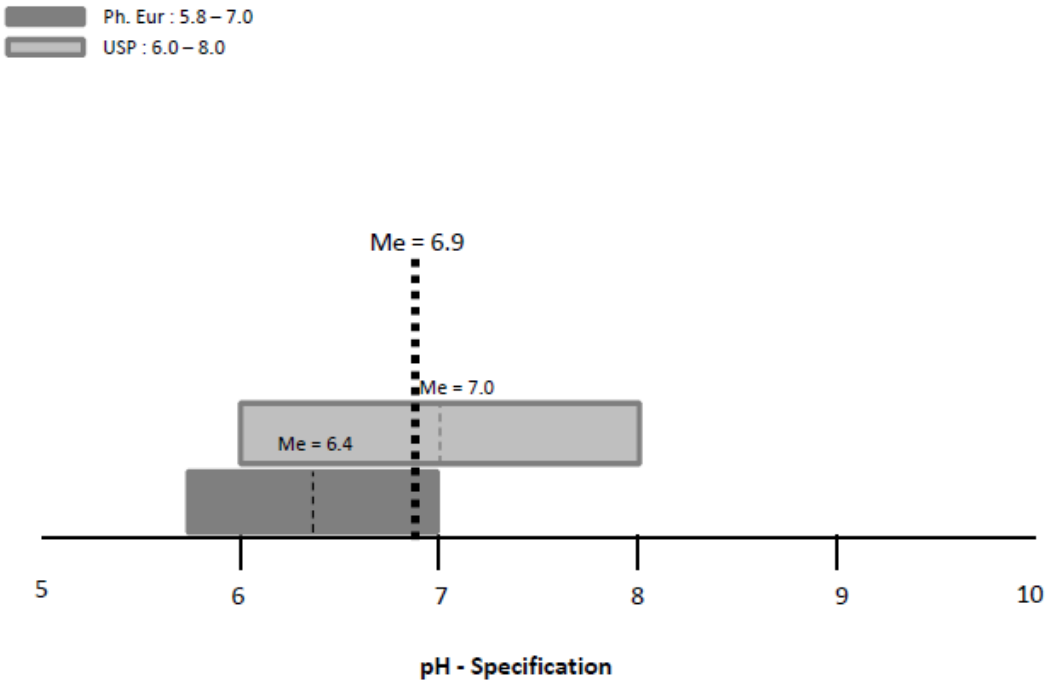


Figure 6.1 Specifications for pH value for zinc acetate dihydrate according to the Ph. Eur. and USP pharmacopoeias (Me = median) (Ph. Eur., 2011; USP, 2011).

No pH specification for zinc gluconate exists in the Ph. Eur. / BP, therefore the USP specification (5.5 – 7.5) using a 0.01 g/ml zinc gluconate solution is recommended for adoption (USP, 2011).

6.2 Determination of pH of zinc acetate dihydrate and zinc gluconate solutions

To illustrate the method's fitness for purpose (i.e. evaluation of repeatability and a degree of robustness of the proposed methods) three different analysts prepared the zinc solutions according to the method described above and determined the pH (in triplicate) on day 0 and day 1 (after 24 hours) using both pH meters specified in Table 6.2. A schematic presentation of the matrix used illustrating the analytical conditions for the pH values determined is depicted in Figure 6.2.

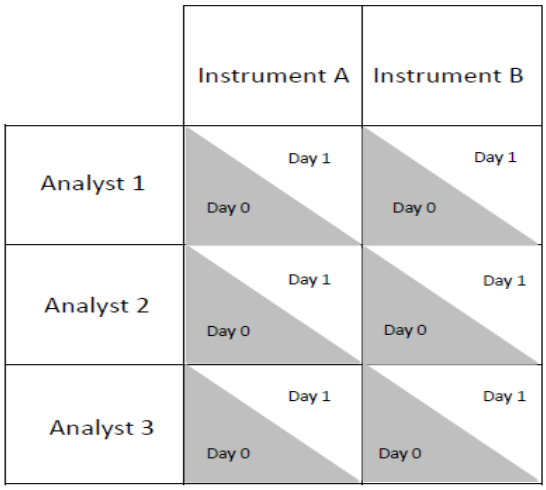


Figure 6.2 Schematic presentation of the matrix used illustrating the analytical conditions for pH values calculated of zinc acetate dihydrate and zinc gluconate solutions.

6.2.1 Materials and equipment

All the materials used in the pH determination of zinc acetate dihydrate and zinc gluconate solutions are tabulated in Table 6.1. A list of the equipment used is tabulated in Table 6.2. Both pH meters were calibrated before use with reference buffer solutions pH 2.0, 4.0 and 7.0 at room temperature.

Table 6.1 Information of materials used in the pH determination of zinc salt solutions

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Buffer solution pH 2.0	220210	Merck	South Africa
Buffer solution pH 4.0	1036987	Merck	South Africa
Buffer solution pH 7.0	1036301	Merck	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

Table 6.2 Information of equipment used in the pH determination of zinc salt solutions

Equipment	Supplier	Country of origin
Sartorius ED623S+ balance	IMP	South Africa
Mettler Toledo Seven Easy pH meter	Microsep	South Africa
Mettler Toledo Seven Multi pH meter	Microsep	South Africa

Mettler Toledo Seven Easy™ was used as *Instrument A* and is equipped with a PolyEther-Imde (PEI) gel HI 1230 electrode (HANNA Instruments, South Africa); and Mettler Toledo Seven Multi™ was used as *Instrument B* and is equipped with a glass 5014 electrode (CRISON Instruments, South Africa).

6.2.2 Procedure

Prepare the following zinc salt solutions in triplicate:

a) Dissolve about 1 g of zinc acetate dihydrate in 10 ml carbon-dioxide-free water R, and further dilute 5 ml of stock solution in 10 ml carbon-dioxide-free water R (final concentration 0.05 g/ml);
b) Dissolve about 1 g of zinc gluconate in 100 ml carbon-dioxide-free water R (final concentration 0.01 g/ml).

Determine the pH of the zinc salt solutions potentiometrically, using a calibrated pH meter, whilst the samples are being stirred by means of a magnetic stirrer. The average pH reading of the three solutions can be considered to be the determined pH value.

6.2.3 Results and discussion

Table 6.3 and Table 6.4 provide a summary of the pH values for the zinc acetate dihydrate and zinc gluconate solutions respectively.

Table 6.3 The average pH values (n=3) and standard deviation (indicated in brackets) for zinc acetate dihydrate solutions

	pH (% RSD)			
	Day 0		**Day 1**	
	Instrument A	**Instrument B**	**Instrument A**	**Instrument B**
Analyst 1	6.4 (0.01)	6.4 (0.01)	6.4 (0.00)	6.4 (0.00)
Analyst 2	6.4 (0.01)	6.4 (0.00)	6.4 (0.00)	6.4 (0.00)
Analyst 3	6.4 (0.00)	6.4 (0.00)	6.4 (0.00)	6.4 (0.01)

The zinc acetate dihydrate pH values (Table 6.3) complied with the recommended specification (5.8 – 7.0) and had an average value of 6.4; which is the same as the median value (me = 6.4) of the Ph. Eur. specification (Figure 6.1).

Table 6.4 The average pH values (n=3) and standard deviation (indicated in brackets) for zinc gluconate solutions

	pH (% RSD)			
	Day 0		Day 1	
	Instrument A	Instrument B	Instrument A	Instrument B
Analyst 1	6.2 (0.01)	6.3 (0.01)	6.3 (0.01)	6.3 (0.01)
Analyst 2	6.3 (0.01)	6.2 (0.01)	6.2 (0.00)	6.2 (0.00)
Analyst 3	6.2 (0.00)	6.2 (0.00)	6.2 (0.01)	6.2 (0.00)

The zinc gluconate pH values (Table 6.4) complied with the recommended specification (5.5 - 7.5) and had an average value of 6.2; which is slightly lower than the median value (me = 6.5) of the USP specification.

An one-way ANOVA (95 % confidence interval) was performed on the data to detect any statistically significant differences in the pH values reported when the analysis was performed by: (i) different analysts, on (ii) different instruments and to detect (iii) potential changes in the pH of the solution when stored for 24 hours at ambient conditions. The p-values obtained were interpreted with the use of Table 6.5.

Table 6.5 Interpretation of the one-way ANOVA results with respect to p-values

Test statistic results	Conclusion
$p \geq 0.05$	Fail to reject the null hypothesis - Accept H_0: $\mu A = \mu B = \mu C$ Thus there is no significant difference between the pH values.
$p \leq 0.05$	Reject the null hypothesis - Accept H_a: $\mu_a \neq \mu_b \neq \mu_c$ Thus there is a significant difference between the pH values.

Based on the data presented in Table 6.3 it can be concluded that there was no difference in the pH values reported for the zinc acetate dihydrate solutions when the analyses were performed by different analysts on different instruments and no changes were detected in the pH of the solutions when stored for 24 hours at ambient conditions.

The data presented in Table 6.4 for zinc gluconate showed diminutive variations. To evaluate the significance of these variations the one-way ANOVA was performed and the p-values obtained are depicted in Figure 6.3.

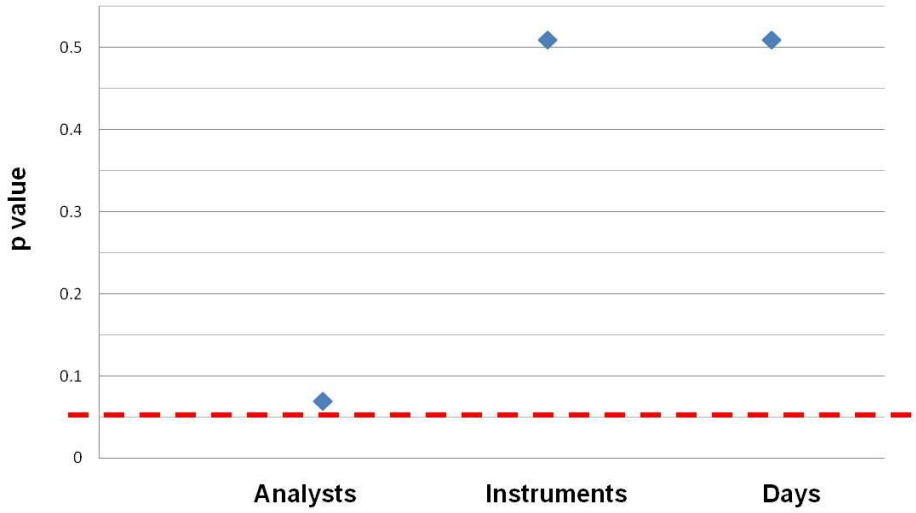


Figure 6.3 Graph of the p-values which illustrates the significance in the variance of the zinc gluconate pH values when the analyses were performed by different analysts, on different instruments over two days.

The p-value obtained when the pH values of the three different analysts were compared was greater than 0.05, which indicated that the results did not differ significantly when the proposed method was executed by different analysts.

ANOVA of the pH values obtained when two different pH meters were used in the analyses revealed that the pH values did not differ significantly ($p > 0.05$).

The pH values of the zinc gluconate samples did not reveal any significant change in pH when stored for 24 hours at ambient condition ($p > 0.05$).

The ability of the proposed method to generate reproducible results was illustrated by the low standard deviations (≤ 0.01) for the average pH values obtained.

6.3 Conclusion

It can thus be concluded that the proposed method for determination of pH of the zinc salt solutions generates reproducible results and shows a suitable degree of robustness, and could be considered suitable for its purpose. It is therefore recommended that this method is to be included in zinc acetate dihydrate and zinc gluconate monographs for *The Ph. Int.*

CHAPTER 7

GENERAL TESTS: MOISTURE DETERMINATION

7.1 Introduction

The quality of pharmaceutical products may be influenced by the water content thereof. Water determination is important in cases where the API is known to be hygroscopic or hydrolysed by water (ICH, 1999:10; WHO, 2007:41). Moisture determination is also necessitated where the assay specification is expressed with reference to the dried substance (BP, 2011; Ph. Eur., 2011; *Ph. Int*., 2011).

Two general procedures are described in pharmacopoeial methods for moisture determination, namely Karl Fischer titrations and loss on drying tests (BP, 2011; Ph. Eur., 2011; *Ph. Int*., 2011). Loss on drying may be considered adequate for moisture determination; however Karl Fischer titration is specific for water and is preferred by ICH guidelines (ICH, 1999:10).

Karl Fischer titrations are based on the quantitative reaction of water with Karl Fischer reagent. The Karl Fischer reagent consists of a mixture of iodine, sulphur dioxide and pyridine in the presence of methanol. The following reactions describe the reaction of water and the Karl Fischer reagent (Kar, 2005:225; Skoog *et al.*, 1997:381):

$$C_5H_5N{\cdot}I_2 + C_5H_5N{\cdot}SO_2 + C_5H_5N + H_2O \rightarrow 2\ C_5H_5N{\cdot}HI + C_5H_5N{\cdot}SO_3$$

$$C_5H_5{\cdot}SO_3 + CH_3OH \rightarrow C_5H_5N(H)SO_4CH_3$$

The end-point detection is made electrometrically and the water content is measured by determining the quantity of electricity required for the production of iodine (Kar, 2005:225).

The loss on drying of a sample is calculated based on the weight loss that the sample exhibited when it was dried to constant mass at specified conditions (*Ph. Int*., 2011).

Zinc acetate dihydrate is chemically stable. The dihydrate crystal lattice is stable at ambient conditions and contains a known amount of moisture (ANON, 2004:3). The

assay specification is expressed with reference to the dihydrate in the Ph. Eur. / BP and USP (BP, 2011; Ph. Eur., 2011; USP, 2011). It can thus be concluded that moisture determination is not required for inclusion in the zinc acetate dihydrate API monograph for *The Ph. Int*.

Zinc gluconate is a known hygroscopic API (Ph. Eur., 2011:3255), thus the assay specification is expressed with reference to the dried substance in the available monographs (BP, 2011; Ph. Eur., 2011; USP, 2011).

The aim of this chapter was to establish a moisture determination method and a moisture content specification for zinc gluconate which is to be included in *The Ph. Int.* monograph for zinc gluconate API.

7.2 Specification for the moisture content of zinc gluconate

The specifications for the moisture content of zinc gluconate API according the Ph. Eur. / BP, and USP are depicted in Figure 7.1

Based on the information provided, a specification for the moisture content of NMT 12 % m/m (120 mg/g) is proposed for zinc gluconate API in *The Ph. Int.*

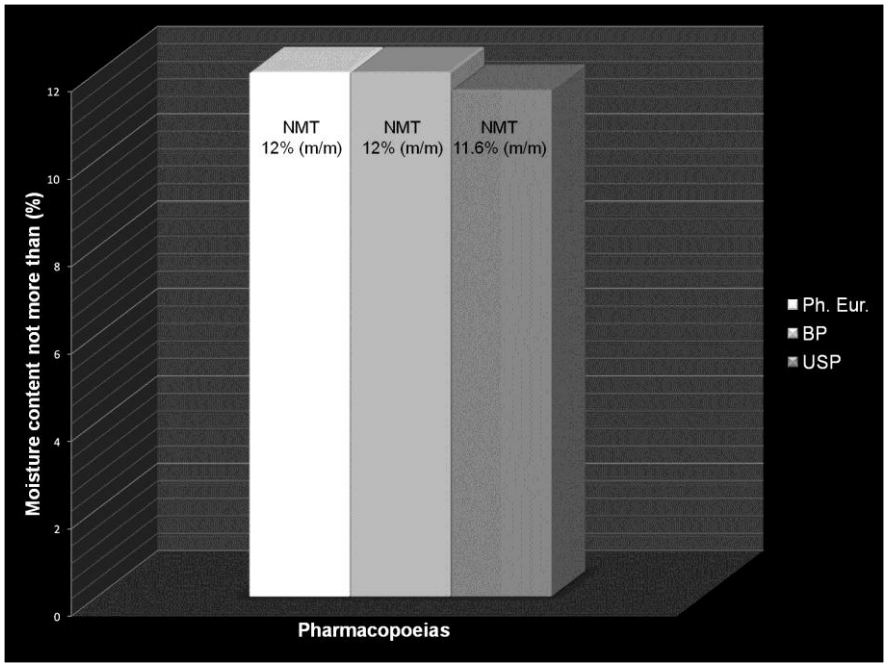


Figure 7.1 Specifications for the moisture content of zinc gluconate API according to the Ph. Eur. / BP and USP (BP, 2011; Ph. Eur., 2011; USP, 2011).

7.3 The determination of the moisture content of zinc gluconate API

As mentioned in the introduction of this chapter, two general procedures are described in the pharmacopoeias for the moisture determination of APIs and final products, namely Karl Fischer titration and loss on drying. The suitability of both these methods was investigated.

7.3.1 Materials and equipment

7.3.1.1 Loss on drying

A 1 g sample was used for the loss on drying analysis, and was based on the general method for drying a sample to constant mass as described in *The Ph. Int.* (*Ph. Int*., 2011). Two different techniques were used for the loss on drying determination, firstly an automated method using a RADWAG MAX 50 moisture analyzer; and secondly, a manual method using a Binder oven and an analytical balance.

The RADWAG MAX 50 moisture analyzer consists of an analytical balance with an internal heating system. The mass of the sample is thus continuously recorded for the duration of the study.

A list of materials used during the loss on drying analysis of zinc gluconate is tabulated in Table 7.1, and Table 7.2 lists the equipment used.

Table 7.1 Material used during the loss on drying analysis of zinc gluconate API

Material	Batch number	Manufacturing company	Country of origin
Zinc gluconate	K38073079 025	Merck KGaA	Germany

Table 7.2 Equipment used during the loss on drying analysis of zinc gluconate API

Equipment	Supplier	Country of origin
Sartorius R200D+ balance	Labotec	South Africa
Sartorius ED623S+ balance	IMP	South Africa
RADWAG MAX 50 moisture analyzer	Lasec	South Africa
Binder oven	Apollo Scientific	South Africa

The equipment setup and parameters for the automated and manual loss on drying analysis are summarised in Table 7.3.

Table 7.3 Information on the equipment setup and parameters used to determine the moisture content of zinc gluconate API

Equipment conditions	RADWAG MAX 50 moisture analyzer	Binder oven
Sample mass	1 g	1 g
Drying temperature	105 °C	105 °C
Time	3 hrs	Up to 8 hrs
Result reporting interval	Every 5 minutes	Hourly

7.3.1.2 Karl Fischer water analysis

The water content of the zinc gluconate samples was determined with a Metrohm 787 KF Titrino autotitrator (Metrohm, Switzerland). The instrument was calibrated using 20 - 30 µl water R. Thereafter the accuracy of the instrument was verified using Hydranal® Water Standard 10.0 (Sigma Aldrich, Germany).

A list of the materials and equipment used for the Karl Fischer analysis is summarised in Table 7.4 and Table 7.5 respectively. Hydranal® Composite 5 was selected for the analysis (where 1 ml of the reagent reacts with 5 mg of water) due to it being best suited for moisture analysis in samples with a moisture content above 2 % (Sigma-Aldrich, 2006:48).

Table 7.4 Materials used for the Karl Fischer water analysis of zinc gluconate API

Material	Batch number	Manufacturing company	Country of origin
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Hydranal® Water Standard 10.0	SZBA2230 SZE92100	Sigma-Aldrich	Germany
Hydranal® Composite 5	8156B	Sigma-Aldrich	Germany
UnivAR® Anhydrous Methanol	SA15F61031	Merck	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

Table 7.5 Equipment used for the Karl Fischer moisture analysis of zinc gluconate API

Equipment	Supplier	Country of origin
Sartorius R200D+ balance	Labotec	South Africa
701 Karl Fischer Titrino Methrohm and 703 Ti Stand	Metrohm	Switzerland

The Karl Fischer autotitrator had a burette with a volume of 10 ml. A 1 g sample (with a theoretical moisture content of 12 % (m/m)) would thus consume 24 ml of the Hydranal® Composite 5 titrant, which exceeds the burette volume. Ideally, the moisture present in the sample size should consume half of the burette's volume of titrant (i.e. 5 ml for this experimental setup) (Sigma-Aldrich, 2006:48). Thus a 0.25 g zinc gluconate sample (with a theoretical moisture content of 12 % (m/m)) would consume approximately 6 ml of Hydranal® Composite 5 titrant. A 250 mg zinc gluconate sample size was thus recommended.

7.3.2 Procedure

7.3.2.1 Automated loss on drying

Set the RADWAG MAX 50 moisture analyzer according to the settings summarised in Table 7.3. Tare the weighing pan and transfer approximately 1.0 g of sample to the pan. Close the lid of the analyzer to start the analysis. The RADWAG MAX 50 moisture analyzer reports the percentage dried content every five minutes. Calculate the percentage loss on drying using the following formula (Beckett & Stenlake, 1975:21):

$$\%\ Loss\ on\ Drying = 100 - \%\ dry\ content$$

This procedure should be executed by three independent analysts.

7.3.2.2 Manual Loss on drying

Dry suitable loss on drying glass containers at 120 °C for approximately one hour, and allow cooling to ambient temperature in a desiccator. Weigh empty glass container and lid. Transfer approximately 1.0 g sample to glass container, and distribute the sample evenly. Place the sample in the oven previously heated to

105 °C. Remove the samples after an hour; allow the samples to cool to ambient temperature in a desiccator containing dried silica. Weigh the samples on an analytical balance. Place samples back into the oven and repeat the process every hour until the result of two consecutive weighings do not differ by more than 0.5 mg or a maximum drying period of four hours has elapsed. Once the constant mass is reached the percentage loss on drying is calculated by means of the following equation (BP, 2011):

$$\%\ Loss\ on\ Drying = \frac{Mass\ of\ sample\ before\ drying - Mass\ of\ sample\ after\ drying}{Mass\ of\ sample\ before\ drying}\ x\ 100$$

This procedure should be executed by three independent analysts.

7.3.2.3 Karl Fischer water analysis

Weigh 250 mg of zinc gluconate in triplicate and perform the Karl Fischer titration on the individual samples.

This procedure should be executed by three independent analysts.

7.3.3 Results and discussion

7.3.3.1 Loss on drying

The loss on drying results obtained from the automated method at five minute intervals are depicted in Figure 7.2.

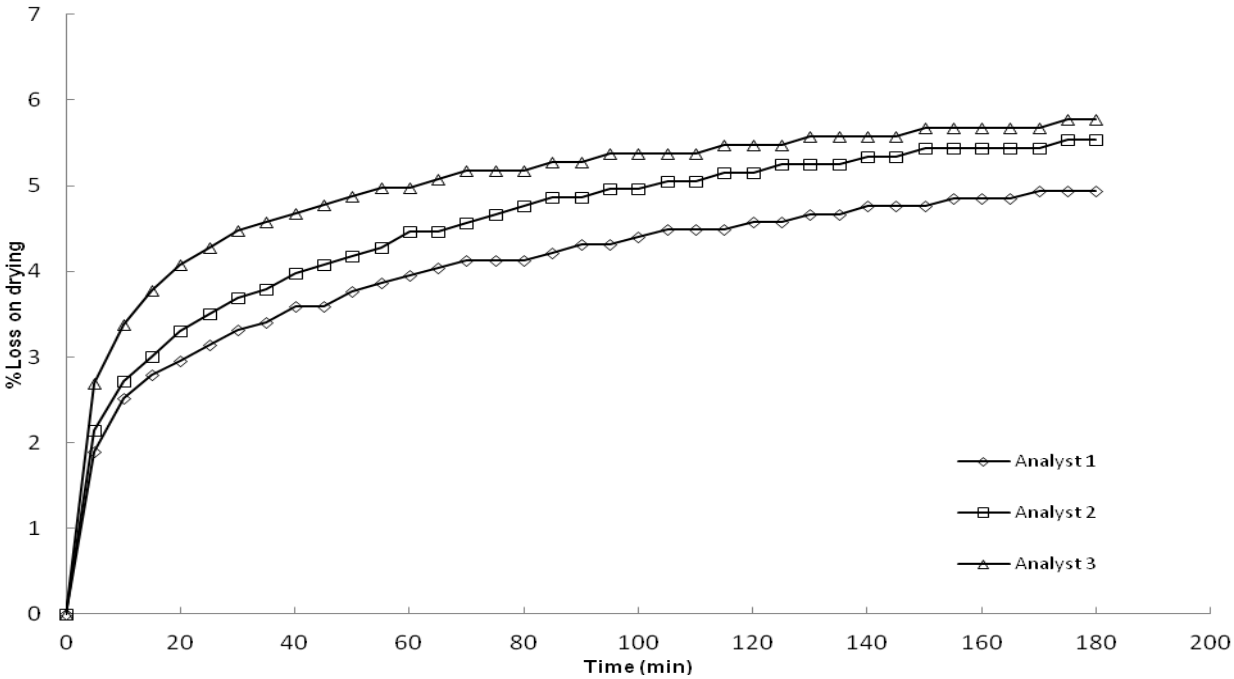


Figure 7.2 The % loss on drying results obtained from the automated loss on drying method at five minute intervals.

From Figure 7.2 it is clear that the % loss on drying varied between 4.9 - 5.8 % m/m (49 - 58 mg/g). The average % loss on drying for the three analysts after 3 hours was 5.4 % m/m (or 54 mg/g) which complied with the proposed specification (NMT 12 % m/m or 120 mg/g).

For loss on drying testing the sample is usually dried at the specified temperature to constant mass. The expression "dry to constant mass" indicates that the drying process should be continued until the results of two consecutive weighings do not differ by more than 0.5 mg, the second weighing being made after an additional hour of drying under the prescribed conditions (*Ph. Int.*, 2011). The differences in the consecutive weighings (as obtained from the Radwag Max 50 analyzer) were determined and are depicted in Figure 7.3.

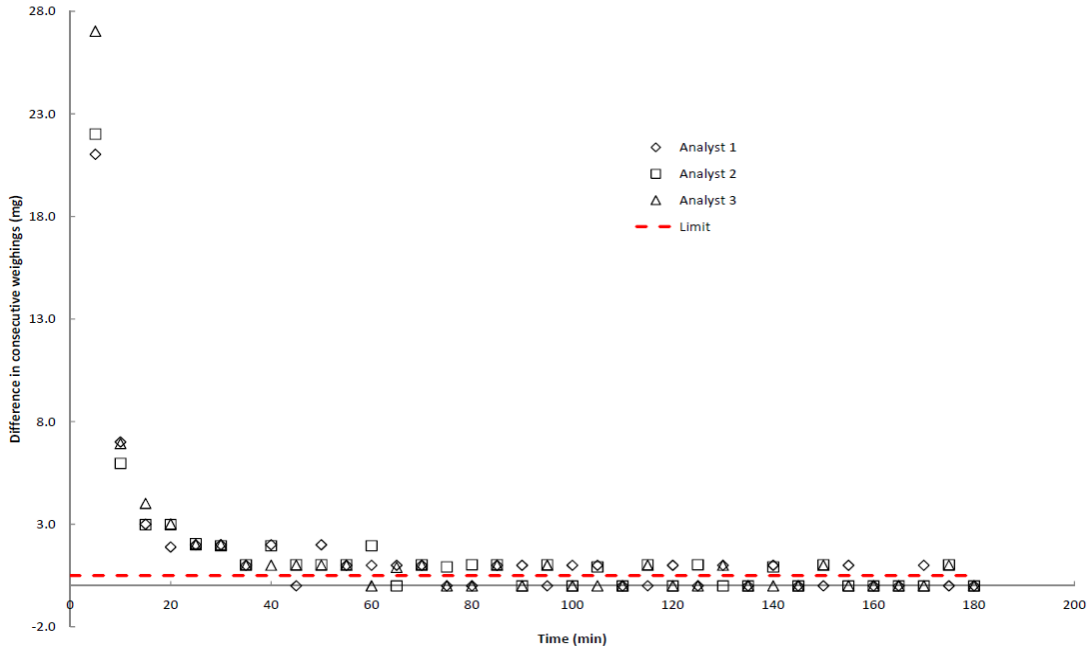


Figure 7.3 The differences in the consecutive weighings at five minute intervals using the automated loss on drying method. The “dried to constant mass” limit (NMT 0.5 mg) is indicated by the dashed line.

It is clear that not all the differences in the consecutive weighings stabilised below the 0.5 mg limit (Figure 7.3), which indicated that the samples did not achieve constant mass, a requirement for loss on drying analysis. It is thus suggested that there is a continuous adsorption-desorption of moisture taking place at the surface of the sample, which could be attributed to the hygroscopic behaviour of zinc gluconate.

The loss on drying results obtained from the manual method at hourly intervals are depicted in Figure 7.4.

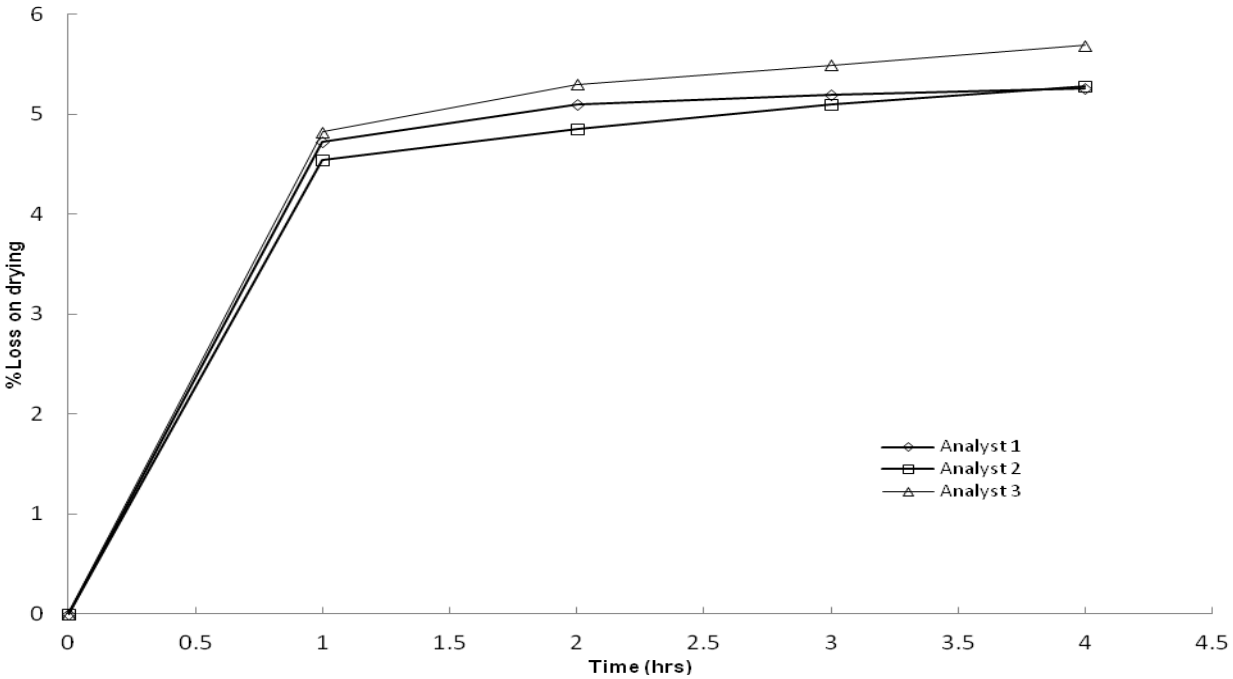


Figure 7.4 The % loss on drying results obtained from the manual loss on drying method at hourly intervals.

The % loss on drying varied between 4.5 - 7.2 % m/m (45 - 72 mg/g). The average % loss on drying for the three analysts after 3 hours was 5.3 % m/m (or 53 mg/g) which complied with the proposed specification (NMT 12 % m/m or 120 mg/g).

The differences in the consecutive weighings were determined and are depicted in Figure 7.5. It is clear from Figure 7.5 that none of the samples achieved constant mass within 4 hours. This confirmed the hygroscopic behaviour observed during the automated loss on drying analysis.

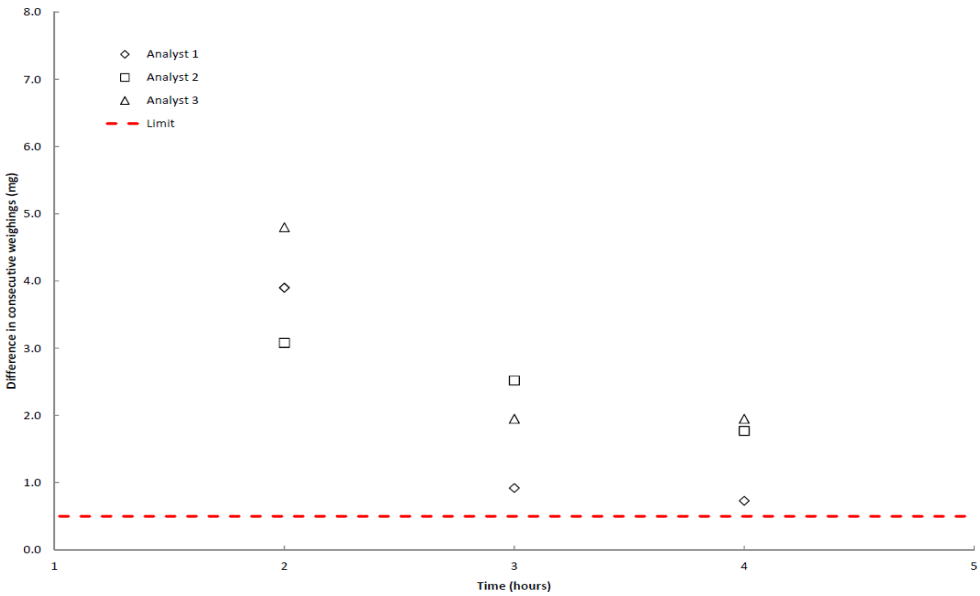


Figure 7.5 The differences in the consecutive weighings at hourly intervals using the manual loss on drying method. The "dried to constant mass" limit (NMT 0.5 mg) is indicated by the dashed line.

The results obtained did not support the application of loss on drying as technique for the establishment of the moisture content of zinc gluconate due to the fact that absolute constant mass was not achieved by the samples in a reasonable time (four hours).

7.3.3.2 Karl Fischer water analysis

Karl Fischer is a specific method for the determination of the water content of a sample (ICH, 1999:10). With the appropriate amount of sample and suitably diluted Karl Fischer reagents, the lower detection limit is approximately 0.05 – 0.1 mg/g (Fischer, 1935:395). The specificity and limit of detection of Karl Fischer titrations are thus suitably defined in the available literature, therefore only repeatability of the proposed method needs to be investigated.

The Karl Fischer water analysis results obtained are presented in Table 7.6 and depicted in Figure 7.6. The values in Figure 7.6 are expressed as mg/g water content as required by *The Ph. Int.* (*Ph. Int.,* 2011).

Three analysts performed the Karl Fischer moisture analysis in triplicate on different days and showed an intra-variability and inter-variability of below 2.5 % (Table 7.6).

This indicated that the Karl Fischer analysis is sufficiently repeatable (Ermer & Miller, 2005:320).

Table 7.6 Karl Fischer results % m/m (mg/g) for zinc gluconate samples reported by the three analysts

% m/m Moisture content (mg/g)			
	Analyst 1	**Analyst 2**	**Analyst 3**
Sample 1	5.0 (50)	5.3 (53)	4.9 (49)
Sample 2	4.9 (49)	5.0 (50)	4.8 (48)
Sample 3	4.9 (49)	5.1 (51)	5.0 (50)
Average	4.9 (49)	5.1 (51)	4.9 (49)
% SD	0.1	0.1	0.1
% RSD	1.2	2.4	1.6

SD = Standard deviation; RSD = Relative standard deviation

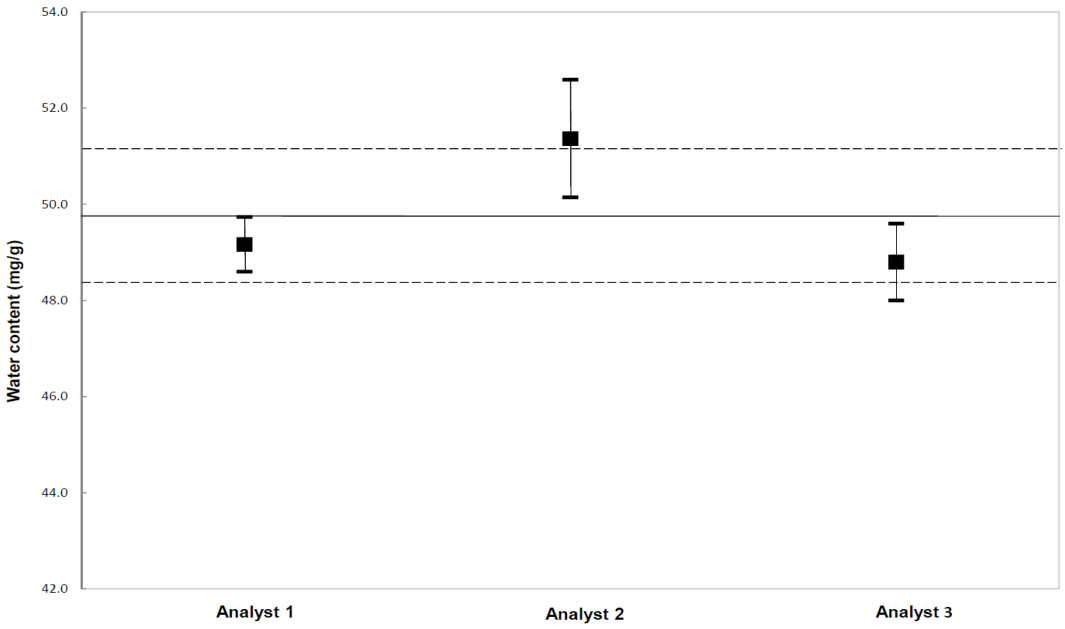


Figure 7.6 Water content results (mg/g) for zinc gluconate samples reported by the three analysts. Error bars are indicated based on 2x standard deviation. The average water content for all the values obtained (n = 12) is indicated by the solid line and the associated error bar is indicated by the two dashed lines.

The water content reported by the three analysts ranged between 48 mg/g and 53 mg/g; which was below the recommended specification of 120 mg/g. The average water content for all the reported values was calculated to be 49.8 mg/g (standard deviation = 1.2 mg/g).

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Analyst 1	3	148.00	49.33	0.33
Analyst 2	3	154.00	51.33	2.33
Analyst 3	3	147.00	49.00	1.00

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.555555556	2	4.777777778	3.909090909	0.081865523	5.14325285
Within Groups	7.333333333	6	1.222222222			
Total	16.88888889	8				

Figure 7.7 One-way ANOVA (95 % confidence interval) results for the Karl Fischer method for moisture determination of the zinc gluconate samples reported by the three analysts.

No significant difference was detected between the water content values reported by the three analysts ($F < F_{crit}$) when the values were submitted to a one-way ANOVA at a 95 % - confidence interval (Figure 7.7).

7.4 Conclusion

A moisture content specification of NMT 120 mg/g is proposed for inclusion in the zinc gluconate API monograph in *The Ph. Int.*

Two general procedures are described in pharmacopoeial methods for moisture determination, namely Karl Fischer titrations and loss on drying tests. From the results obtained in this chapter loss on drying was found not to be suitable for the determination of the moisture content of zinc gluconate API due to the hygroscopic nature of the API. The hygroscopic behaviour of zinc gluconate prevented the samples to be dried to constant mass, thus the results could not be utilised with absolute confidence.

Karl Fischer moisture analysis on a 250 mg sample was found to be a suitable technique for the determination of the moisture content of zinc gluconate API and is recommended to be included in zinc gluconate API monograph in *The Ph. Int*. The zinc gluconate API tested contained 49.8 mg/g water which complied with the proposed moisture content limit (NMT 120 mg/g).

CHAPTER 8

ASSAY

8.1 Introduction

Pharmaceutical substances which essentially contain bivalent or polyvalent ions (i.e. Zn^{2+}, Ca^{2+}, Mg^{2+}, Bi^{2+}, Al^{3+}, etc.) were initially assayed by means of gravimetric methods. However, these methods have become more or less obsolete due to the time consuming and tiresome steps associated with it, for example: precipitation, filtration, washing, drying and finally ignition to constant weight. The next step in the "evolution of the assay of bivalent / polyvalent ions" was the introduction of the faster oxalate-permanganate titrations. Soon thereafter, ethylenediaminetetra-acetic acid (EDTA) complexometric titration was introduced and is, till today, the method of choice for the quantitative analysis of several bivalent or polyvalent ions (Kar, 2005:161).

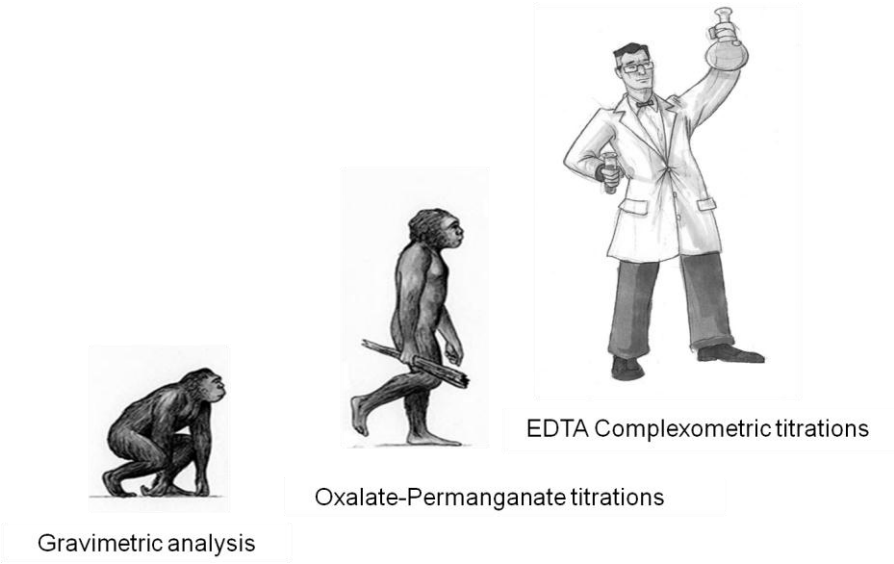


Figure 8.1 Evolution of the assay of bivalent or polyvalent ions.

The aims of the chapter were:

(i) To establish criteria for the assay of zinc acetate dihydrate and zinc gluconate APIs; and

(ii) To develop an assay method for zinc acetate dihydrate and zinc gluconate by means of a zinc-EDTA complexometric titration for inclusion in *The Ph. Int*.

8.2 Specifications for the assay of zinc acetate dihydrate and zinc gluconate APIs

The specifications for the assay of zinc acetate dihydrate and zinc gluconate APIs according the Ph. Eur. / BP and USP are depicted in Figure 8.2. From Figure 8.2 it is clear that the assay specifications of the Ph. Eur. and the BP are tighter when compared with that of the USP.

The USP reports an asymmetric assay specification (97.0 – 102.0 %) for zinc gluconate (USP, 2011). The prevalence of asymmetric specifications for the assay of pharmaceuticals, which are tested by means of titrations, is not uncommon (Ermer & Miller, 2005:327).

Based on the collaboration and harmonisation of *The Ph. Int.* and the BP, it is proposed that the assay specifications of the BP are to be adopted:

- Zinc acetate dihydrate contains not less than 99.0 % and not more than 101.0 % of $C_4H_6O_4Zn,2H_2O$;
- Zinc gluconate contains not less than 98.0 % and not more than 102.0 % of $C_{12}H_{22}ZnO_{14}$, calculated with reference to the dried substance.

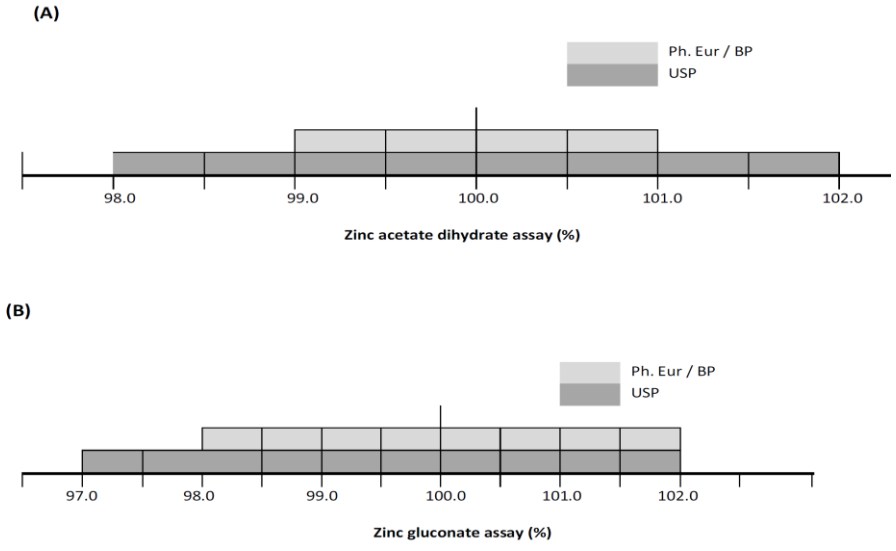


Figure 8.2 Specifications for the assay of (A) zinc acetate dihydrate and (B) zinc gluconate APIs according the BP, Ph. Eur. and USP (BP, 2011; Ph. Eur., 2011; USP, 2011).

8.3 Development of an assay method for the quantitative analysis of zinc acetate dihydrate and zinc gluconate APIs

Complexometric titrations are also known as chelometric titrations because the titrant, a ligand, reacts with the analyte, a metal ion, to form a complex, more specifically a chelate in this case (Husain, 2007:2).

The following complexometric titration method appears in the *2.5 Complexometric titrations* general chapter of *The Ph. Int.* for the quantitative analysis of zinc:

"Dissolve the quantity of substance, accurately weighed, as specified in the monograph, in 5 - 10 ml of water R, acidified with a minimum quantity of acetic acid (~300 g/l) TS if necessary, and then dilute to about 50 ml with water R. Add about 50 mg of xylenol orange indicator mixture R and sufficient methenamine R (about 5 g) to turn the solution pink-violet and titrate with disodium edetate (0.05 mol/l) volumetric solution (VS) until the solution turns from pink-violet to full yellow. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 3.268 mg of Zn." (*Ph. Int.,* 2011).

This general method was used as reference for the development of a method for the assay of zinc acetate dihydrate and zinc gluconate APIs based on the fact that:

a) Ethylenediaminetetra-acetic acid (also known as disodium edetate) (EDTA) is the most suitable ligand for the complexometric titrations with zinc cations:

The ligand, EDTA, which is used in this titration, is the most suitable ligand for complexometric titrations with metal cations based on the following properties thereof:

- EDTA forms 1:1 metal-EDTA complexes with most metal ions, regardless of the cation (Mendham *et al.*, 1987:222-229). The following complex is formed when EDTA chelates zinc metal ions in water:

$$Zn^{2+}_{(aq)} + EDTA^{4-}_{(aq)} \rightarrow Zn(EDTA)^{2-}_{(aq)}$$

- EDTA forms stable complexes with zinc metal ions (Figure 8.3), where the bonding is reinforced by the chelate effect. The formation constant of the

zinc-EDTA complex at 20 °C and ionic strength of pM = 0.1 is $K_f = 3.2 \times 10^{16}$ (Skoog *et al.,* 1997:280).

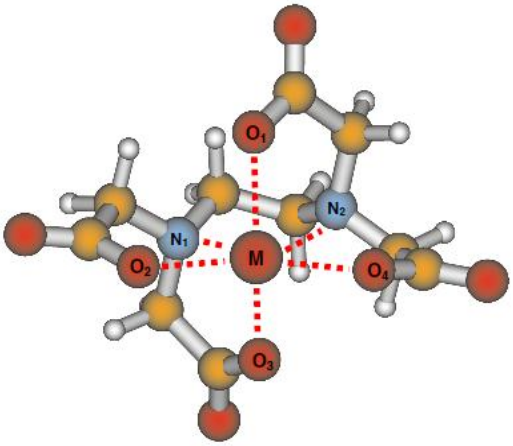


Figure 8.3 Schematic presentation of the computed structure of the EDTA complex with metal cations reported by Kovács *et al.* (2010:94).

- EDTA reacts instantaneously with zinc, producing accurate and repeatable endpoint determinations (Mendham *et al.*, 1987:222-229).

- EDTA forms a Zn-EDTA complex which is ionic, and is freely soluble in water (Mendham *et al.*, 1987:222-229).

b) An EDTA complexometric titration shows suitable reactivity with zinc:

Suitable reactivity for zinc cations in an EDTA complexometric titration can be accomplished by judicious choice of the pH. EDTA is an acidic substance with four weak acid dissociations ($k_1 = 1.02 \times 10^{-2}$, $k_2 = 2.14 \times 10^{-3}$, $k_3 = 6.92 \times 10^{-7}$, and $k_4 = 5.50 \times 10^{-11}$), thus the reaction with the metal ions is pH sensitive. Zinc reacts readily and sturdy with EDTA, therefore allowing it to be titrated in an acidic solution (in a pH range 3 - 4) (Skoog *et al.,* 1997:289-290).

The minimum permissible pH for a satisfactory end point in the titration of zinc ranges between 3.3 and 4.0 (Figure 8.4) (Skoog *et al.,* 1997:290). When 100 mg zinc acetate dihydrate is dissolved in 50 ml of acetic acid (~300 g/l) TS and about 5 g methenamine R is added, the pH of the zinc solution ranges from 3.5 to 4.0, which is ideal for the complexometric titration with EDTA. When 200 mg zinc gluconate is dissolved in 50 ml of acetic acid (~300 g/l) TS and about 5 g

methenamine R is added the pH of the zinc solution ranges between 3.3 – 3.6 which is also suitable for the complexometric titration.

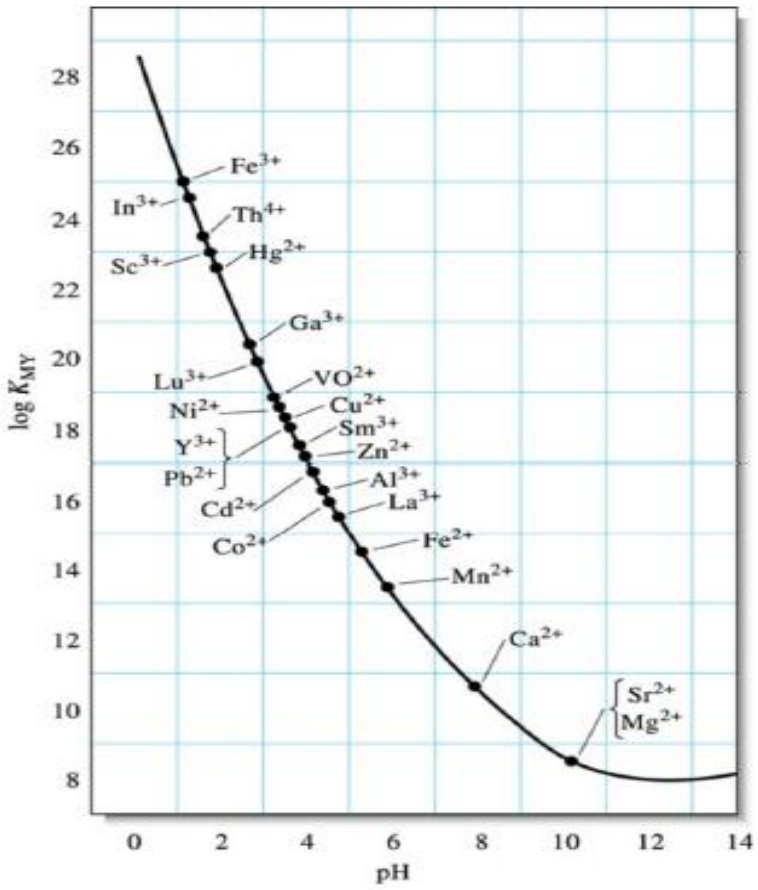


Figure 8.4 Minimum pH required for satisfactory titration of various cations with EDTA (Skoog *et al.,*1997:290).

c) Xylenol orange is a suitable indicator for the identification of the end point of the complexometric titration:

Xylenol orange (Figure 8.5) has a pH operation range of 1 – 6, making it a suitable metal-ion indicator for this titration (Harris, 2007:242). Xylenol orange has a red colour when it is bound to zinc metal cations (*Ph. Int*., 2011). However, the colour of the unbound indicator depends on pH of the solution (Table 8.1).

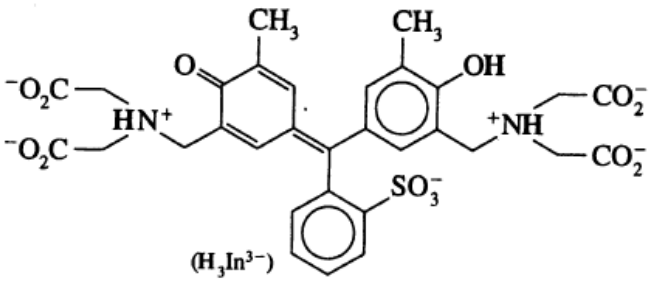


Figure 8.5 Chemical structure of xylenol orange (Harris, 2007:242).

Table 8.1 Xylenol orange metal-ion indicator's pK_a values, colour of the free indicator, as well as colour of the metal-ion complex (Harris, 2007:242)

pK_a	Colour of free indicator	Colour of metal-ion complex
$pK_2 = 2.32$ $pK_3 = 2.85$ $pK_4 = 6.70$ $pK_5 = 10.47$ $pK_6 = 12.23$	H_5In^- - yellow H_4In^{2-} - yellow H_3In^{3-} - yellow H_2In^{4-} - violet HIn^{5-} - violet In^{6-} - violet	Red

Based on Table 8.1, the end point colour of the proposed titration solutions (pH ~ 3 - 4) would be a bright yellow colour, which confirms the colour reported in the general method of *The Ph. Int.* (2011). The change from pink-violet to bright yellow is a clear and acceptable indication of the end point.

8.3.1 Proposed methods for the assay of zinc acetate dihydrate and zinc gluconate APIs

Based on the information presented in section 8.3, the following two methods are proposed for the assay of zinc acetate dihydrate and zinc gluconate APIs.

8.3.1.1 Assay of zinc acetate dihydrate API

Dissolve about 100 mg, accurately weighed, in 50 ml of acetic acid (~10 g/l) TS and proceed with the titration as described under 2.5 Complexometric titrations for zinc. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 10.98 mg of $C_4H_6O_4Zn,2H_2O$.

8.3.1.2 Assay of zinc gluconate API

Dissolve about 200 mg, accurately weighed, in 50 ml of acetic acid (~10 g/l) TS and proceed with the titration as described under 2.5 Complexometric titrations for zinc. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 22.78 mg of $C_{12}H_{22}ZnO_{14}$.

The disodium edetate (0.05 mol/ml) VS should be standardised using the general method of standardisation specified in the "*Reagents, test solutions and volumetric solutions*" section of *The Ph. Int.* (*Ph. Int.*, 2011).

Since the recommended methods are based on the established *Ph. Int.* zinc-EDTA complexometric titration method, method validations were performed to ensure the fitness for purpose of the proposed methods (Ermer & Miller, 2005:327).

8.4 Materials and equipment

The information of the materials used in the complexometric titrations of zinc acetate dihydrate and zinc gluconate APIs is presented in Table 8.2. A Sartorius R200D+ balance (Labotec, SA) and a Sartorius ED623S+ balance (IMP, SA) were used during this test.

Table 8.2 Information of materials used for the complexometric titrations

Name	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Calcium carbonate	A827266 820	Merck KGaA	Germany
Disodium edetate	058 K0115	Sigma-Aldrich	Germany
Hydrochloric acid	1037916	Merck (Pty) Ltd.	South Africa
Sodium hydroxide	ME9M591063	Merck (Pty) Ltd.	South Africa
Sodium sulfate anhydrous	1032038	Merck (Pty) Ltd.	South Africa
Erichrome black T	71770	Sigma-Aldrich	Germany
Glacial acetic acid	K42028063 107	Merck KGaA	Germany
Xylenol orange tetrasodium salt	06022PD	Sigma-Aldrich	Germany
Potassium nitrate	1037476	Merck (Pty) Ltd.	South Africa
Hexamethylene tetramine	S5410912 935 S6252512 130	Merck Schuchardt OHG	Germany
Methenamine (GR for analysis)	K41563543 101	Merck KGaA	Germany
Water R	N/A	RIIP®/CENQAM®	South Africa

OHG = German: General partnership

8.5 Method validation of the assay method for zinc acetate dihydrate API

The validation parameters investigated for the assay method of zinc acetate dihydrate API are listed in Table 8.3.

Table 8.3 The validation parameters investigated for the assay method of zinc acetate dihydrate API

Validation parameter	Applicability
Specificity	+
Linearity	+
Range	+
Accuracy	+
Precision:	
Repeatability	+
Intermediate precision	+
Robustness	+

8.5.1 Specificity

The complexometric titrations with EDTA could be regarded as a non-specific method due to the complexation with most metal ions (Husain, 2007:5); therefore no specificity testing was performed. However, impurity testing is performed along with the assay in order to support the assay by titration, which validates the use of the complexometric titration (Ermer & Miller, 2005:327). These impurity tests will be discussed in Chapters 9, 10 and 11.

To exclude the potential effect of metal ions in the solvent used (i.e. acetic acid ~10g/l TS), a blank sample (consisting of only the acetic acid (~10 g/l) TS, applicable indicator and buffer agent) was prepared and titrated.

8.5.1.1 Procedure

Prepare a blank sample by transferring 50 ml acetic acid (~10 g/l) TS into a 100 ml Erlyn-meyer flask and add 50 mg of xylenol orange indicator mixture R. Add about 5 g of methenamine R and swirl to dissolve.

8.5.1.2 Acceptance criteria

i. The zinc acetate dihydrate API should comply with the impurity tests.
ii. The blank solution should turn yellow after the addition of the indicator and buffer agent, indicating the absence of metal ions.

8.5.1.3 Results and discussion

The zinc acetate dihydrate API complied with all the impurity tests – refer to Chapters 9, 10 and 11.

The blank sample prepared turned yellow immediately after the addition of the indicator and buffer agent, which confirmed the absence of any traceable metal ions (Figure 8.6).

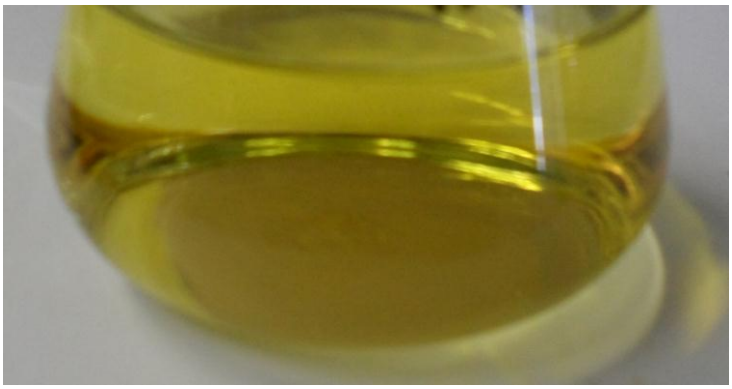


Figure 8.6 Photograph of the blank sample after the addition of the indicator and buffer agent.

8.5.2 Range & linearity

The target concentration of the test solution (based on the method described in section 8.3.1.1) was 2.0 mg/ml zinc acetate dihydrate. For the purpose of this validation a range of 1.6 - 2.4 mg/ml zinc acetate dihydrate was selected. The linearity was investigated by measuring the response (i.e. titer volume) of five samples with a concentration range of ± 80 - 120 % of the target concentration (2.0 mg/ml).

8.5.2.1 Procedure

Prepare five test solutions (in triplicate) with final concentrations ranging from 1.6 mg/ml to 2.4 mg/ml of zinc acetate dihydrate, with the target concentration of 2.0 mg/ml zinc acetate dihydrate, by accurately weighing the amounts of zinc acetate dihydrate specified in Table 8.4 and diluting each sample as follows:

Transfer the specified zinc acetate dihydrate samples into separate 100 ml Erlyn-meyer flasks and add 50 ml acetic acid (~10 g/l) TS. Swirl the solutions to dissolve, add 50 mg of xylenol orange indicator mixture R to each solution. Add about 5 g of methenamine R to each solution to turn the solutions pink-violet.

Table 8.4 Zinc acetate dihydrate concentrations used for the linear regression analysis

Mass zinc acetate dihydrate (mg)	Zinc acetate dihydrate concentration (mg/ml)	% of Target concentration
~80	~1.6	~80
~90	~1.8	~90
~100	~2.0	~100
~110	~2.2	~110
~120	~2.4	~120

Determine the content of zinc acetate dihydrate in the respective test solutions by titrating with standardised EDTA (0.05 mol/l) VS until the solution turns from pink-violet to full yellow. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 10.98 mg of zinc acetate dihydrate (use the standardised concentration of EDTA in the final calculations).

Calculate the theoretical concentration (mg/ml) of zinc acetate dihydrate in the prepared test solutions using the following equation:

$$Theorectical\ concentration\ (mg/ml) = \frac{sample\ mass\ (mg)}{50\ ml}$$

Calculate the experimental concentration (mg/ml) of zinc acetate dihydrate in the prepared test solutions using the following equation:

$$Experimental\ concentration\ (mg/ml) = \frac{titer\ volume\ (ml) \times equivalence\ factor}{50\ ml}$$

Where: the equivalence factor is calculated with the following equation:

$$Equivalence\ factor = \frac{10.98\ x\ cEDTA}{0.05}$$

Where: cEDTA is the concentration of standardised EDTA (mol/l) obtained from the standardisation procedure.

Plot the titer volumes as a function of the theoretical concentration (mg/ml) of zinc acetate dihydrate in the various test solutions. Perform a linear regression analysis to determine the correlation coefficient, y-intercept and slope of the regression line.

8.5.2.2 Acceptance criteria

i. A linear relationship should exist between the theoretical concentration (mg/ml) of the analyte and the titer volume (ml) in the range 1.6 - 2.4 mg/ml with a linear correlation coefficient (r^2) not less than 0.99 ($r^2 \geq 0.99$).
ii. When a linear regression equation is applied to the results, it should have an intercept not significantly different from zero.

8.5.2.3 Results and discussion

The standardised concentration of the disodium edetate (0.05 mol/l) VS was determined as 0.0446 mol/l (n = 5; % RSD = 0.75 %). Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 10.98 mg $C_4H_6O_4Zn,2H_2O$, thus each ml of disodium edetate (0.0446 mol/l) VS is equivalent to 9.79 mg $C_4H_6O_4Zn,2H_2O$.

A summary of the results obtained is tabulated in Table 8.5.

Table 8.5 Tabulated results of the zinc acetate dihydrate titration results obtained for linearity and range

Test solution	Mass of $C_4H_6O_4Zn{\cdot}2H_2O$ (mg)	Theoretical concentration (mg/ml)	Titer volume (ml)	Experimental concentration (mg/ml)
1 (80 %)	81.81	1.636	8.4	1.645
	80.57	1.611	8.3	1.626
	80.68	1.614	8.2	1.606
2 (90 %)	90.54	1.811	9.2	1.802
	89.77	1.795	9.1	1.783
	90.02	1.800	9.2	1.802
3 (100 %)	100.40	2.008	10.2	1.998
	102.16	2.043	10.4	2.037
	101.53	2.031	10.4	2.037
4 (110 %)	109.86	2.197	11.2	2.194
	109.99	2.200	11.3	2.204
	110.13	2.203	11.2	2.194
5 (120 %)	119.19	2.384	12.2	2.390
	119.85	2.397	12.2	2.390
	120.67	2.413	12.3	2.409

The titer volumes were plotted as a function of the theoretical concentration of zinc acetate dihydrate in the test solutions (Figure 8.7). A linear regression analysis was performed using STATISTICA 10 (2011) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

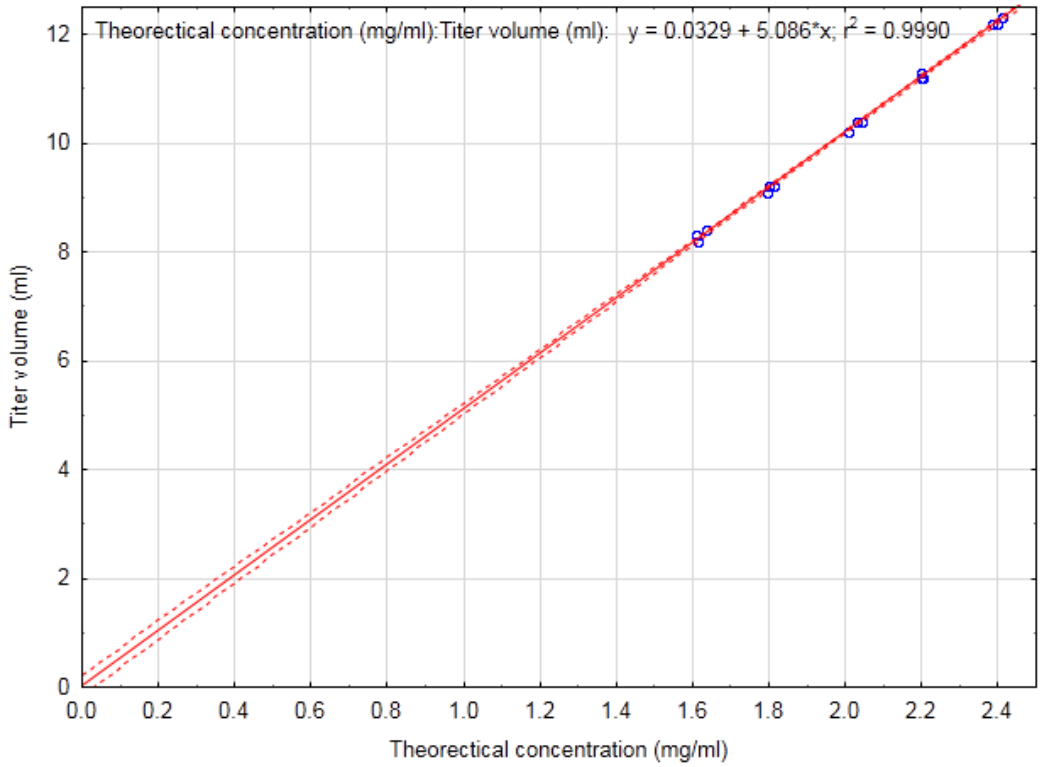


Figure 8.7 The titer volumes (ml) plotted as a function of the theoretical concentration of zinc acetate dihydrate (mg/ml) in the test solutions.

The linear regression analysis reported r^2 = 0.9990, therefore a linear relationship existed between the zinc acetate dihydrate concentration and the titer volume in the range 1.6 - 2.4 mg/ml. The intercept of the regression line in Figure 8.7 was calculated as 0.0329 (±0.0897), which did not differ significantly from zero. It can thus be concluded that this method is linear over the concentration range of 1.6 - 2.4 mg/ml zinc acetate dihydrate.

8.5.3 Accuracy

The accuracy of the method was evaluated using the results of the fifteen test solutions prepared in section 8.5.2.1 (as specified in Table 8.4) which covered the range of the analytical method.

The accuracy was reported as the % recovery from the assay of the zinc acetate dihydrate test solutions with known theoretical concentrations.

8.5.3.1 Procedure

Use the theoretical concentrations and experimental concentrations calculated in section 8.5.2.3 (Table 8.5) to calculate the % recovery using the following equation:

$$\% \text{ Recovery} = \frac{\text{Experimental concentration (mg/ml)}}{\text{Theoretical concentration (mg/ml)}} \text{ x } 100$$

8.5.3.2 Acceptance criterion

i. The % recovery values should range between: 99.0 % - 101.0 %.

8.5.3.3 Results and discussion

The theoretical- and experimental zinc acetate dihydrate concentrations, and the % recovery for each of the test solutions, were calculated. The results obtained are tabulated in Table 8.6.

Table 8.6 Zinc acetate dihydrate titration results for the determination of the % recovery

Test solutions	Replicates	Theoretical concentration (mg/ml)	Experimental concentration (mg/ml)	% Recovery
80 %	**1**	1.636	1.645	100.6
	2	1.611	1.626	100.9
	3	1.614	1.606	99.5
90 %	**1**	1.811	1.802	99.5
	2	1.795	1.783	99.3
	3	1.800	1.802	100.1
100 %	**1**	2.008	1.998	99.5
	2	2.043	2.037	99.7
	3	2.031	2.037	100.3
110 %	**1**	2.197	2.194	99.9
	2	2.200	2.204	100.2
	3	2.203	2.194	99.6
120 %	**1**	2.384	2.390	100.3
	2	2.397	2.390	99.7
	3	2.413	2.409	99.8
			Average	**99.9**
			SD	**0.45**
			% RSD	**0.45**

The % recovery was calculated over a concentration range of 1.6 - 2.4 mg/ml zinc acetate dihydrate. The individual % recovery values ranged between 99.3 % and 100.9 %. The average % recovery over the mentioned concentration range was 99.9 %. Both the individual and the average % recovery values complied with the acceptance criterion (99.0 - 101.0 %).

8.5.4 Repeatability and intermediate precision

Repeatability was assessed by estimating the variance in the triplicate % recovery values (section 8.5.3.3) reported for each concentration interval (i.e. 1.6 mg/ml, 1.8 mg/ml, 2.0 mg/ml, 2.2 mg/ml and 2.4 mg/ml) which covered the entire analytical range of the method.

The intermediate precision was assessed by evaluating the variance in assay values when the assay procedure was executed by three different analysts on different days in different laboratories on the same sample.

8.5.4.1 Procedure

Calculate the analytical variability of the % recovery results reported in section 8.5.3.3 (Table 8.6) by calculating:

i. The % RSD of the triplicate determinations at the respective concentrations, i.e. 1.6 mg/ml, 1.8 mg/ml, 2.0 mg/ml, 2.2 mg/ml and 2.4 mg/ml; and
ii. The % RSD for the whole analytical range (i.e. for all 15 % recovery values); using the following equation:

$$\%\ RSD = \frac{SD}{\left(\frac{\sum x}{n}\right)} \times 100$$

Where: n = 3 for the respective concentrations, n = 15 for the whole analytical range; and SD is the standard deviation, which can be calculated using the following equation:

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

Where: x represents the respective % recovery values; $\bar{x}$ represents the average of the triplicate values for the respective concentrations or the average of the 15 values for the whole analytical range, and n is the sample size (i.e. n = 3 or n = 15).

For the investigation of the intermediate precision, three different analysts should execute the assay procedure (described in section 8.3.1.1) in triplicate, on different days and in different laboratories.

Calculate the theoretical- and experimental concentrations of the abovementioned test solutions using the equations presented in section 8.5.2.1. Calculate the % assay values using the following equation:

$$\%\ \text{Assay} = \frac{\text{Experimental concentration (mg/ml)}}{\text{Theoretical concentration (mg/ml)}}\ \text{x}\ 100$$

Perform a one-way analysis of variance (ANOVA) on the % assay values, using a 95 % confidence interval.

8.5.4.2 Acceptance criteria

i. For repeatability: the % RSD of the triplicate determinations at the respective concentrations: i.e. 1.6 mg/ml, 1.8 mg/ml, 2.0 mg/ml, 2.2 mg/ml and 2.4 mg/ml and that for the whole analytical range (i.e. for all 15 - % recovery values) should not exceed 1.0 %.

ii. For intermediate precision: the % assay values reported by the three analysts should be comparable ($F < F_{crit}$).

8.5.4.3 Results and discussion

The % RSD of the triplicate determinations at the respective concentrations: i.e. 1.6 mg/ml, 1.8 mg/ml, 2.0 mg/ml, 2.2 mg/ml and 2.4 mg/ml did not exceed 1.0 %, as illustrated in Figure 8.8. The % RSD for the whole analytical range (i.e. for all 15 % recovery values) was calculated to be 0.45 %, which also complied with the specification (NMT 1.0 %) (Table 8.6).

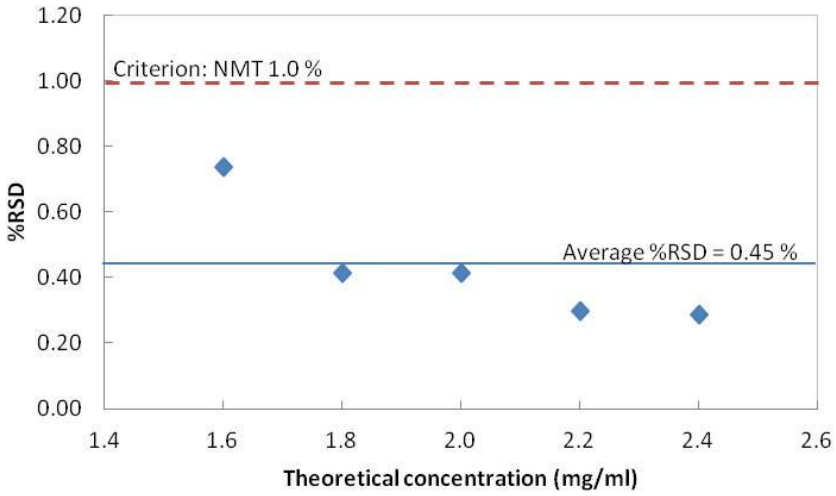


Figure 8.8 Graph of % RSD values calculated of the triplicate determinations at the respective zinc acetate dihydrate concentrations, i.e. 1.6 mg/ml, 1.8 mg/ml, 2.0 mg/ml, 2.2 mg/ml and 2.4 mg/ml. The solid line illustrates the % RSD for the whole analytical range (i.e. for all 15 % recovery values) and the dashed line illustrates the acceptance criterion.

The complexometric titration was executed by the three analysts and the results obtained are tabulated in Table 8.7.

Table 8.7 Tabulated results of the % assay zinc acetate dihydrate for determination of intermediate precision

Analyst	% Assay	Average %	SD	% RSD
1	99.7	99.7	0.05	0.05
	99.7			
	99.8			
2	99.5	99.8	0.41	0.41
	99.7			
	100.3			
3	100.1	100.3	0.42	0.42
	100.8			
	100.1			
Average %		**100.0**		
SD		**0.32**		
% RSD		**0.32**		

The average % assay for each analyst varied between 99.7 % and 100.3 %, well within the range of 99.0 - 101.0 %. The one-way ANOVA was used to investigate the significance of variances in the % assays obtained by the three analysts; the results obtained can be seen in Figure 8.9.

The null hypothesis states that there is no significant difference between the means of the % assay values reported by the three analysts: H_0: $\mu_A = \mu_B = \mu_C$ (Bolton, 1997:273). The alternative hypothesis states that there is a difference between any two of the means: H_a: $\mu_i \neq \mu_j$. If the calculated F value is equal to or greater than F_{crit} at the specified α (confidence interval), the null hypothesis can be rejected (Table 4.13 in Chapter 4) (Bolton, 1997:273).

The F value (2.7) calculated is smaller than F_{crit} (5.1) which illustrated that there is no significant difference between the means of the % assay values reported by the three analysts (Figure 8.9). Thus, the means of the % assay values of the three analysts are comparable.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Analyst 1	3	299.20	99.73	0.0033
Analyst 2	3	299.50	99.83	0.1733
Analyst 3	3	301.00	100.33	0.1633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.62	2	0.31	2.73529412	0.143118798	5.14325285
Within Groups	0.68	6	0.1133333			
Total	1.3	8				

Figure 8.9 One-way ANOVA results for the means of the % assay values obtained by the three analysts for the complexometric titration of zinc acetate dihydrate.

8.5.5 Robustness

Factors which may influence EDTA metal titrations according to Husain (2007:5) include:

- The metal ion's nature and activity.
- The pH at which the titration is carried out.
- The presence of interfering ions such as CN^-, citrate, tartrate, F^- and other complex forming agents.
- The stability of the complex may be increased by the use of organic solvents.

Zinc's nature and activity is a non-variable in this case and the presence of interfering ions was avoided by using purified water; where the absence of interfering ions was proved by preparing a blank solution (section 8.5.1). Thus, the only variable that could play a role is the pH of the solution. The robustness of the proposed method was investigated by determining the influence of variances in the pH of the solvent (acetic acid TS).

8.5.5.1 Procedure

Prepare three acetic acid TS with the following concentrations: ~5g/l, ~10g/l and ~15g/l. Prepare three test solutions (in triplicate), with the target concentration of 2.0 mg/ml zinc acetate dihydrate. Transfer the weighed zinc acetate dihydrate

samples into separate 100 ml Erlyn-meyer flasks and add 50 ml of the respective acetic acid (~5, ~10 or ~15 g/l) TS, swirl the solutions to dissolve. Add 50 mg of xylenol orange indicator mixture R to each test solution. Add about 5 g of methenamine R to the respective test solutions to turn the solutions pink-violet. Determine and record the average pH of the test solutions.

Determine the content of zinc acetate dihydrate in the respective test solutions by titrating with standardised EDTA (0.05 mol/l) VS until the solution turns from pink-violet to full yellow. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 10.98 mg of zinc acetate dihydrate.

Calculate the theoretical and experimental concentrations, as well as the % assay using the formulas in section 8.5.2.1.

Perform a one-way ANOVA on the % assay values, using a 95 % confidence interval to investigate the significance of variances in the pH of the solvent on the robustness of the proposed method.

8.5.5.2 Acceptance criteria

The method of analysis should remain unaffected by deliberate changes in the pH of the solvent (acetic acid TS) used in the complexometric titration; where:

i. The % assay values should be within the range: 99.0 - 101.0 %; and
ii. The % assay values obtained using the three solvents with different pH should be comparable ($F < F_{crit}$).

8.5.5.3 Results and discussion

The pH of the three different solvents (~5 g/l acetic acid TS, ~10 g/l acetic acid TS and ~15 g/l acetic acid TS) and that of the final test solutions (before commencement of the titration) were measured and are tabulated in Table 8.8.

The complexometric titrations in the three different solvents were executed and the results obtained are tabulated in Table 8.9.

Table 8.8 Average pH values of solvent and test solutions during robustness investigation of the assay for zinc acetate dihydrate

Solution	pH value
~5 g/l acetic acid TS	2.89
~10 g/l acetic acid TS	2.74
~15 g/l acetic acid TS	2.64
Zinc acetate dihydrate in ~5 g/l acetic acid TS	3.95
Zinc acetate dihydrate in ~10 g/l acetic acid TS	3.62
Zinc acetate dihydrate in ~15 g/l acetic acid TS	3.46

Table 8.9 The results of the complexometric titrations with the three different solvents to investigate the robustness of the proposed zinc acetate dihydrate assay method

Acetic acid TS	Replicates	Theoretical zinc acetate dihydrate concentration (mg/ml)	Experimental zinc acetate dihydrate concentration (mg/ml)	% Assay
~5 g/l	**1**	2.017	2.018	100.0
	2	2.012	1.998	99.3
	3	2.004	1.988	99.2
~10 g/l	**1**	2.008	1.998	99.5
	2	2.043	2.037	99.7
	3	2.031	2.037	100.3
~15 g/l	**1**	2.014	2.018	100.2
	2	2.005	1.988	99.2
	3	2.001	1.988	99.4
			Average	**99.6**
			SD	**0.43**
			% RSD	**0.43**

The one-way ANOVA was used to investigate the significance of variances in the pH of the solvent on the robustness of the proposed method. The results obtained are summarised in Figure 8.10.

The null hypothesis states that there is no significant difference between the means of the % assay values in the three different solvents: H_0: $\mu_A = \mu_B = \mu_C$ (Bolton, 1997:273). The alternative hypothesis states that there is a difference between any two of the means: H_a: $\mu_i \neq \mu_j$. If the calculated F value is equal to or greater than F_{crit}

at the specified α (confidence interval), the null hypothesis can be rejected (Table 4.13 in Chapter 4) (Bolton, 1997:273).

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
5g/L	3	298.50	99.50	0.190
10g/L	3	299.53	99.84	0.183
15g/L	3	298.68	99.56	0.276

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.20236101	2	0.10118	0.467494974	0.64761402	5.1432528
Within Groups	1.29858728	6	0.21643			
Total	1.50094829	8				

Figure 8.10 One-way ANOVA results for the means of the % assay values in the three different solvents used in the complexometric titration of zinc acetate dihydrate.

The F value (0.5) calculated is smaller than F_{crit} (5.1) (see Figure 8.10) which illustrated that there is no significant difference between the means of the % assay values in the three different solvents. Thus, it can be concluded that the results obtained from the method of analysis remained unaffected by the deliberate changes in the pH of the solvent (acetic acid TS) used for the complexometric titration.

8.5.6 Conclusion

The assay of zinc acetate dihydrate API by means of the proposed EDTA complexometric titration method was validated with regards to the following parameters: specificity, linearity and range, accuracy, precision (intermediate precision and repeatability), and robustness. The acceptance criteria for each validation parameter were met, therefore the method can be considered fit for its purpose to determine the content / assay of zinc acetate dihydrate API.

8.6 Method validation of the assay method for zinc gluconate API

The validation parameters investigated for the assay method of zinc gluconate API are listed in Table 8.10.

Table 8.10 Zinc gluconate assay method validation parameters for complexometric titrations

Validation parameter	Applicability
Specificity	+
Linearity	+
Range	+
Accuracy	+
Precision:	
Repeatability	+
Intermediate precision	+
Robustness	+

8.6.1 Specificity

The same rationale is applicable for specificity as in the case of zinc acetate dihydrate (refer to section 8.5.1).

8.6.1.1 Procedure

Prepare a blank sample by transferring 50 ml acetic acid (~10 g/l) TS into a 100 ml Erlyn-meyer flask and add 50 mg of xylenol orange indicator mixture R. Add about 5 g of methenamine R and swirl to dissolve.

8.6.1.2 Acceptance criteria

i. The zinc gluconate API should comply with the impurity tests.
ii. The blank solution should turn yellow after the addition of the indicator and buffer agent, indicating the absence of metal ions.

8.6.1.3 Results and discussion

The zinc gluconate API complied with the impurity tests – refer to Chapters 9, 10 and 11 on impurity tests.

The blank sample prepared turned yellow after the addition of the indicator and buffer agent, confirming the absence of any metal ions (Figure 8.6).

8.6.2. Range & linearity

The target concentration of the test solution (based on the method described in section 8.3.1.2) was 4.0 mg/ml zinc gluconate. For the purpose of this validation the range: 3.2 - 4.8 mg/ml zinc gluconate was considered. The linearity was determined by measuring the response (i.e. titer volume) of five samples with a concentration range of ± 80 - 120 % of the target concentration (4.0 mg/ml).

8.6.2.1 Procedure

Prepare five test solutions (in triplicate) ranging from 3.2 mg/ml to 4.8 mg/ml, with the target concentration (100 % test solution) of 4.0 mg/ml zinc gluconate. Prepare the test solutions by using the amount of zinc gluconate as specified in Table 8.11. Transfer the respective zinc gluconate samples weighed into separate 100 ml Erlyn-meyer flasks and add 50 ml acetic acid (~10 g/l) TS. Swirl the solutions to dissolve, add 50 mg of xylenol orange indicator mixture R to each test solution. Add about 5 g of methenamine R to the respective test solutions to turn the solutions pink-violet.

Table 8.11 Zinc gluconate concentrations used for linear regression analysis

Mass zinc gluconate (mg)	Zinc gluconate concentration (mg/ml)	% of Target concentration
~160	~3.2	~80
~180	~3.6	~90
~200	~4.0	~100
~220	~4.4	~110
~240	~4.8	~120

Determine the content of zinc gluconate in the respective test solutions by titrating with standardised EDTA (0.05 mol/l) VS until the solution turns from pink-violet to full yellow. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 22.78 mg of zinc gluconate (utilise standardised concentration of EDTA).

Calculate the theoretical concentration (mg/ml) of zinc gluconate in the prepared test solutions using the following equation:

$$Theorectical\ concentration\ (mg/ml) = \frac{sample\ mass\ (mg) \times (100 - \%m)}{50\ ml \times 100}$$

Where: % m = percentage moisture present in zinc gluconate sample (determined in Chapter 7).

Calculate the experimental zinc concentration (expressed as mg/ml) in the prepared test solutions using the following equation:

$$Experimental\ concentration\ (mg/ml) = \frac{titer\ volume\ (ml) \times equivalence\ factor}{50\ ml}$$

The equivalence factor is calculated with the following equation:

$$Equivalence\ factor = \frac{22.78\ x\ cEDTA}{0.05}$$

Where: cEDTA = concentration of standardised EDTA (mol/l) obtained from the standardisation procedure.

Plot the titer volumes as a function of the theoretical concentration (mg/ml) zinc gluconate in the various test solutions. Perform a linear regression analysis to determine the correlation coefficient, y-intercept and slope of the regression line.

8.6.2.2 Acceptance criteria

i. A linear relationship should exist between the theoretical concentration (mg/ml) of the analyte and the titer volume (ml) in the range: 3.2 - 4.8 mg/ml with a linear correlation not less than 0.99 ($r^2 \geq 0.99$).
ii. When a linear regression equation is applied to the results, it should have an intercept not significantly different from zero.

8.6.2.3 Results and discussion

The standardised concentration of the EDTA (0.05 mol/l) VS was determined as 0.0446 mol/l (n = 5; % RSD = 0.75 %). Each ml of EDTA (0.05 mol/l) VS is equivalent to 22.78 mg zinc gluconate, meaning that each ml of EDTA (0.0446 mol/l) VS is equivalent to 20.320 mg zinc gluconate.

A summary of the results obtained is tabulated in Table 8.12.

Table 8.12 Tabulated results of zinc gluconate titration results obtained for linearity and range

Test solution	Mass of $C_{12}H_{22}O_{14}Zn$ (mg)	Theoretical concentration (mg/ml)	Titer volume (ml)	Experimental concentration (mg/ml)
1 (80 %)	160.82	3.056	7.6	3.089
	160.16	3.044	7.5	3.048
	160.57	3.051	7.5	3.048
2 (90 %)	180.74	3.435	8.4	3.393
	180.37	3.428	8.4	3.414
	180.03	3.421	8.5	3.434
3 (100 %)	200.38	3.808	9.4	3.800
	200.19	3.804	9.3	3.779
	200.50	3.810	9.3	3.779
4 (110 %)	220.91	4.198	10.3	4.186
	220.72	4.195	10.3	4.186
	220.77	4.196	10.3	4.186
5 (120 %)	240.37	4.568	11.3	4.592
	240.59	4.572	11.2	4.552
	240.23	4.565	11.2	4.552

The titer volumes were plotted as a function of the theoretical concentration of zinc gluconate in the test solutions (Figure 8.11). A linear regression analysis was performed using STATISTICA 10 (2011) software (StatSoft, Inc., USA) and the correlation coefficient, y-intercept and slope of the regression line determined.

The linear regression analysis reported $r^2 = 0.9987$. A linear relationship therefore existed between the zinc gluconate concentration and the titer volume in the range 3.2 - 4.8 mg/ml. The intercept of the regression line in Figure 8.11 was calculated as 0.0866 (± 0.0928), which did not differ significantly from zero. It can thus be concluded that this method is linear over the concentration range of 3.2 - 4.8 mg/ml zinc gluconate.

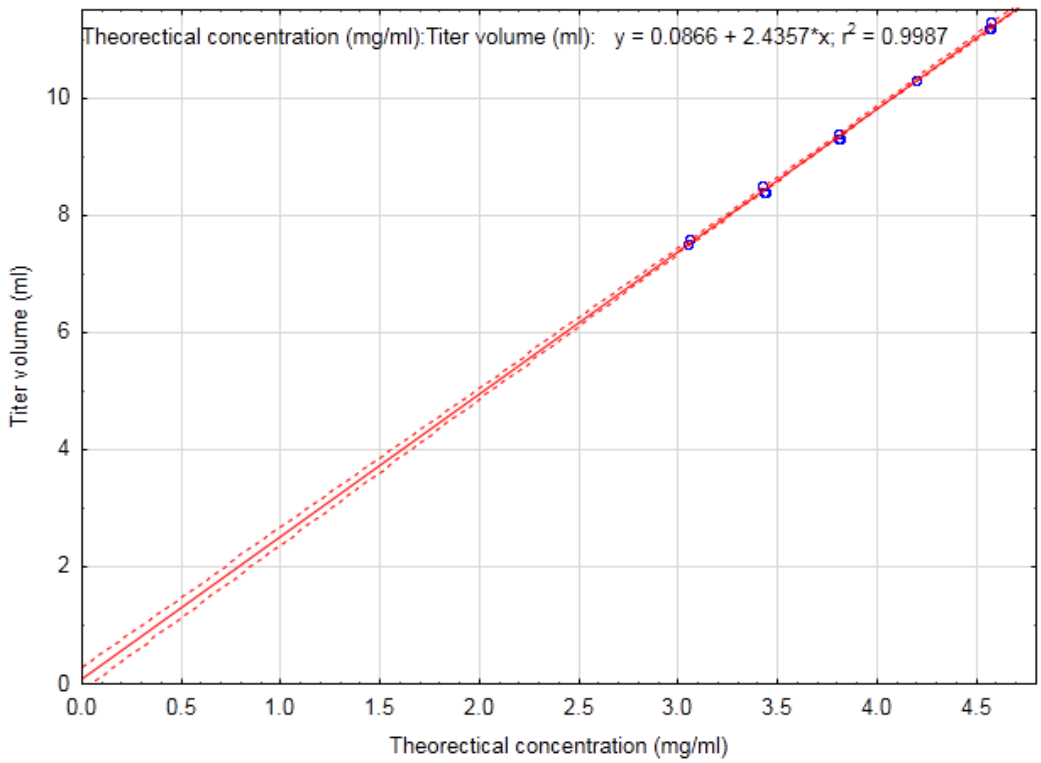


Figure 8.11 The titer volumes (ml) plotted as a function of the theoretical concentration of zinc gluconate (mg/ml) in the test solutions.

8.6.3 Accuracy

The accuracy of the method was evaluated using the results of the fifteen test solutions prepared in section 8.6.2.1 (as specified in Table 8.11) which covered the range of the analytical method.

The accuracy was reported as the percentage recovery (% recovery) from the assay of the zinc gluconate test solutions with known theoretical concentrations.

8.6.3.1 Procedure

Using the theoretical concentration and experimental concentration as calculated in section 8.6.2.3 (Table 8.12), calculate the % recovery using the following equation:

$$\%\ \text{Recovery} = \frac{\text{Experimental concentration (mg/ml)}}{\text{Theoretical concentration (mg/ml)}}\ \text{x}\ 100$$

8.6.3.2 Acceptance criterion

i. The % recovery values should range between: 98.0 % - 102.0 %.

This criterion is not as tight as for the zinc acetate dihydrate assay (99.0 - 101.0 % - section 8.3.3.2) due to the fact that the uncertainty of measurement of the moisture content determination should also be taken into account. However, the proposed acceptance criterion is still within the recommended specifications reported in literature (Ermer & Miller, 2005:327).

8.6.3.3 Results and discussion

The theoretical- and experimental zinc gluconate concentrations, and the % recovery for each of the test solutions were calculated. The results obtained are tabulated in Table 8.13.

Table 8.13 Zinc gluconate % recovery results

Test solutions	Replicates	Theoretical concentration (mg/ml)	Experimental concentration (mg/ml)	% Recovery
80 %	1	3.056	3.089	101.1
	2	3.044	3.048	100.1
	3	3.051	3.048	99.9
90 %	1	3.435	3.393	98.8
	2	3.428	3.414	99.6
	3	3.421	3.434	100.4
100 %	1	3.808	3.800	99.8
	2	3.804	3.779	99.3
	3	3.810	3.779	99.2
110 %	1	4.198	4.186	99.7
	2	4.195	4.186	99.8
	3	4.196	4.186	99.8
120 %	1	4.568	4.592	100.5
	2	4.572	4.552	99.6
	3	4.565	4.552	99.7
			Average	**99.8**
			SD	**0.56**
			% RSD	**0.56**

The % recovery was calculated over a concentration range: 3.2 – 4.8 mg/ml zinc gluconate. The individual % recovery values ranged between 98.8 % and 101.1 %. The average % recovery over the mentioned range was 99.8 %, and found to be within the acceptance criterion (98.0 – 102.0 %).

8.6.4 Repeatability and intermediate precision

Repeatability was assessed by estimating the variance in the triplicate % recovery values (section 8.6.3.3) reported for each concentration interval (i.e. 3.2 mg/ml, 3.6 mg/ml, 4.0 mg/ml, 4.4 mg/ml and 4.8 mg/ml) which covered the entire analytical range of the method.

The intermediate precision was assessed by evaluating the variance in assay values when the assay procedure was executed by three different analysts on different days in different laboratories on the same sample.

8.6.4.1 Procedure

Calculate the analytical variability of the % recovery results reported in section 8.6.3.3 (Table 8.13) by calculating:

i. The % RSD of the triplicate determinations at the respective concentrations: i.e. 3.2 mg/ml, 3.6 mg/ml, 4.0 mg/ml, 4.4 mg/ml and 4.8 mg/ml; and
ii. The % RSD for the whole analytical range (i.e. for all 15 % recovery values), using the following equation:

$$\% \, RSD = \frac{SD}{\left(\frac{\sum x}{n}\right)} \times 100$$

Where: n = 3 for the respective concentrations, n = 15 for the whole analytical range and SD is the standard deviation, which can be calculated using the following equation:

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

Where: x represents the respective % recovery values; $\bar{x}$ represents the average of the triplicate values for the respective concentrations or the average of the 15 values for the whole analytical range, and n is the sample size (i.e. n = 3 or n = 15).

For the investigation of the intermediate precision, three different analysts should execute the assay procedure (described in section 8.3.1.2) in triplicate, on different days and in different laboratories.

Calculate the theoretical- and experimental concentrations of the abovementioned test solutions using the equations presented in section 8.6.2.1. Calculate the % assay values using the following equation:

$$\% \text{ Assay} = \frac{\text{Experimental concentration (mg/ml)}}{\text{Theoretical concentration (mg/ml)}} \text{ x } 100$$

Perform a one-way ANOVA on the % assay values, using a 95 % confidence interval.

8.6.4.2 Acceptance criteria

i. For repeatability: the % RSD of the triplicate determinations at the respective concentrations: i.e. 3.2 mg/ml, 3.6 mg/ml, 4.0 mg/ml, 4.4 mg/ml and 4.8 mg/ml and that for the whole analytical range (i.e. for all 15 % recovery values) should not exceed 1.0 %.
ii. For intermediate precision: the % assay values reported by the three analysts should be comparable ($F < F_{crit}$).

8.6.4.3 Results and discussion

The % RSD of the triplicate determinations at the respective concentrations: i.e. 3.2 mg/ml, 3.6 mg/ml, 4.0 mg/ml, 4.4 mg/ml and 4.8 mg/ml did not exceed 1.0 %, as illustrated in Figure 8.12. The % RSD for the whole analytical range (i.e. for all 15 % recovery values) was calculated to be 0.56 %, which also complied with the specification (NMT 1.0 %) (Table 8.13).

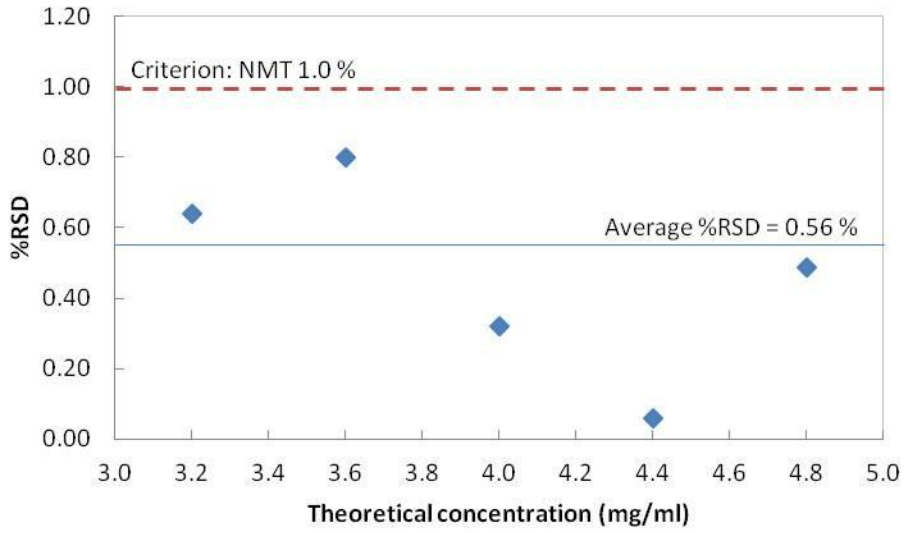


Figure 8.12 Graph of % RSD values calculated of the triplicate determinations at the respective zinc gluconate concentrations, i.e. 3.2 mg/ml, 3.6 mg/ml, 4.0 mg/ml, 4.4 mg/ml and 4.8 mg/ml. The solid line illustrates the % RSD for the whole analytical range (i.e. for all 15 % recovery values) and the dashed line illustrates the acceptance criterion.

Table 8.14 Tabulated results of the % assay of zinc gluconate for determination of intermediate precision

Analyst	% Assay	Average %	SD	% RSD
1	100.3	100.0	0.31	0.31
	100.1			
	99.7			
2	99.8	99.4	0.32	0.32
	99.3			
	99.2			
3	99.5	99.9	0.64	0.64
	100.6			
	99.5			
Average %		**99.8**		
SD		**0.31**		
% RSD		**0.31**		

The average % assay for each analyst varied between 99.4 % and 100.0 %, and is thus well within the range of 98.0 - 102.0 % (Table 8.14).

The one-way ANOVA was used to investigate the significance of variances in the % assays obtained by the three analysts; the results obtained can be seen in Figure 8.13.

The null hypothesis states that there is no significant difference between the means of the % assay values reported by the three analysts: H_0: $\mu_A = \mu_B = \mu_C$ (Bolton, 1997:273). The alternative hypothesis state that there is a difference between any two of the means: H_a: $\mu_i \neq \mu_j$. If the calculated F value is equal to or greater than F_{crit} at the specified α (confidence interval), the null hypothesis can be rejected (Table 4.13 in Chapter 4) (Bolton, 1997:273).

The F value (1.4) calculated is smaller than F_{crit} (5.1) which illustrated that there is no significant difference between the means of the % assay values reported by the three analysts (Figure 8.13). Thus, the means of the % assay values of the three analysts are comparable.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Analyst 1	3	300.10	100.03	0.0933
Analyst 2	3	298.30	99.43	0.1033
Analyst 3	3	299.60	99.87	0.4033

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.58	2	0.28778	1.43888889	0.308703065	5.14325285
Within Groups	1.20	6	0.20000			
Total	1.775556	8				

Figure 8.13 One-way ANOVA results for the means of the % assay values obtained by the three analysts for the complexometric titration of zinc gluconate.

8.6.5 Robustness

The same reasoning is applicable for the robustness, as in the case of zinc acetate dihydrate (refer to section 8.5.5), therefore the robustness of the method was investigated by determining the influence of variances in the pH of the solvent (acetic acid TS).

8.6.5.1 Procedure

Prepare three acetic acid TS with the following concentrations: ~5 g/l, ~10 g/l and ~15 g/l. Prepare three test solutions (in triplicate), with the target concentration of 4.0 mg/ml zinc gluconate. Transfer the weighed zinc gluconate samples into separate 100 ml Erlyn-meyer flasks and add 50 ml of the respective acetic acid (~5, ~10 or ~15 g/l) TS, swirl the solutions to dissolve. Add 50 mg of xylenol orange indicator mixture R to each test solution. Add about 5 g of methenamine R to the respective test solutions to turn the solutions pink-violet. Determine and record the average pH of the test solutions.

Determine the content of zinc gluconate in the respective test solutions by titrating with standardised EDTA (0.05 mol/l) VS until the solution turns from pink-violet to full yellow. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 22.78 mg of zinc gluconate (utilise standardised concentration of EDTA).

Calculate the theoretical and experimental concentrations, as well as the % assay using the formulas in section 8.6.2.1.

Perform a one-way ANOVA on the % assay values, using a 95 % confidence interval to investigate the significance of variances in the pH of the solvent on the robustness of the proposed method.

8.6.5.2 Acceptance criteria

The method of analysis should remain unaffected by deliberate changes in the pH of the solvent (acetic acid TS) used in the complexometric titration, where:

i. The % assay values should be within the range: 98.0 - 102.0 %; and
ii. The % assay values obtained using the three solvents with different pH should be comparable ($F < F_{crit}$).

8.6.5.3 Results and discussion

The pH values of the three different solvents (~5 g/l acetic acid TS, ~10 g/l acetic acid TS and ~15 g/l acetic acid TS), and that of the final test solutions (before commencement of the titration), were measured and are tabulated in Table 8.15.

The complexometric titrations in the three different solvents were executed and the results obtained are tabulated in Table 8.16.

Table 8.15 Average pH values of solvent and test solutions during robustness investigation of the assay for zinc gluconate

Solution	pH value
~5 g/l acetic acid TS	2.89
~10 g/l acetic acid TS	2.74
~15 g/l acetic acid TS	2.64
Zinc gluconate in ~5 g/l acetic acid TS	3.58
Zinc gluconate in ~10 g/l acetic acid TS	3.36
Zinc gluconate in ~15 g/l acetic acid TS	3.24

Table 8.16 The results of the complexometric titrations with the three different solvents to investigate the robustness of the proposed zinc gluconate assay method

Acetic acid TS	Replicates	Theoretical zinc gluconate concentration (mg/ml)	Experimental zinc gluconate concentration (mg/ml)	% Recovery
~5 g/l	1	3.806	3.800	99.8
	2	3.805	3.800	99.9
	3	3.810	3.779	99.2
~10 g/l	1	3.808	3.800	99.8
	2	3.804	3.779	99.3
	3	3.810	3.779	99.2
~15 g/l	1	3.817	3.800	99.6
	2	3.814	3.820	100.2
	3	3.808	3.779	99.3
			Average	**99.6**
			SD	**0.35**
			% RSD	**0.35**

The one-way ANOVA was used to investigate the significance of variances in the pH of the solvent on the robustness of the proposed method. The results obtained are summarised in Figure 8.14.

The null hypothesis states that there is no significant difference between the means of the % assay values in the three different solvents: H_0: $\mu_A = \mu_B = \mu_C$ (Bolton, 1997:273). The alternative hypothesis states that there is a difference between any two of the means: H_a: $\mu_i \neq \mu_j$. If the calculated F value is equal to or greater than F_{crit}

at the specified α (confidence interval), the null hypothesis can be rejected (Table 4.13 in Chapter 4) (Bolton, 1997:273).

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
~5g/L	3	298.90	99.63	0.1433
~10g/L	3	298.30	99.43	0.1033
~15g/L	3	299.00	99.67	0.2533

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.09555556	2	0.04777778	0.286666667	0.76049574	5.14325285
Within Groups	1	6	0.16666667			
Total	1.09555556	8				

Figure 8.14 One-way ANOVA results of the means of the % assay values in the three different solvents used in the complexometric titration of zinc gluconate.

The F value (0.3) calculated is smaller than F_{crit} (5.1) which illustrated that there is no significant difference between the means of the % assay values in the three different solvents (Figure 8.14). Thus, it can be concluded that the results obtained from the method of analysis remained unaffected by the deliberate changes in the pH of the solvent (acetic acid TS) used for the complexometric titration.

8.6.6 Conclusion

To determine the zinc gluconate content by means of complexometric titrations the method was validated with regards to the following parameters: specificity, linearity and range, accuracy, precision (repeatability and intermediate precision), and robustness. The acceptance criteria for each validation parameter were met, therefore the method can be considered fit for its purpose to determine the content / assay of zinc gluconate API.

8.7 Assay determination of commercially available zinc acetate dihydrate and zinc gluconate APIs using the proposed methods

The proposed assay methods for zinc acetate dihydrate (section 8.3.1.1) and zinc gluconate (section 8.3.1.2) were successfully validated by means of method

validations. These methods are thus deemed suitable, and were utilised for the assay determination of commercially available zinc acetate dihydrate and zinc gluconate APIs.

8.7.1 Procedure

Prepare the following test solutions (in triplicate):

a) Dissolve about 100 mg zinc acetate dihydrate, accurately weighed, in 50 ml of acetic acid (~10 g/l) TS.
b) Dissolve about 200 mg zinc gluconate, accurately weighed, in 50 ml of acetic acid (~10 g/l) TS.

Proceed with the titration as described under section 8.5.2.1 for the zinc acetate dihydrate test solution, and section 8.6.2.1 for the zinc gluconate test solution.

8.7.2 Results

The standardised concentration of the EDTA (0.05 mol/l) VS was determined as 0.0446 mol/l (n = 5; % RSD = 0.75 %). Each ml of EDTA (0.05 mol/l) VS is equivalent to 10.98 mg zinc acetate dihydrate, meaning that each ml of EDTA (0.0446 mol/l) VS is equivalent to 9.794 mg zinc acetate dihydrate. Each ml of EDTA (0.05 mol/l) VS is equivalent to 22.78 mg zinc gluconate, meaning that each ml of EDTA (0.0446 mol/l) VS is equivalent to 20.320 mg zinc gluconate.

The assay results for zinc acetate dihydrate are summarised in Table 8.17, and that for zinc gluconate are in Table 8.18.

Table 8.17 Assay results of commercially available zinc acetate dihydrate API

Test solution	Mass of sample (mg)	Theoretical concentration (mg/ml)	Titer volume (ml)	Experimental concentration (mg/ml)	% Assay
1	100.40	2.008	10.2	1.998	99.5
2	102.16	2.043	10.4	2.037	99.7
3	101.53	2.031	10.4	2.037	100.3
				Average	99.8
				SD	0.43
				% RSD	0.43

Table 8.18 Assay results of commercially available zinc gluconate API

Test solution	Mass of sample (mg)	Theoretical concentration (mg/ml)	Titer volume (ml)	Experimental concentration (mg/ml)	% Assay
1	200.38	3.808	9.4	3.800	99.8
2	200.19	3.804	9.3	3.779	99.3
3	200.50	3.810	9.3	3.779	99.2
				Average	99.4
				SD	0.31
				% RSD	0.31

8.7.3 Discussion

The assays of zinc acetate dihydrate and zinc gluconate APIs were determined by means of complexometric titration. The complexometric titration was carried out using EDTA as the titrant (ligand) and the end-point was determined by using xylenol orange as indicator. The samples were assayed in triplicate, and the theoretical concentration, experimental concentration and % assay calculated for each sample.

The average % assay for zinc acetate dihydrate was 99.8 %, and varied between 99.5 % and 100.3 %, with the % RSD of 0.43 %. The sample thus complied with the specified limits (99.0 % - 101.0 %).

The average % assay for zinc gluconate was 99.4 %, and varied between 99.2 % and 99.8 %, with the % RSD of 0.31 %. The sample thus complied with the specified limits of 98.0 % - 102.0 %.

8.8 Conclusion

Criteria were established for the assay of zinc acetate dihydrate and zinc gluconate APIs based on the information provided in this Chapter.

Two complexometric titration methods were developed for the assay of zinc acetate dihydrate and zinc gluconate APIs respectively, based on the current zinc-EDTA complexometric titration method available in *The Ph. Int*.

To illustrate the fitness for purpose of the two methods, both methods were validated. All the validation parameters were met, deeming the methods suitable for use.

Commercially available zinc acetate dihydrate and zinc gluconate APIs were tested using the developed methods and it was found that both APIs complied with the proposed criteria.

The proposed assay methods are therefore recommended for inclusion in the zinc acetate dihydrate and zinc gluconate API monographs of *The Ph. Int*.

CHAPTER 9

ORGANIC IMPURITIES

9.1 Introduction

Ahuja (1998:1) defines an impurity as any material that affects the purity of the material of interest. Impurity testing is required due to the presence thereof in APIs and Inactive Pharmaceutical Ingredients (IPIs) which may be responsible for toxic effects, chemical interference during the analysis of the API and it's ability to potentiate the instability of the final product (Kar, 2005:4).

The requirements of monographs are not framed to detect all possible impurities. The monographs presented in *The Ph. Int.* are designed to determine impurities on which attention should be focused, to fix the limits of those that are tolerable to a certain extent, and to provide methods to control or to ensure the absence of those that are undesired. It is, therefore, not to be presumed that an impurity can be tolerated because it has not been precluded by the prescribed tests in *The Ph. Int.* (*Ph. Int*., 2011).

According to ICH Q3A (R2) (ICH, 2008:1) impurities can be classified into three categories:

- Organic impurities (process- and API related);
- Inorganic impurities; and
- Residual solvents.

The Ph. Int. does not include residual solvent testing specifications in their API monographs (*Ph. Int.,* 2011), thus only organic- and inorganic impurities will be considered in the following three chapters.

The aims of this chapter were to:

i) Identify the potential impurities that could be present in zinc acetate dihydrate and zinc gluconate APIs;
ii) Set specifications for the identified impurities; and

iii) Develop or propose limit testing methods for organic impurities which are to be included into *The Ph. Int.* monographs.

9.2 Identification of potential impurities that could be present in zinc acetate dihydrate and zinc gluconate APIs based on available literature

Impurities which are likely to be present in a given pharmaceutical substance can be determined / compiled from prior art knowledge of the starting materials employed during the manufacturing, the manufacturing process and the stability of the final API. Impurities may also arise from physical contamination and improper storage conditions (Goel, 2007:2).

About 70 % of the world's zinc originates from mining, while the remaining 30 % is obtained from the recycling of secondary zinc. Zinc is mined from base metal ores, that also contain sulfates, arsenic, cadmium, copper, iron, lead, and nickel in various concentrations (World Bank Group, 1998:270). The aforementioned can thus all be considered as possible impurities in zinc raw materials, which are used as starting materials for the synthesis of zinc acetate dihydrate and zinc gluconate. Cadmium is a known impurity of zinc metal and zinc salts (Calvert, 2002:1).

Limited information is available in the literature with regards to the routes of synthesis of zinc acetate dihydrate and zinc gluconate APIs. The route of synthesis of zinc acetate dihydrate consists of the reaction of zinc oxide with glacial acetic acid, consequent crystallisation, separation of the crystals by centrifugation, followed by the drying and milling of the final API crystals (Figure 9.1) (Anon, 2004:2).

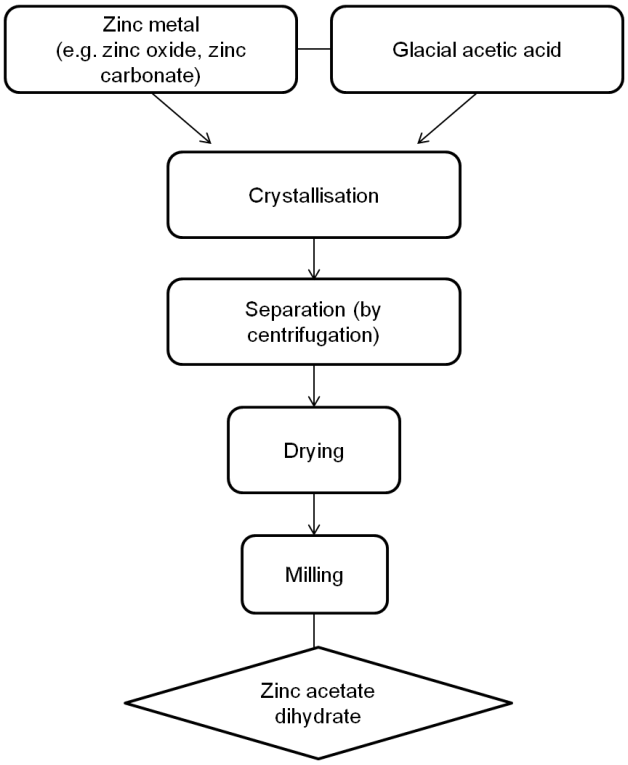


Figure 9.1 Flowchart of the synthesis of zinc acetate dihydrate (Anon, 2004:2).

Zinc gluconate is synthesised by the reaction of zinc metal base with gluconic acid. Gluconic acid is prepared by means of batch fermentation or using suitable enzymes. In a United States Patent by Chatterjee *et al.* (2004:8) a method is proposed for the production of metal gluconates, which includes zinc gluconate. The proposed method entails the following:

a) Adding a glucose solution to a preparation comprising glucose oxidase in a reaction chamber and adding a metal base to the reaction chamber to form a reaction mixture containing metal gluconate.
b) Removing a portion of the reaction mixture from the reaction chamber.
c) Separating a permeate from the portion of the reaction mixture by ultra filtration; and
d) Separating metal gluconate from the permeate to form a residual solvent.

Due to the abovementioned fermentation process it is clear that microbial contamination testing should be included in the monograph for zinc gluconate API (ICH, 1999:5). It is therefore recommended that the microbial contamination test of the Ph. Eur. / BP should be included in *The Ph. Int.* monograph (if deemed

necessary by *The Ph. Int.*). Unfortunately microbial testing is not performed at the RIIP® incorporating CENQAM®, thus it will not be covered in this dissertation.

Zinc acetate dihydrate is a stable compound, is not hygroscopic and the formation of zinc oxide is unlikely during storage (Anon, 2004:3). Zinc gluconate is chemically stable under standard ambient conditions (Merck Chemicals Ltd, 2011:6).

9.3 Specifications for the impurities present in zinc acetate dihydrate and zinc gluconate APIs

The EMA states that harmonised pharmacopoeial specifications may be utilised where the procedure and acceptance criteria defined are acceptable to regulatory authorities in all regions (EMA, 2000:6).

The International Conference on Harmonisation of Technical Requirements for registration of Pharmaceuticals for Human Use is currently in the process of developing a Q3D guideline, namely “Impurities: Guideline for Metal Impurities”, in order to address some of the issues which are not addressed in Q3A(R2) (ICH, 2009:1). According to the ICH Harmonised tripartite guideline on Impurities in New Drug Substances Q3A (R2) (ICH, 2008:3), the acceptance criteria for inorganic impurities could be based on pharmacopoeial standards or known safety data.

Based on the abovementioned recommendation, a summary of the zinc salt impurities and their respective limits / specifications was constructed from the information available in current pharmacopoeias (Table 9.1).

Table 9.1 Summary of impurity tests and their respective limits / specifications for zinc acetate dihydrate and zinc gluconate according to the available pharmacopoeias (BP, 2011; Ph. Eur., 2011; USP, 2011)

Impurity	Zinc acetate dihydrate		Zinc gluconate	
	Ph. Eur. / BP	USP	Ph. Eur. / BP	USP
Reducing substances	Pink colour remains	-	-	-
Reducing sugars	-	-	No red precipitate is formed	NMT 1.0 %
Chlorides	50 ppm	0.005 %	500 ppm	0.05 %
Sulfates	100 ppm	0.010 %	500 ppm	0.05 %
Arsenic	2 ppm	3 ppm	-	3 ppm
Heavy metals	-	-	10 ppm	-
Aluminium	5.0 ppm	-	-	-
Cadmium	2.0 ppm	-	2.0 ppm	5 ppm
Copper	50.0 ppm	-	-	-
Iron	50.0 ppm	-	-	-
Lead	10.0 ppm	0.002 %	-	0.001 %

Based on the information reported in section 9.2, available certificates of analysis from various manufacturers of zinc acetate dihydrate and zinc gluconate and the harmonisation of *The Ph. Int.* and BP, it was decided to adopt the Ph. Eur. / BP list of impurities and their specified limits / specifications.

The abovementioned impurities can be classified into three groups as depicted in Figure 9.2. This chapter will focus on the organic impurities, Chapter 10 on the acid radical impurities and Chapter 11 on the metallic impurities.

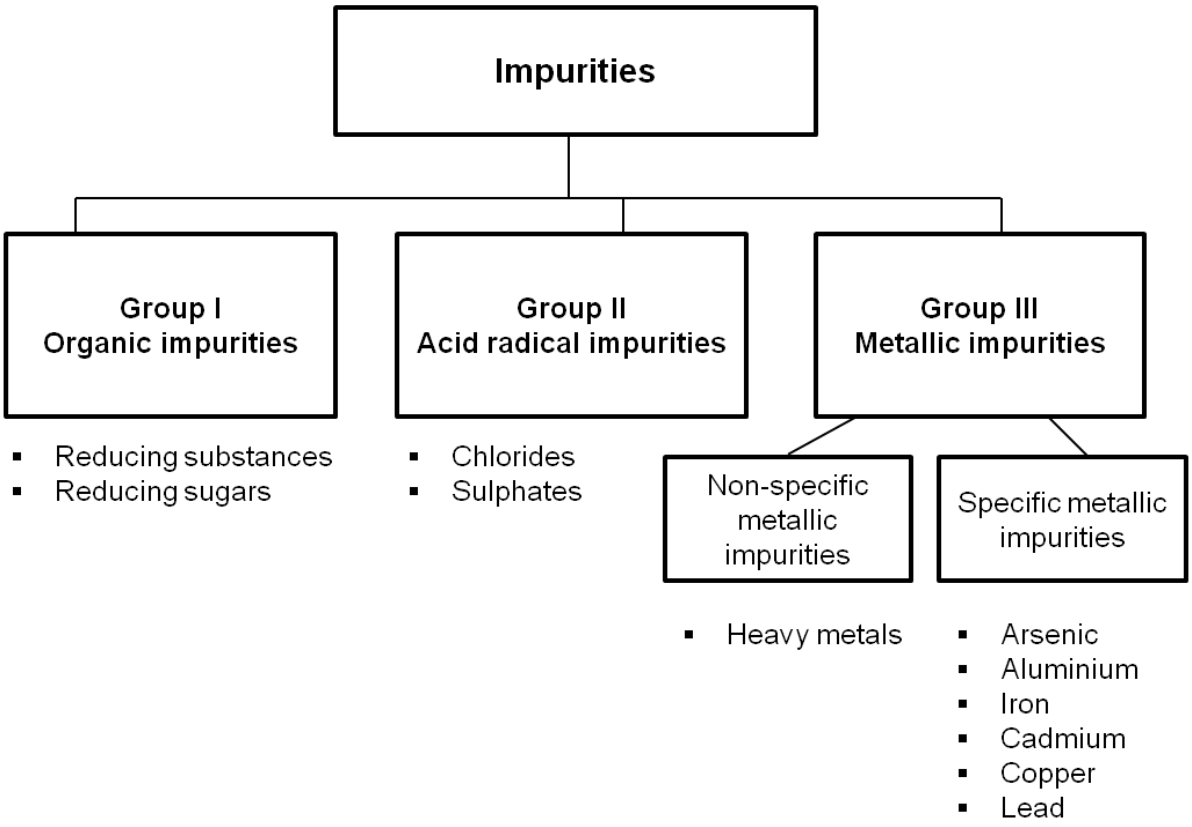


Figure 9.2 Classification of the impurities to be studied in Chapters 9 - 11.

9.4 Development of methods for Group 1 - Organic impurities

Reducing substances donate electrons for other substances and are oxidised in the process (Harris, 2007:271). Reducing substances comprise of all the sugars exhibiting ketonic and aldehydic functions and are detected by their reducing action on an alkaline solution of a copper salt (OVI, 2009:1).

The Ph. Int. and the Ph. Eur. / BP recommend the use of colorimetric limit tests to test for the presence of any reducing substance / reducing sugars in APIs.

Since the recommended methods for the detection of these two organic impurities are already established in *The Ph. Int.* and / or the Ph. Eur. / BP, only method verifications were performed to ensure the fitness for purpose of the proposed methods, instead of complete revalidation of the methods (Ermer & Miller, 2005:302).

9.4.1 Proposed method to test for the presence of reducing substances in zinc acetate dihydrate API

The following method (adopted from Ph. Eur. / BP) is proposed to test for the presence of reducing substances in zinc acetate dihydrate API:

Dissolve 1 g in 10 ml of water R. Boil with 90 ml of water R, 5 ml of sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~0.3 g/l) TS; the pink colour of the solution remains.

For this method to be adopted by *The Ph. Int.* the potassium permanganate (~ 0.3 g/l) TS needs to be defined and included into the "*Reagents, test solutions and volumetric solutions*" section of *The Ph. Int.* The following text is recommended for inclusion:

Potassium permanganate (~0.3 g/l) TS.

A solution of potassium permanganate R containing about 0.3 g of $KMnO_4$ per litre.

9.4.2 Method verification of the reducing substances test method for zinc acetate dihydrate API

This colorimetric method is based on disappearance of the pink colour of potassium permanganate in the presence of a reducing substance (Ph. Eur., 2011; BP, 2011). To ensure this method's fitness for purpose, method verification was performed based on the recommendations presented in the literature (Ermer & Miller, 2005:311). The specificity and a relative detection limit of the method were investigated. No specific limit is reported in the pharmacopoeias for reducing substances, therefore the ability of the method to detect reducing substances in a range: 0.1, 0.3 and 0.6 % (m/m) was evaluated.

9.4.2.1 Materials and equipment

The information of the materials used in the reducing substances test of zinc acetate dihydrate is tabulated in Table 9.2. A Sartorius ED623S+ balance (IMP, SA) was used during this test.

Table 9.2 Information of materials used in the reducing substances test of zinc acetate dihydrate

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Sulfuric acid	1035329	Merck Chemicals Pty. Ltd.	South Africa
Potassium permanganate	A892302 020	Merck KGaA	Germany
D-(+) Dextrose	052 K0002	Sigma-Aldrich, Inc.	Germany
Water R	N/A	RIIP®/CENQAM®	South Africa

To investigate the selectivity / specificity of the method three solutions were utilised:

- Solution A: A blank solution (water R) that did not contain any reducing substances.
- Solution B: An aqueous solution which contained 0.005 g dextrose.
- Solution C: The test solution which was spiked with dextrose (approximately 0.005 g) to contain 0.6 % m/m dextrose in zinc acetate dihydrate.

The absence of reducing substances in a test solution is illustrated in section 9.4.3.

To investigate the relative limit of detection (using a range of 0.1 - 0.6 % m/m) three test solutions (solutions D - F) were prepared (as described in section 9.4.1). The three test solutions were spiked with an aqueous dextrose solution in order to evaluate the ability of the method to detect 0.1, 0.3 and 0.6 % m/m dextrose in zinc acetate dihydrate.

9.4.2.2 Procedure

In six separate flasks boil the following solutions for 5 minutes:
A) A mixture of 100 ml of water R, 5 ml of dilute sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~ 0.3 g/l) TS.

B) A mixture of 1 ml of 0.5 % dextrose solution, 99 ml of water R, 5 ml of dilute sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~ 0.3 g/l) TS.

C) Dissolve 1 g zinc acetate dihydrate in 10 ml of water R. To 9.0 ml of this test solution add 1 ml of a 0.5 % dextrose solution, 90 ml of water R, 5 ml of dilute sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~0.3 g/l) TS.

D) Dissolve 1 g zinc acetate dihydrate in 10 ml of water R. To 9.0 ml of this test solution add 1 ml of a 0.5 % dextrose solution, 90 ml of water R, 5 ml of dilute sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~0.3 g/l) TS.

E) Dissolve 1 g zinc acetate dihydrate in 10 ml of water R. To 9.0 ml of this test solution add 1 ml of a 0.25 % dextrose solution, 90 ml of water R, 5 ml of dilute sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~0.3 g/l) TS.

F) Dissolve 1 g zinc acetate dihydrate in 10 ml of water R. To 9.0 ml of this test solution add 1 ml of a 0.05 % dextrose solution, 90 ml of water R, 5 ml of dilute sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~0.3 g/l) TS.

9.4.2.3 Acceptance criteria

i. A sufficient level of specificity is illustrated when the pink colour of solution A remains; and that of solutions B and C disappear.
ii. The relative detection limit can be assigned to the lowest concentration of dextrose in solutions D - F, where the pink colour of the solution disappears.

9.4.2.4 Results and discussion

When the procedure for specificity was executed the pink colour of solution A remained; and that of solutions B and C disappeared; see Figure 9.3 for a photograph of the results.

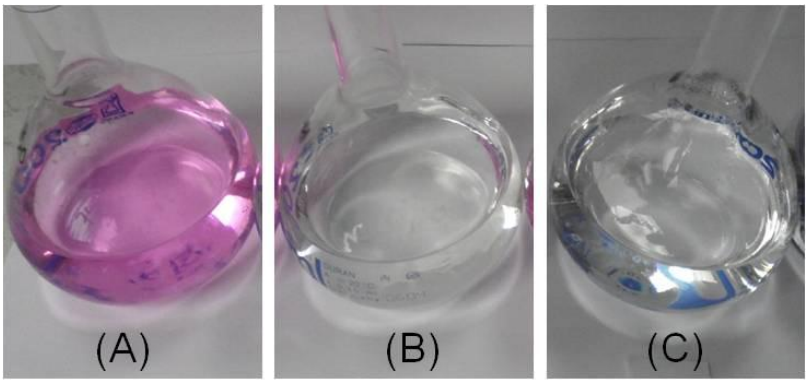


Figure 9.3 Photograph of selectivity results: (A) Solution A (water R), (B) Solution B (aqueous solution containing 0.005 g dextrose), (C) Solution C (test solution spiked to contain 0.6 % m/m dextrose).

The pink colour of solutions D, E and F disappeared completely (Figure 9.4) indicating that 0.1 – 0.6 % (m/m) of the reducing substance can be detected in zinc acetate dihydrate API.

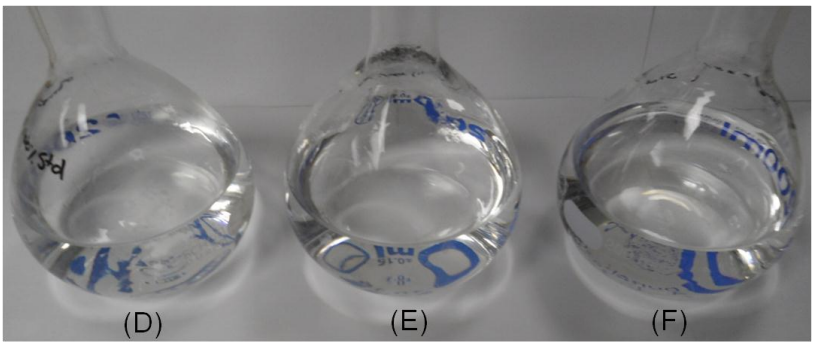


Figure 9.4 Photograph of the relative detection limit results: (D) zinc acetate dihydrate solution spiked to contain 0.6 % m/m dextrose, (E) zinc acetate dihydrate solution spiked to contain 0.3 % m/m dextrose, (F) zinc acetate dihydrate solution spiked to contain 0.1 % dextrose.

9.4.2.5 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The reducing substance in the spiked zinc acetate dihydrate samples was detected in a quantity of up to 0.1 % m/m in zinc acetate dihydrate.

It can thus be concluded that this limit test is fit for the limiting of reducing substances in zinc acetate dihydrate API.

9.4.3 Detection of reducing substances in commercially available zinc acetate dihydrate using the proposed method

The proposed method (described in section 9.4.1) was applied to test a commercially available sample of zinc acetate dihydrate for the presence of reducing substances. The product information is summarised in Table 9.2.

9.4.3.1 Procedure

Dissolve 1 g zinc acetate dihydrate in 10 ml of water R. Boil with 90 ml of water R, 5 ml of sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~0.3 g/l) TS; the pink colour of the solution remains.

9.4.3.2 Results and discussion

The test solution mixture remained pink after the sample was boiled for 5 minutes; a photograph of the mixture is illustrated in Figure 9.5.

Figure 9.5 Photograph of the reducing substances impurity test solution on a commercially available sample of zinc acetate dihydrate.

The commercially available sample of zinc acetate dihydrate API complied with the specification of the test for reducing substances, which indicated the absence of any reducing substance.

9.4.4 Proposed method to test for the presence of reducing sugars in zinc gluconate API

The following method (adopted from Ph. Eur. / BP) is proposed to test for the presence of reducing sugars in zinc gluconate API:

Dissolve 0.5 g in a mixture of 2 ml of hydrochloric acid (~330 g/l) TS and 10 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (~10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes; no red-brown precipitate is formed.

For this method to be adopted by *The Ph. Int.* the cupri-tartaric TS needs to be defined and included into the "*Reagents, test solutions and volumetric solutions*" section of *The Ph. Int.* The following text is recommended for inclusion:

Cupri-tartaric TS.
Procedure. Dissolve 34.6 g copper (II) sulfate R in sufficient water to produce 100 ml. Separately dissolve 173 g of potassium sodium tartrate R and 50 g sodium hydroxide in 400 ml water R; heat to boiling, allow to cool and dilute to 500 ml with water R. Shortly before use, mix together equal volumes of both solutions.

9.4.5 Method verification of the reducing sugar testing method for zinc gluconate API

This method is based on the appearance of a red precipitate in the presence of a reducing sugar (BP, 2011; Ph. Eur., 2011). To ensure this method's fitness for purpose, method verification was performed based on the recommendations presented in the literature (Ermer & Miller, 2005:311). The specificity and a relative detection limit of the method were investigated.

9.4.5.1 Materials and equipment

The information of the materials used in the reducing sugar test of zinc gluconate API is tabulated in Table 9.3. A Sartorius ED623S+ balance (IMP, SA) was used during this test.

Table 9.3 Information of the materials used in the test for reducing sugars of zinc gluconate API

Material	Batch number	Manufacturing company	Country of origin
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Hydrochloric acid	K42075217 111	Merck KGaA	Germany
Sodium carbonate	088 K0038	Sigma-Aldrich, Inc.	USA
Copper (II) sulfate	1035121	Merck Chemicals (Pty) Ltd.	South Africa
Potassium sodium tartrate	1032774	Merck Chemicals (Pty) Ltd.	South Africa
Sodium hydroxide	MB0M600313	Merck Chemicals (Pty) Ltd.	South Africa
D-(+) Dextrose	052 K0002	Sigma-Aldrich, Inc.	Germany
Water R	N/A	RIIP®/CENQAM®	South Africa

To investigate the selectivity / specificity of the method three solutions were utilised:

- Solution A: A blank solution that did not contain any API or reducing sugars.
- Solution B: An aqueous solution containing 0.005 g dextrose.
- Solution C: The test solution which was spiked with dextrose (approximately 0.005 g) to contain 1.0 % m/m dextrose in zinc gluconate.

The absence of reducing sugars in a test solution is illustrated in section 9.4.6.

To investigate the relative limit of detection (using a range of 1.0 - 0.1 % m/m) three test solutions (solutions D - F) were prepared (as described in section 9.4.4). The three test solutions were spiked with an aqueous dextrose solution in order to evaluate the ability of the method to detect 0.1, 0.5 and 1.0 % (m/m) dextrose in zinc gluconate.

9.4.5.2 Procedure

Prepare the following six solutions:

A) Prepare a mixture of 2 ml of hydrochloric acid (~330 g/l) TS and 10 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes.

B) Transfer 1 ml of a 0.5 % dextrose solution into a mixture of 2 ml of hydrochloric acid (~330 g/l) TS and 10 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml

of sodium carbonate (10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes.

C) Dissolve 0.5 g zinc gluconate in a mixture of 1 ml of a 0.5 % dextrose solution, 2 ml of hydrochloric acid (~330 g/l) TS and 9 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (~10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes.

D) Dissolve 0.5 g zinc gluconate in a mixture of 1 ml of a 0.5 % dextrose solution, 2 ml of hydrochloric acid (~330 g/l) TS and 9 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (~10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes.

E) Dissolve 0.5 g zinc gluconate in a mixture of 1 ml of a 0.25 % dextrose solution, 2 ml of hydrochloric acid (~330 g/l) TS and 9 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (~10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes.

F) Dissolve 0.5 g zinc gluconate in a mixture of 1 ml of a 0.05 % dextrose solution, 2 ml of hydrochloric acid (~330 g/l) TS and 9 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (~10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes.

9.4.5.3 Acceptance criteria

i. A sufficient level of specificity is illustrated when no red precipitate is formed by solution A; but a red precipitate if formed by solutions B and C.
ii. The relative detection limit can be assigned to the lowest concentration of dextrose in solutions D - F, where a red precipitate is formed.

9.4.5.4 Results and discussion

When the procedure for specificity was executed no red precipitate was formed by solution A; but a red precipitate was formed by solutions B and C; see Figure 9.6 for a photograph of the results.

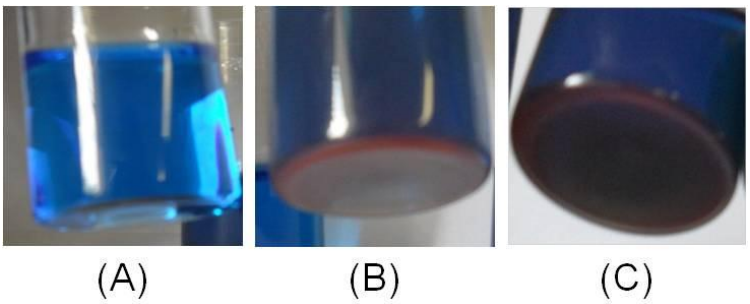


Figure 9.6 Photograph of selectivity results: (A) Solution A (blank solution), (B) Solution B (aqueous solution containing 0.005 g dextrose), (C) Solution C (test solution spiked to contain 1.0 % m/m dextrose).

A red precipitate formed in solutions D, E and F (Figure 9.7). The intensity of the red precipitate decreased with the decreasing concentrations of dextrose present in the sample. The 0.1 % m/m dextrose spiked sample showed a faint red precipitate when compared to the negative reference (Figure 9.6) which revealed that 0.1 - 1.0 % m/m of the reducing sugars can be detected in zinc gluconate API.

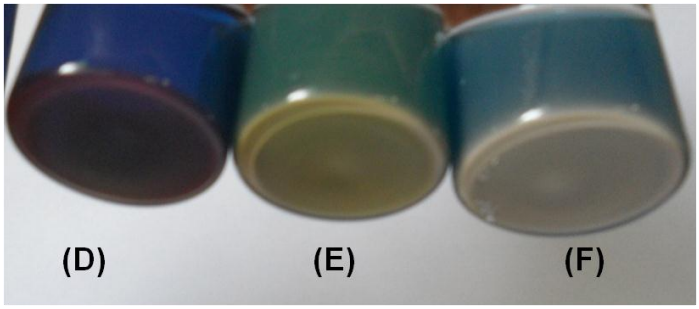


Figure 9.7 Photograph of the relative detection limit results: (D) zinc gluconate solution spiked to contain 1.0 % m/m dextrose, (E) zinc gluconate solution spiked to contain 0.5 % m/m dextrose, (F) zinc gluconate solution spiked to contain 0.1 % m/m dextrose.

9.4.5.5 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The reducing sugar in the spiked zinc gluconate samples was detected in a quantity of up to 0.1 % m/m dextrose in zinc gluconate.

It can thus be concluded that this limit test is fit for the limiting of reducing sugars in zinc gluconate API.

9.4.6 Detection of reducing sugars in commercially available zinc gluconate using the proposed method

The proposed method (described in section 9.4.4) was applied to test a commercially available sample of zinc gluconate for the presence of reducing sugars. The product information is summarised in Table 9.3.

9.4.6.1 Procedure

Dissolve 0.5 g in a mixture of 2 ml of hydrochloric acid (~330 g/l) TS and 10 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (~10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes; no red-brown precipitate is formed.

9.4.6.2 Results and discussion

No red precipitate formed in the test solution as illustrated in the photograph in Figure 9.8.

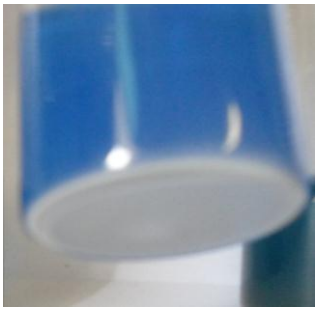


Figure 9.8 Photograph of the reducing sugar impurity test solution on a commercially available sample of zinc gluconate.

The commercially available sample of zinc gluconate API complied with the specification of the test for reducing sugars.

9.5 Conclusion

Nine potential impurities were identified for zinc acetate dihydrate API and six potential impurity types for zinc gluconate API which need to be controlled by means of suitable limit tests. The limits / specifications set for the respective limit tests were adopted from the available literature (i.e. Ph. Eur. / BP monographs).

The recommended limit tests and their specifications are summarised in Table 9.4.

Table 9.4 Summary of limit tests and their respective limits / specifications for zinc acetate dihydrate and zinc gluconate APIs to be included in *The Ph. Int.*

Impurity test	Zinc acetate dihydrate	Zinc gluconate
Reducing substances/ sugars	Pink colour remains	No red precipitate is formed
Chlorides	50 µg/g	500 µg/g
Sulfates	100 µg/g	500 µg/g
Arsenic	2 µg/g	-
Heavy metals	-	10 µg/g
Aluminium	5.0 µg/g	-
Cadmium	2.0 µg/g	2.0 µg/g
Copper	50.0 µg/g	-
Iron	50.0 µg/g	-
Lead	10.0 µg/g	-
Microbial contamination testing	-	To be established

The reducing substances and reducing sugars tests (Table 9.4) can be classified as organic impurities. To limit these two organic impurities, the reducing substances limit test (for zinc acetate dihydrate API) and the reducing sugars limit test (for zinc gluconate API) were adopted from the Ph. Eur. / BP. The fitness for purpose of these two methods was illustrated by means of suitable method verification procedures.

The aforementioned two methods will therefore be recommended to be included into *The Ph. Int.* monographs.

CHAPTER 10

ACID RADICAL IMPURITIES

10.1 Introduction

Acid radical impurities arise from the use of tap water during the manufacturing of APIs. The two most commonly found acid radical impurities are chloride (Cl^-) and sulfate (SO_4^{2-}) (Beckett & Stenlake, 1975:35). Due to the fact that these two acid radical impurities are commonly found, the pharmacopoeias categorically stipulate limit tests for them (Kar, 2005:30).

The aim of this chapter was to develop or propose limit testing methods for acid radical impurities for inclusion in *The Ph. Int.* monographs.

10.2 Specifications for the acid radical impurities present in zinc acetate dihydrate and zinc gluconate APIs

Chlorides and sulfates can be classified as acid radical impurities, and were both identified in Chapter 9 as potential impurities that could be present in zinc acetate dihydrate and zinc gluconate APIs (refer to Figure 9.2). Specifications for the chloride and sulfate limit tests were derived from those set for these specific zinc salts as published in the Ph. Eur. / BP (BP, 2011; Ph. Eur., 2011) (Table 10.1).

Table 10.1 Acid radical impurities limit tests and specifications applicable to zinc acetate dihydrate and zinc gluconate APIs (BP, 2011; Ph. Eur., 2011)

Impurity test	Zinc acetate dihydrate	Zinc gluconate
Chlorides	50 µg/g	500 µg/g
Sulfates	100 µg/g	500 µg/g

The differences in the specifications for the two APIs can be attributed to the fact that the acceptance criteria of the impurity tests are based on knowledge of the manufacturing process (EMA, 2000:11). The manufacturing process of zinc acetate dihydrate and zinc gluconate differs significantly (Chapter 9 – section 9.2). The higher chloride and sulfate limits for zinc gluconate might be attributed to the fact that

it is exposed to higher levels of chloride and sulfate during the manufacturing process, compared to zinc acetate dihydrate.

There is no absolute limit set for acid radical impurities in APIs. The specification for chloride limit test for APIs in *The Ph. Int*. ranges between 10 - 4000 µg/g (Figure 10.1) and for sulfates between 20 - 10000 µg/g (Figure 10.2).

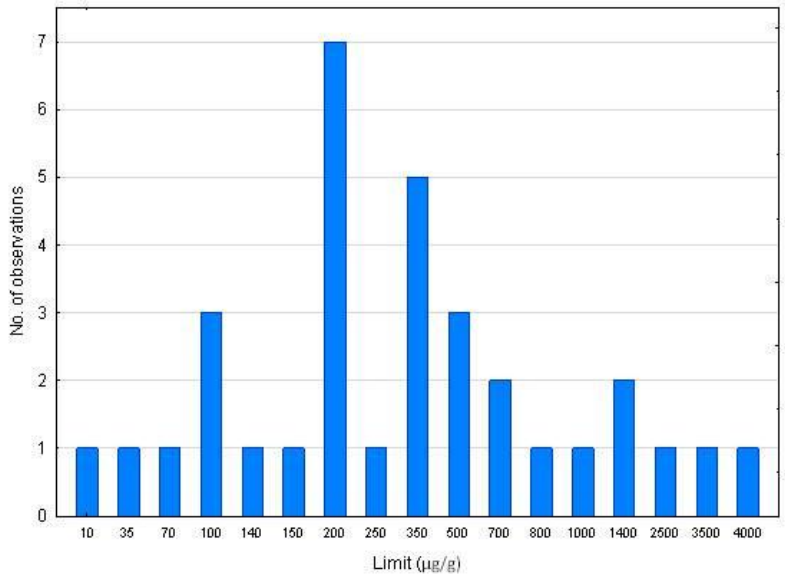


Figure 10.1 Limit test for chlorides specifications currently available for API monographs of *The Ph. Int.* (*Ph. Int*., 2011).

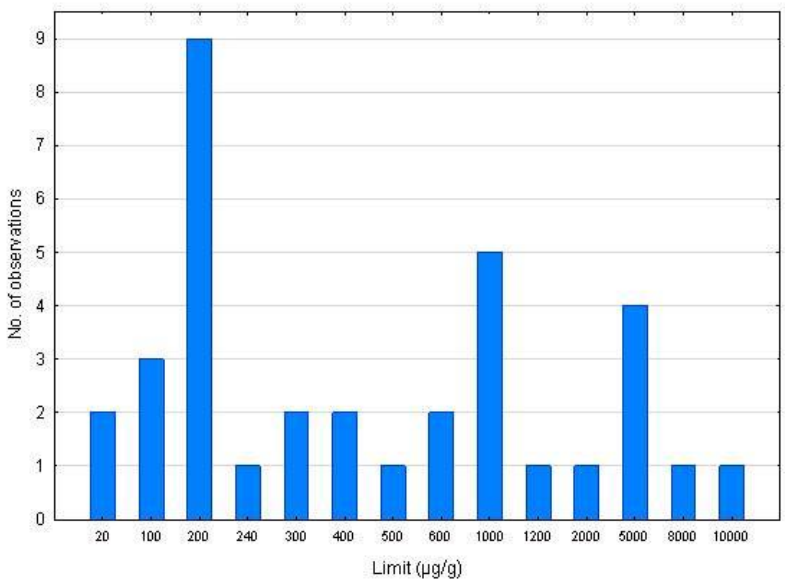


Figure 10.2 Limit test for sulfates specifications currently available for API monographs of *The Ph. Int.* (*Ph. Int*., 2011).

Based on the limited information available on the manufacturing of zinc acetate dihydrate and zinc gluconate, and the harmonisation between *The Ph. Int*. and BP, the BP specification limits for chloride and sulfate limit test (Table 10.1) are proposed for adoption by *The Ph. Int*.

10.3 Development of methods for Group II Acid radical impurities – Chlorides

The well established chloride limit test in section 2.2.1 Limit test for chlorides, of *The Ph. Int.*, was applied on zinc acetate dihydrate and zinc gluconate APIs.

This method is based on the precipitation of chloride with silver nitrate in the presence of dilute nitric acid (Kar, 2005:30):

$$NaCl + AgNO_3 \rightarrow AgCl \downarrow + NaNO_3$$

A comparison is made between the opalescence of the test solution and a standard opalescence solution (hydrochloric acid-chloride test solution / hydrochloric acid ClTS), to establish whether the chloride content of the test solution exceeds the limit reported in the specific monograph in terms of micrograms of chloride ions per gram of the substance being tested. The standard solution against which the comparison of opalescence is made contains 250 µg of Cl^- ions (*Ph. Int.*, 2011). The following recommended procedure and standard opalescence solution are described in *The Ph. Int*. in section 2.2.1 Limit test for chlorides:

"Recommended procedure

Carry out the test in matched flat-bottomed comparison tubes of transparent glass of about 70 ml capacity and about 23 mm internal diameter bearing a 45 ml and a 50 ml mark. Nessler cylinders complying with the above dimensions are suitable. The expression "matched tubes" means tubes that are matched as closely as possible in internal diameter and in all other respects.

Prepare a solution as specified in the monograph, transfer to a comparison tube, dilute to 50 ml with water and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight. The opalescence produced is not greater than the similarly prepared standard

opalescence when viewed down the vertical axis of the tube in diffused light against a black background.

Standard opalescence

Measure 5.0 ml of hydrochloric acid CITS and 10 ml of nitric acid (~130 g/l) TS into a comparison tube. Dilute to 50 ml with water, and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod and set aside for 5 minutes, protected from direct sunlight." (*The Ph. Int.*, 2011).

Since the recommended methods are the established methods in *The Ph. Int.*, only method verifications were performed to ensure the fitness for purpose of the proposed methods, instead of complete revalidation of the methods (Ermer & Miller, 2005:302).

10.3.1 Proposed method to test for the presence of chloride ions in zinc acetate dihydrate and zinc gluconate APIs

The following methods (derived from *The Ph. Int.*) are proposed to test for the presence of chlorides in zinc acetate dihydrate and zinc gluconate APIs.

The sample masses / sizes were determined by taking into consideration the limit of the test (Table 10.1) and the concentration of the standard opalescence solution (250 µg chloride ions). For example:

The standard opalescence solution contains 250 µg chloride ions. If the zinc acetate dihydrate contains at least 50 µg/g chloride, *x* g of the zinc acetate dihydrate should be used in the test solution to produce a solution with opalescence similar to that of the standard opalescence solution.

Therefore if 1 g of zinc acetate dihydrate contains at least 50 µg chloride, then *x* g zinc acetate dihydrate will contain at least 250 µg chloride. Thus,

$$x = \frac{1\text{ g x }250\text{ µg}}{50\text{ µg}} = 5\text{ g}$$

A similar approach was followed to calculate the required mass of zinc gluconate for the test.

10.3.1.1 Chloride limit test for zinc acetate dihydrate API

Chlorides. Dissolve 5.0 g in 25 ml of water R, and proceed as described under 2.2.1 Limit test for chlorides; the chloride content is not more than 50 µg/g.

10.3.1.2 Chloride limit test for zinc gluconate API

Chlorides. Dissolve 0.5 g in 25 ml of water R, and proceed as described under 2.2.1 Limit test for chlorides; the chloride content is not more than 500 µg/g.

10.4 Materials and equipment

The information of the materials used in the limit test for chlorides of zinc acetate dihydrate and zinc gluconate APIs can be seen in Table 10.2. A Sartorius R200D+ balance (Labotec, SA) and a Sartorius ED623S+ balance (IMP, SA) were used during these tests.

Table 10.2 Information of materials used in the limit test for chlorides of zinc acetate dihydrate and zinc gluconate APIs

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Silver nitrate	1033933	Merck Chemicals Pty. Ltd.	South Africa
Hydrochloric acid	1037916	Merck Chemicals Pty. Ltd.	South Africa
Nitric acid	1033014	Merck Chemicals Pty. Ltd.	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

10.5 Method verification of the chloride limit test method for zinc acetate dihydrate API

To ensure this method's fitness for purpose, specificity and a relative detection limit of the method were investigated.

To investigate the specificity of the method three solutions were utilised:

- Solution A: A blank solution (water R) that did not contain any chloride ions.
- Solution B: A standard opalescence solution that contained 250 µg of chloride ions (hydrochloric acid ClTS).
- Solution C: A test solution (as described in section 10.3.1.1), which was spiked to contain 250 µg of chloride ions.

To investigate the relative limit of detection three test solutions (solutions D - F) were prepared (as described in section 10.3.1.1). The three test solutions were spiked with 50 µg, 125 µg and 250 µg chloride ions, which is equivalent to the zinc acetate dihydrate API containing 10 µg/g, 25 µg/g and 50 µg/g chloride ions.

10.5.1 Procedure

Prepare the following solutions:

A) To 50 ml of water R add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

B) Measure 5.0 ml of hydrochloric acid ClTS and 10 ml of nitric acid (~130 g/l) TS into a comparison tube. Dilute to 50 ml with water R, and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod and set aside for 5 minutes, protected from direct sunlight.

C) Dissolve 5.0 g zinc acetate dihydrate in 25 ml of water R; add 5.0 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

D) Dissolve 5.0 g zinc acetate dihydrate in 25 ml of water R; add 5.0 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

E) Dissolve 5.0 g zinc acetate dihydrate in 25 ml of water R; add 2.5 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS.

Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

F) Dissolve 5.0 g zinc acetate dihydrate in 25 ml of water R; add 1.0 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

Compare the opalescence produced when viewed down the vertical axis of the tubes in diffused light against a black background.

10.5.2 Acceptance criteria

i. A sufficient level of specificity is illustrated when solution A does not produce any opalescence (remains clear); and solution C produces an opalescence equal to the opalescence produced by solution B.
ii. A relative detection limit can be assigned for the lowest concentration of chlorides where the opalescence produced by solutions D - F can be readily distinguished from solution A. The relative detection limit is at or below the threshold of the limit test.

10.5.3 Results and discussion

When the procedure for specificity was executed solution A remained clear, and the opalescence produced by solution C was comparable to the opalescence produced by solution B (Figure 10.3).

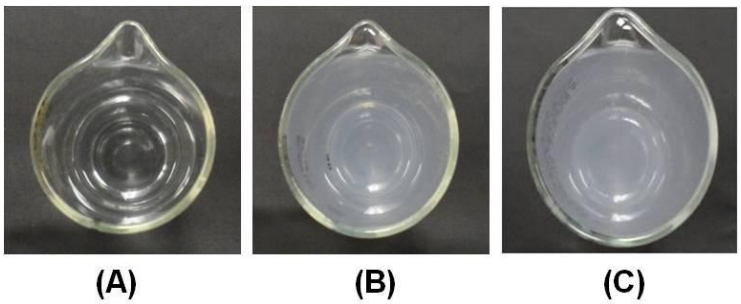


Figure 10.3 Photograph of specificity results: (A) solution A (water R), (B) solution B (hydrochloric acid ClTS), (C) solution C (test solution spiked to contain 250 µg chloride ions).

The opalescence of all three chloride-spiked zinc acetate dihydrate test solutions (solutions D - F) could readily be distinguished from solution A (water R). The relative detection limit could thus be considered being lower than 10 µg/g, which is below the threshold of the limit test. Photographs of the results can be seen in Figure 10.4.

Figure 10.4 Photographs of the relative detection limit results: (D) zinc acetate dihydrate solution spiked to contain 50 µg/g chloride ions, (E) zinc acetate dihydrate solution spiked to contain 25 µg/g chloride ions, (F) zinc acetate dihydrate solution spiked to contain 10 µg/g chloride ions; and (A) solution A (water R).

10.5.4 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The chloride limit in the spiked zinc acetate dihydrate samples was detected in a quantity of up to 10 µg/g.

It can thus be concluded that this limit test is fit for the limiting of chlorides in zinc acetate dihydrate API.

10.6 Method verification of the chloride limit test method for zinc gluconate API

To ensure this method's fitness for purpose, specificity and a relative detection limit of the method were investigated.

To investigate the specificity of the method three solutions were utilised:

- Solution A: A blank solution (water R) that did not contain any chloride ions.

- Solution B: A standard opalescence solution that contained 250 µg of chloride ions.
- Solution C: A test solution (as described in section 10.3.1.2), which was spiked with 250 µg of chloride ions.

To investigate the relative limit of detection three test solutions (solutions D - F) were prepared (as described in section 10.3.1.2). The three test solutions were spiked with 50 µg, 125 µg and 250 µg chloride ions, which is equivalent to the zinc gluconate API containing 100 µg/g, 250 µg/g and 500 µg/g chloride ions.

10.6.1 Procedure

Prepare the following solutions:

A) To 50 ml of water R add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

B) Measure 5.0 ml of hydrochloric acid ClTS and 10 ml of nitric acid (~130 g/l) TS into a comparison tube. Dilute to 50 ml with water R, and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod and set aside for 5 minutes, protected from direct sunlight.

C) Dissolve 0.5 g zinc gluconate in 25 ml of water R; add 5.0 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

D) Dissolve 0.5 g zinc gluconate in 25 ml of water R; add 5.0 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

E) Dissolve 0.5 g zinc gluconate in 25 ml of water R; add 2.5 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

F) Dissolve 0.5 g zinc gluconate in 25 ml of water R; add 1.0 ml hydrochloric acid ClTS; dilute to 50 ml with water R and add 1 ml of silver nitrate (40 g/l) TS. Stir immediately with a glass rod, and set aside for 5 minutes, protected from direct sunlight.

Compare the opalescence produced when viewed down the vertical axis of the tubes in diffused light against a black background.

10.6.2 Acceptance criteria

i. A sufficient level of specificity is illustrated when solution A does not produce any opalescence (remains clear); and solution C produces an opalescence equal to the opalescence produced by solution B.
ii. A relative detection limit can be assigned for the lowest concentration of chlorides, where the opalescence produced by solutions D - F can be readily distinguished from solution A. The relative detection limit is at or below the threshold of the limit test.

10.6.3 Results and discussion

When the procedure for specificity was executed solution A remained clear, and the opalescence produced by solution C was comparable to the opalescence produced by solution B (Figure 10.5).

Figure 10.5 Photograph of specificity results: (A) solution A (water R), (B) solution B (hydrochloric acid ClTS), (C) solution C (test solution spiked to contain 250 µg chloride ions).

The opalescence of all three chloride-spiked zinc gluconate test solutions (solutions D - F) could readily be distinguished from solution A (water R). The relative

detection limit could thus be considered being lower than 100 µg/g, which is below the threshold of the limit test. Photographs of the results can be seen in Figure 10.6.

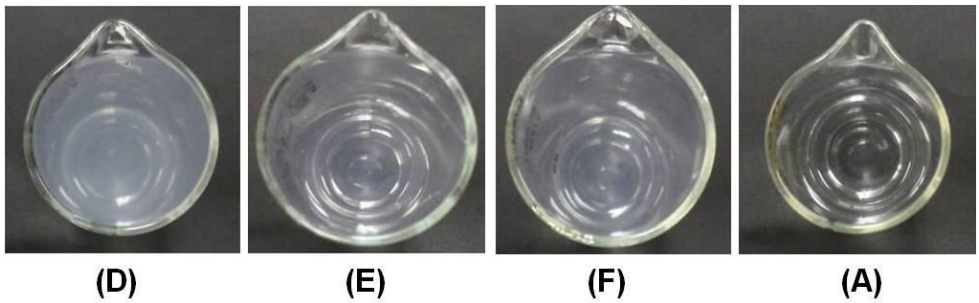


Figure 10.6 Photographs of the relative detection limit results: (D) zinc gluconate solution spiked to contain 500 µg/g chloride ions, (E) zinc gluconate solution spiked to contain 250 µg/g chloride ions, (F) zinc gluconate solution spiked to contain 100 µg/g chloride ions; and (A) solution A (water R).

10.6.4 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The chloride limit in the spiked zinc gluconate samples was detected in a quantity of up to 100 µg/g.

It can thus be concluded that this limit test is fit for the limiting of chlorides in zinc gluconate API.

10.7 Chloride limit test for commercially available zinc acetate dihydrate and zinc gluconate APIs using the proposed methods

The proposed methods, for both zinc acetate dihydrate (section 10.3.1.1) and zinc gluconate (section 10.3.1.2) were successfully verified by means of method verifications. These methods are thus deemed suitable, and were utilised for the chloride limit test for commercially available zinc acetate dihydrate and zinc gluconate APIs.

10.7.1 Procedure

Prepare the following test solutions by:
A) Dissolve 5.0 g zinc acetate dihydrate in 25 ml of water R; and

B) Dissolve 0.5 g zinc gluconate in 25 ml of water R.

Proceed with the chloride limit test as described under section 2.2.1 Limit test for chlorides of *The Ph. Int*. The test complies if the opalescence produced is not greater than the similarly prepared standard solution opalescence when viewed down the vertical axis of the tube in diffused light against a black background.

10.7.2 Results and discussion

Neither the zinc acetate dihydrate, nor the zinc gluconate opalescence produced was greater than the standard opalescence, when viewed down the vertical axis of the tube in diffused light against a black background. The results can be seen in Figure 10.7.

Figure 10.7 Photographs of the chloride limit test for commercially available samples, (A) zinc acetate dihydrate API test solution, (B) standard solution (hydrochloric acid ClTS), (C) zinc gluconate API test solution.

10.7.3 Conclusion

The opalescence of the zinc acetate dihydrate API test solution was not greater than the opalescence of the standard solution. Zinc acetate dihydrate API therefore complied with the limit test for chlorides, meaning that the API did not contain more than 50 µg/g of chloride ions.

Also, the opalescence of the zinc gluconate API test solution was not greater than the opalescence of the standard solution. Zinc gluconate API therefore complied with the limit test for chlorides, meaning that the API did not contain more than 500 µg/g of chloride ions.

10.8 Development of methods for Group II Acid radical impurities – Sulfates

The well established sulfate limit test available in the section 2.2.2 Limit test for sulfates of *The Ph. Int.*, was applied on zinc acetate dihydrate and zinc gluconate APIs.

This method is based on the precipitation of sulfates as barium sulfate in the presence of barium chloride, hydrochloric acid and traces of barium sulfate (Beckett & Stenlake, 1975:38):

$$SO_4^{2-}{}_{(aq)} + Ba^{2+}{}_{(aq)} + Cl^-{}_{(aq)} \rightarrow BaSO_4 \downarrow + Cl^-{}_{(aq)}$$

A comparison is made between the turbidity of the test solution and a standard turbidity solution, to establish whether the sulfate content of the test sample exceeds the limit reported in the specific monograph in terms of micrograms of sulfate ions per gram (µg/g) of the substance being tested. The standard solution contains 480 µg of SO_4^{2-} ions, which is used to make the comparison of turbidity (*Ph. Int.*, 2011). The following recommended procedure and standard turbidity solution are described in *The Ph. Int*. in section 2.2.2 Limit test for sulfates:

"Recommended procedure

Carry out the test in matched flat-bottomed comparison tubes of transparent glass of about 70 ml capacity and about 23 mm internal diameter bearing a 45 ml and a 50 ml mark. Nessler cylinders complying with the above dimensions are suitable. The expression "matched tubes" means tubes that are matched as closely as possible in internal diameter and in all other respects.

Prepare a solution as specified in the monograph, transfer to a comparison tube, dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes. The turbidity produced is not greater than the similarly prepared standard turbidity when viewed down the vertical axis of the tube in diffused light against a black background.

Standard turbidity

Measure 1.00 ml of sulfuric acid (0.005 mol/l) VS and 3 ml of hydrochloric acid (~70 g/l) TS into a comparison tube. Dilute to 45 ml with water, and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod and set aside for 10 minutes." (*The Ph. Int.*, 2011).

Since the recommended methods are the established methods in *The Ph. Int.,* only method verifications were performed to ensure the fitness for purpose of the proposed methods, instead of complete revalidation of the methods (Ermer & Miller, 2005:302).

10.8.1 Proposed method to test for the presence of sulfate ions in zinc acetate dihydrate and zinc gluconate APIs

The following methods (derived from *The Ph. Int.*) are proposed to test for the presence of sulfates in zinc acetate dihydrate and zinc gluconate APIs.

The sample masses / sizes were determined by the limit set and the concentration of the standard solution (refer to section 10.3.1).

10.8.1.1 Sulfate limit test for zinc acetate dihydrate API

Sulfates. Dissolve 4.8 g in 25 ml of water R, and proceed as described under 2.2.2 Limit test for sulfates; the sulfate content is not more than 100 µg/g.

10.8.1.2 Sulfate limit test for zinc gluconate API

Sulfates. Dissolve 0.96 g in 25 ml of water R, and proceed as described under 2.2.2 Limit test for sulfates; the sulfate content is not more than 500 µg/g.

10.9 Materials and equipment

The information of materials used in the sulfate limit test for zinc acetate dihydrate and zinc gluconate can be seen in Table 10.3. A Sartorius R200D+ balance (Labotec, SA) and a Sartorius ED623S+ balance (IMP, SA) were used during this test.

Table 10.3 Information of materials used in the limit test for sulfates of zinc acetate dihydrate and zinc gluconate APIs

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Barium chloride	MKMOM603122	Merck Chemicals Pty. Ltd.	South Africa
Hydrochloric acid	1037916	Merck Chemicals Pty. Ltd.	South Africa
Potassium sulfate	1032805	Merck Chemicals Pty. Ltd.	South Africa
Ethanol	1036688	Merck Chemicals Pty. Ltd.	South Africa
Sulfuric acid	1035329	Merck Chemicals Pty. Ltd.	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

10.10 Method verification of the sulfate limit test method for zinc acetate dihydrate API

To ensure this method's fitness for purpose, specificity and a relative detection limit of the method were investigated.

To investigate the specificity of the method three solutions were utilised:

- Solution A: A blank solution (water R) that did not contain any sulfate ions.
- Solution B: A standard opalescence solution that contained 480 µg of sulfate ions.
- Solution C: A test solution (as described in section 10.8.1.1), which was spiked to contain 480 µg of sulfate ions.

To investigate the relative limit of detection three test solutions (solutions D - F) were prepared (as described in section 10.8.1.1). The three test solutions were spiked with 96 µg, 240 µg and 480 µg sulfate ions, which is equivalent to zinc acetate dihydrate API containing 20 µg/g, 50 µg/g and 100 µg/g sulfate ions.

10.10.1 Procedure

Prepare the following solutions:

A) To 45 ml of water R add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

B) Measure 1.0 ml of sulfuric acid (0.005 mol/l) VS and 3 ml of hydrochloric acid (~70 g/l) TS into a comparison tube. Dilute to 45 ml with water R, and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod and set aside for 10 minutes.

C) Dissolve 4.80 g zinc acetate dihydrate in 25 ml of water R; add 1.0 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

D) Dissolve 4.80 g zinc acetate dihydrate in 25 ml of water R; add 1.0 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

E) Dissolve 4.80 g zinc acetate dihydrate in 25 ml of water R; add 0.5 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

F) Dissolve 4.80 g zinc acetate dihydrate in 25 ml of water R; add 0.2 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

Compare the turbidity produced when viewed down the vertical axis of the tubes in diffused light against a black background.

10.10.2 Acceptance criteria

i. A sufficient level of specificity is illustrated when solution A does not produce any turbidity (remains clear); and solution C produces turbidity equal to the turbidity produced by solution B.
ii. A relative detection limit can be assigned for the lowest concentration of sulfates, where the turbidity produced by solutions D - F can be readily distinguished from solution A. The relative detection limit is at or below the threshold of the limit test.

10.10.3 Results and discussion

When the procedure for specificity was executed solution A remained clear, and the turbidity produced by solution C was comparable to the turbidity produced by solution B (Figure 10.8).

Figure 10.8 Photograph of specificity results: (A) solution A (water R), (B) solution B (sulfuric acid 0.005 mol/l VS), (C) solution C (test solution spiked to contain 480 µg sulfate ions).

The turbidity of the 100 µg/g and 50 µg/g sulfate-spiked zinc acetate dihydrate test solutions could readily be distinguished from solution A (water R). However, the 20 µg/g sulfate-spiked zinc acetate dihydrate test solution could not readily be distinguished from solution A (water R). The relative detection limit could thus be considered being 50 µg/g, which is below the threshold of the limit test. Photographs of the results can be seen in Figure 10.9.

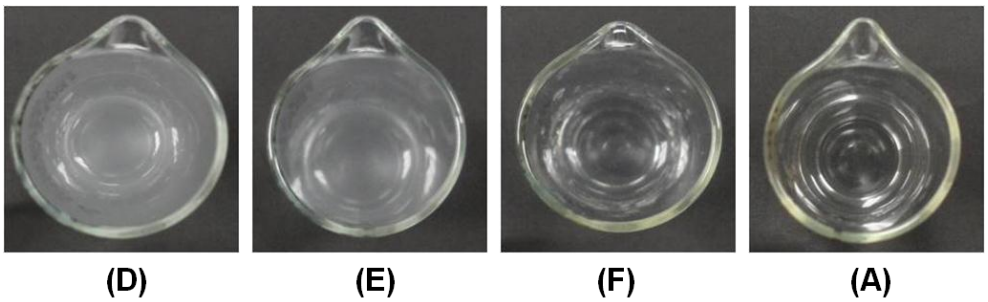


Figure 10.9 Photographs of the relative detection limit results: (D) zinc acetate dihydrate solution spiked to contain 100 µg/g sulfate ions, (E) zinc acetate dihydrate solution spiked to contain 50 µg/g sulfate ions, (F) zinc acetate dihydrate solution spiked to contain 20 µg/g sulfate ions; and (A) solution A (water R).

10.10.4 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The sulfates limit in the spiked zinc acetate dihydrate samples was detected in a quantity of up to 50 µg/g.

It can thus be concluded that this limit test is fit for the limiting of sulfates in zinc acetate dihydrate API.

10.11 Method verification of the sulfate limit test method for zinc gluconate API

To ensure this method's fitness for purpose, specificity and a relative detection limit of the method were investigated.

To investigate the specificity of the method three solutions were utilised:

- Solution A: A blank solution (water R) that did not contain any sulfate ions.
- Solution B: A standard opalescence solution that contained 480 µg of sulfate ions.
- Solution C: A test solution (as described in section 10.8.1.2), which was spiked with 480 µg of sulfate ions.

To investigate the relative limit of detection (using a range of 100 µg/g - 500 µg/g) three test solutions (solutions D - F) were prepared (as described in section 10.8.1.2). The three test solutions were spiked with 96 µg, 240 µg and 480 µg sulfate ions, which is equivalent to zinc gluconate API containing 100 µg/g, 250 µg/g and 500 µg/g sulfate ions.

10.11.1 Procedure

Prepare the following solutions:

A) To 45 ml of water R add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

B) Measure 1.0 ml of sulfuric acid (0.005 mol/l) VS and 3 ml of hydrochloric acid (~70 g/l) TS into a comparison tube. Dilute to 45 ml with water R, and add

5 ml of barium sulfate suspension TS. Stir immediately with a glass rod and set aside for 10 minutes.

C) Dissolve 0.96 g zinc gluconate in 25 ml of water R; add 1.0 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

D) Dissolve 0.96 g zinc gluconate in 25 ml of water R; add 1.0 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

E) Dissolve 0.96 g zinc gluconate in 25 ml of water R; add 0.5 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

F) Dissolve 0.96 g zinc gluconate in 25 ml of water R; add 0.2 ml sulfuric acid (0.005 mol/l) VS; dilute to 45 ml with water R and add 5 ml of barium sulfate suspension TS. Stir immediately with a glass rod, and set aside for 10 minutes.

Compare the turbidity produced when viewed down the vertical axis of the tubes in diffused light against a black background.

10.11.2 Acceptance criteria

i. A sufficient level of specificity is illustrated when solution A does not produce any turbidity (remains clear); and solution C produces turbidity equal to the turbidity produced by solution B.
ii. A relative detection limit can be assigned for the lowest concentration of sulfates, where the turbidity produced by solutions D - F can be readily distinguished from solution A. The relative detection limit is at or below the threshold of the limit test.

10.11.3 Results and discussion

When the procedure for specificity was executed solution A remained clear, and the turbidity produced by solution C was comparable to the turbidity produced by solution B (Figure 10.10).

Figure 10.10 Photograph of specificity results: (A) solution A (water R), (B) solution B (sulfuric acid 0.005 mol/l VS), (C) solution C (test solution spiked to contain 480 µg sulfate ions).

The turbidity of the 500 µg/g and 250 µg/g sulfate-spiked zinc gluconate test solutions could readily be distinguished from solution A (water R). However, the 100 µg/g sulfate-spiked zinc gluconate test solution could not readily be distinguished from solution A (water R). The relative detection limit could thus be considered being 250 µg/g, which is below the threshold of the limit test. Photographs of the results can be seen in Figure 10.11.

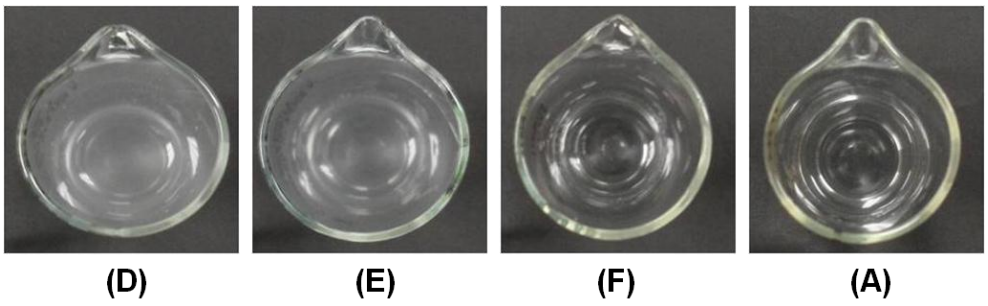


Figure 10.11 Photographs of the relative detection limit results: (D) zinc gluconate solution spiked to contain 500 µg/g sulfate ions, (E) zinc gluconate solution spiked to contain 250 µg/g sulfate ions, (F) zinc gluconate solution spiked to contain 100 µg/g sulfate ions; and (A) solution A (water R).

10.11.4 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The sulfate limit in the spiked zinc gluconate samples was detected in a quantity of up to 250 µg/g.

It can thus be concluded that this limit test is fit for the limiting of sulfates in zinc gluconate API.

10.12 Sulfate limit test for commercially available zinc acetate dihydrate and zinc gluconate APIs using the proposed methods

The proposed methods, for both zinc acetate dihydrate (section 10.8.1.1) and zinc gluconate (section 10.8.1.2) were successfully verified by means of method verifications. These methods are thus deemed suitable, and were utilised for the sulfate limit test for commercially available zinc acetate dihydrate and zinc gluconate APIs.

10.12.1 Procedure

Prepare the following test solutions by:
A) Dissolving 4.8 g zinc acetate dihydrate in 25 ml of water R; and
B) Dissolving 0.96 g zinc gluconate in 25 ml of water R.

Proceed with the sulfate limit test as described under section 2.2.2 Limit test for sulfates of *The Ph. Int*. The test complies if the turbidity produced is not greater than the similarly prepared standard turbidity solution when viewed down the vertical axis of the tube in diffused light against a black background.

10.12.2 Results and discussion

Neither the zinc acetate dihydrate, nor the zinc gluconate test solutions produced turbidity that was greater than that of the standard turbidity solution, when viewed down the vertical axis of the tube in diffused light against a black background. The results can be seen in Figure 10.12.

Figure 10.12 Photographs of the sulfate limit test for commercially available samples: (A) zinc acetate dihydrate API test solution, (B) standard solution (sulfuric acid 0.005 mol/l VS), (C) zinc gluconate API test solution.

10.12.3 Conclusion

The turbidity of the zinc acetate dihydrate API test solution was not greater than the turbidity of the standard solution. Zinc acetate dihydrate API therefore complied with the limit test for sulfates, meaning that the API did not contain more than 100 µg/g of sulfate ions.

Also, the turbidity of the zinc gluconate API test solution was not greater than the turbidity of the standard solution. Zinc gluconate API therefore complied with the limit test for sulfates, meaning that the API did not contain more than 500 µg/g of sulfate ions.

10.13 Chapter conclusion

To limit the acid radical impurities (i.e. chlorides and sulfates), the limit test for chlorides and limit test for sulfates of *The Ph. Int.* were utilised for both zinc acetate dihydrate and zinc gluconate APIs. The fitness of purpose for these two methods was illustrated by means of suitable method verification procedures.

The aforementioned two methods are therefore recommended for inclusion in *The Ph. Int.* monographs.

CHAPTER 11

METALLIC IMPURITIES

11.1 Introduction

A great deal of emphasis is placed by regulatory agencies on the control of physiologically harmful impurities. Arsenic and lead contamination is widespread, mainly as a result of atmospheric pollution, and limit tests of wide general applicability are specified for them (Beckett & Stenlake, 1975:24). According to Waterman *et al*., metallic impurities can be brought into pharmaceutical products during processing or be leached from packaging. Metallic impurities need to be controlled for the following reasons: inherent toxicity of the metal, formation of insoluble metal complexes, and oxidative and hydrolytic catalytic activity (Waterman *et al.*, 2004:81).

The aim of this chapter was to develop limit test methods to control metallic impurities in zinc acetate dihydrate and zinc gluconate APIs, which are to be included in *The Ph. Int*.

11.2 Specifications for metallic impurities present in zinc acetate dihydrate and zinc gluconate APIs

The metallic impurities of Group III can be divided into two categories, namely non-specific metallic impurities and specific metallic impurities (Figure 9.2). The Ph. Eur. and BP monographs require that six specific metallic impurities (i.e. arsenic, aluminium, cadmium, copper, iron and lead) need to be controlled in zinc acetate dihydrate API; whereas only the heavy metals (non-specific metallic impurities) and cadmium (specific metallic impurity) are to be controlled in zinc gluconate API (BP, 2011; Ph. Eur., 2011). It is clear that there are differences between the metallic impurity testing profiles of the mentioned two APIs, which may be attributed to differences in the routes of synthesis and purification processes. No information was available from the manufacturers of the two zinc salts to elaborate on the aforementioned, thus the proposed metallic impurity testing profiles of the Ph. Eur. / BP were adopted.

The metallic impurities which should be controlled in zinc acetate dihydrate API and the proposed specifications are summarised in Table 11.1.

Table 11.1 Specific metallic impurity limit tests and specifications applicable to zinc acetate dihydrate API based on Ph. Eur. / BP (BP, 2011; Ph. Eur., 2011)

Metallic impurity limit test	**Specification** (not more than)
Arsenic	2 µg/g
Aluminium	5.0 µg/g
Cadmium	2.0 µg/g
Copper	50.0 µg/g
Iron	50.0 µg/g
Lead	10.0 µg/g

The metallic impurities which should be controlled in zinc gluconate API (Table 11.2) and the proposed specifications for these limit tests were adopted from the Ph. Eur. / BP (BP, 2011; Ph. Eur., 2011).

Table 11.2 Metallic impurity limits test and specifications applicable to zinc gluconate API based on Ph. Eur. / BP (BP, 2011; Ph. Eur., 2011)

Metallic impurity limit test	**Specification** (not more than)
Heavy metals	10 µg/g
Cadmium	2.0 µg/g

The non-specific metallic impurity limit test will be discussed first, followed by specific metallic impurity limit tests.

11.3 Development of methods for Group III non-specific metallic impurities – Heavy metals

Heavy metals can be defined as the group of metals having a density / specific gravity of 4.0 or more. However, heavy metals that are poisonous to man, such as Ag^{+}, Hg^{2+}, Pb^{2+}, Bi^{2+}, Cu^{2+}, As^{3+}, Sb^{3+} and Sn^{4+}, are the heavy metals which became the object of the pharmaceutical heavy metals limit test (KYOWA, 2010). This method is based on heavy metal ions that quantitatively form sulfides in acidic solutions (with a pH of about 3) to turn the solutions brown or black (KYOWA, 2010):

$$Pb^{2+}_{(aq)} + S^{2-}_{(aq)} \rightarrow PbS \downarrow$$

In order to propose a heavy metal limit test for zinc gluconate API for inclusion in *The Ph. Int*., a series of methods were evaluated. The suitability of the methods was evaluated based on the formation of a clearly visible brown colour (due to the formation of lead sulfide) using a lead-spiked zinc gluconate test solution (spiked to contain 10 µg lead ions) that was prepared according to the procedure. A summary of the approach followed to identify the most suitable method is illustrated in Figure 11.1, and will be discussed in detail.

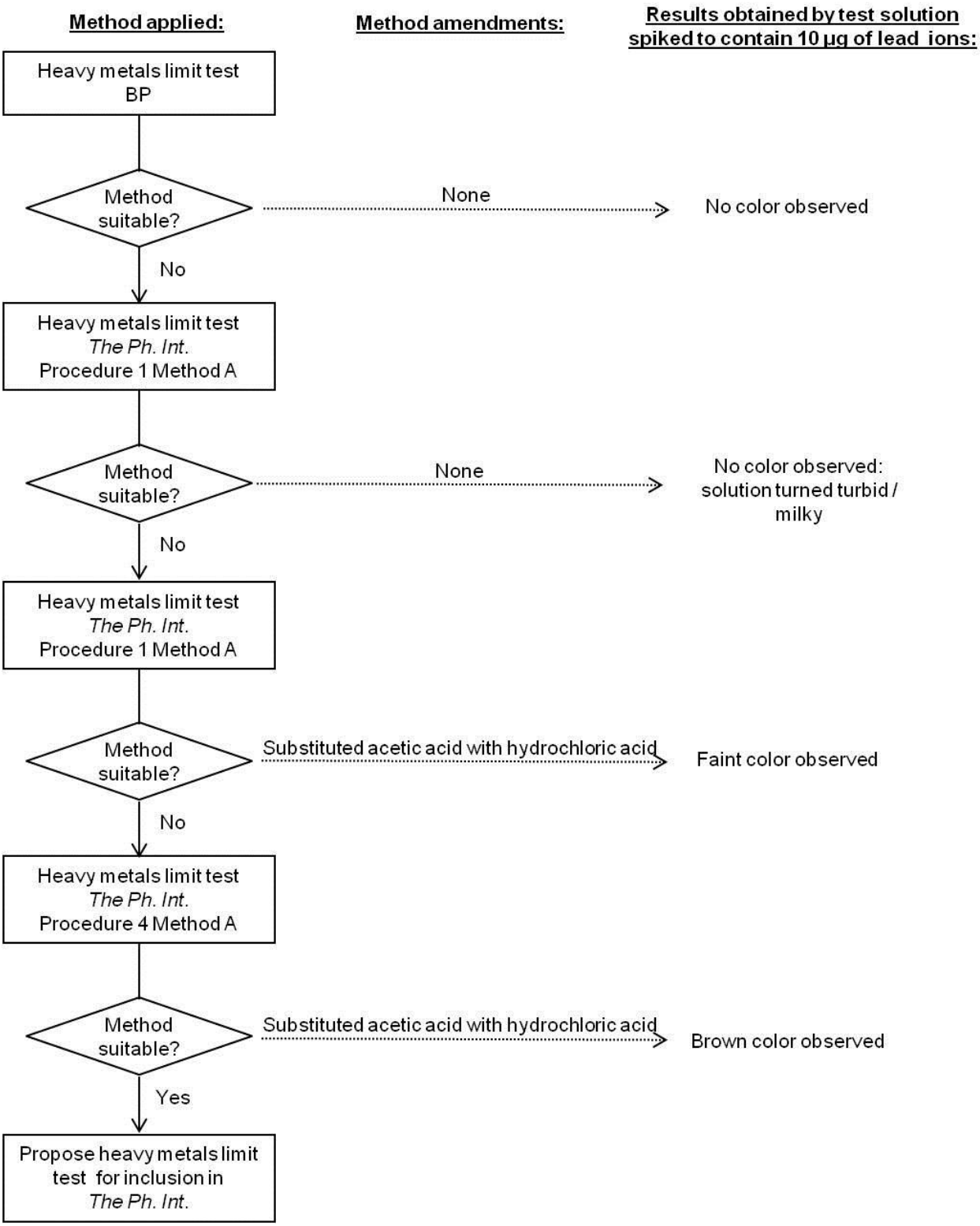


Figure 11.1 Illustration of the approach followed to identify the most suitable heavy metal limit test for zinc gluconate API.

The general heavy metal limit test as described in the BP was investigated as a potential method for the detection of heavy metals in zinc gluconate APIs. The recommended procedure as described in the BP is as follows:

*"**Heavy metals (2.4.8)***

Maximum 10 ppm.

Dissolve 2.0 g in 20 ml of water R, heating in a water-bath at 60 °C. 12 ml of the solution complies with test A. Prepare the reference solution using lead standard solution (1 ppm Pb) R.

Limit Test for Heavy Metals

(Ph. Eur. method 2.4.8)

The methods described below require the use of thioacetamide reagent R. As an alternative, sodium sulfide solution R1 (0.1 ml) is usually suitable. Since tests prescribed in monographs have been developed using thioacetamide reagent R, if sodium sulfide solution R1 is used instead, it is necessary to include also for methods A, B and H a monitor solution, prepared from the quantity of the substance to be examined prescribed for the test, to which has been added the volume of lead standard solution prescribed for preparation of the reference solution. The test is invalid if the monitor solution is not at least as intense as the reference solution.

Method A

Test solution: 12 ml of the prescribed aqueous solution of the substance to be examined.

Reference solution (standard) A: mixture of 10 ml of lead standard solution (1 ppm Pb) R or lead standard solution (2 ppm Pb) R, as prescribed, and 2 ml of the prescribed aqueous solution of the substance to be examined.

Blank solution A: mixture of 10 ml of water R and 2 ml of the prescribed aqueous solution of the substance to be examined.

To each solution, add 2 ml of buffer solution pH 3.5 R. Mix and add to 1.2 ml of thioacetamide reagent R. Mix immediately. Examine the solutions after 2 min.

System suitability: The reference solution shows a slight brown colour compared to the blank solution.

Result: Any brown colour in the test solution is not more intense than that in the reference solution." (BP, 2011).

When the abovementioned procedure was executed, the "system suitability" failed. The reference solution did not show a slight brown colour. This indicated that there was an interference with the colour production / precipitation of lead sulfide. This might be due to a compound present that has a higher affinity for the sulfide ions, competing therefore and preventing lead sulfide to form.

The heavy metals limit test in *The Ph. Int.* (Procedure 1, Method A) was then investigated as an alternative method to detect the presence of heavy metals in zinc gluconate API. The method is described as follows (*Ph. Int*., 2011):

*"**Recommended procedure***

Preparation of test solution

Procedure 1. Weigh the quantity (1.0 g) *of substance specified in the monograph, dissolve it in 25 ml of water, adjust the pH of the solution to 3 - 4 with acetic acid (~60 g/l) PbTS, or with ammonia (~100 g/l) PbTS, as necessary, then dilute to 40 ml with water and mix.*

Colour development and measurement

Method A

To 40 ml of the liquid contained in the comparison tube add 10 ml of freshly prepared hydrogen sulfide TS, mix and allow to stand for 5 minutes.

In another comparison tube place a volume of solution of dilute lead PbTS, containing the lead equivalent of the heavy metals limit specified in the monograph,

dilute with water, adjust the pH with ammonia (~100 g/l) PbTS and acetic acid (~60 g/l) PbTS to 3 - 4; dilute with water or the solvent used to 40 ml, mix, add 10 ml of freshly prepared hydrogen sulfide TS, mix and allow to stand for 5 minutes.

Compare the colours by viewing down the vertical axis of the tube in diffused light against a white background, or by another suitable method. The colour of the test solution is not darker than that of the lead standard." (*Ph. Int.*, 2011).

The test was executed utilising *Procedure 1* and *Method A* of *The Ph. Int*. and a zinc gluconate test solution (spiked to contain 10 µg lead ions). The lead standard solution used in the test, dilute lead PbTS, contains 10 µg of lead in 1 ml.

A brown colour was produced by the lead reference solution. No colour was observed for the lead-spiked zinc gluconate API test solution. However, the spiked solution turned turbid / milky, which indicated that the zinc gluconate interfered with the colour production. Thus the method was not found suitable for use.

The differences in the appearance of the two solutions were more prominent during the visual inspection of the solutions. Photography of the solutions did not manage to exemplify the differences as clearly. Photographs of the results can be seen in Figure 11.2.

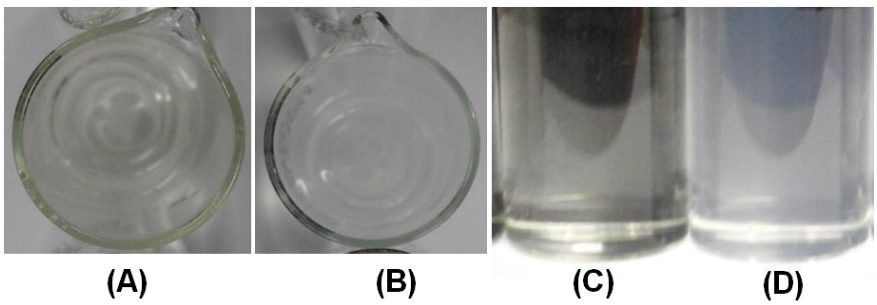


Figure 11.2 Photographs of heavy metal limit test executed on zinc gluconate API utilising procedure 1 of the heavy metal limit test provided in *The Ph. Int.*: viewed down the vertical axis (A) reference solution, and (B) zinc gluconate API test solution spiked to contain 10 µg of lead ions; and viewed down the horizontal axis (C) reference solution, and (D) zinc gluconate API test solution spiked to contain 10 µg of lead ions.

Zinc, nickel and cobalt are precipitated in the presence of weak acids, i.e. acetic acid during the heavy metal test (Beckett & Stenlake, 1975:33). Acetic acid was utilised in the heavy metal limit test to adjust the pH of the solutions, thus the milky appearance might be attributed to the precipitation of zinc sulfide.

Qualitative analytical Group I and II, i.e. Ag^{+}, Hg^{2+}, Pb^{2+}, Bi^{2+}, Cu^{2+}, As^{3+}, Sb^{3+} and Sn^{4+}, are precipitated in the presence of mineral acids during the heavy metal test. It was then decided to substitute the acetic acid with hydrochloric acid. The recommended procedure was amended as follows:

Weigh the quantity (1.0 g) *of substance specified in the monograph, dissolve it in 25 ml of water, adjust the pH of the solution to 3 - 4 with* hydrochloric acid (~70 g/l) TS*, or with ammonia (~100 g/l) PbTS, as necessary, then dilute to 40 ml with water and mix.*

To 40 ml of the liquid contained in the comparison tube add 10 ml of freshly prepared hydrogen sulfide TS, mix and allow to stand for 5 minutes.

In another comparison tube place a volume of solution of dilute lead PbTS, containing the lead equivalent of the heavy metals limit specified in the monograph, dilute with water, adjust the pH with ammonia (~100 g/l) PbTS and hydrochloric acid (~70 g/l) TS *to 3 - 4; dilute with water or the solvent used to 40 ml, mix, add 10 ml of freshly prepared hydrogen sulfide TS, mix and allow to stand for 5 minutes.*

Compare the colours by viewing down the vertical axis of the tube in diffused light against a white background, or by another suitable method. The colour of the test solution is not darker than that of the lead standard.

The reference solution produced a brown colour with the abovementioned procedure, and the lead-spiked test solution produced a faint brown colour with no precipitation. However, the brown colour produced by the lead-spiked test solution was not comparable to the brown colour of the reference solution (Figure 11.3) which suggested that organic material present in the spiked test solution still interfered with the colour development.

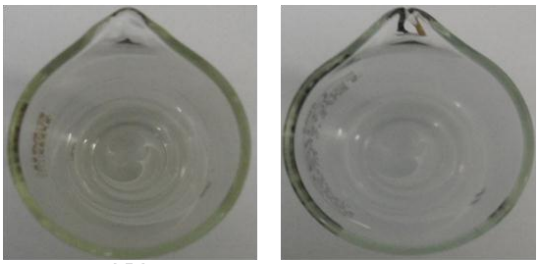


Figure 11.3 Photograph of the heavy metal limit test executed on zinc gluconate API utilising the recommended procedure (Procedure 1- without the use of acetic acid); (A) reference solution, and (B) zinc gluconate API test solution spiked to contain 10 µg of lead ions.

The differences in the visual appearance of the two solutions were more prominent during the visual inspection of the solution compared to that observed in the photographs.

In procedure 4 of the heavy metal limit test in *The Ph. Int*., the sample is incinerated before the test solution is prepared to eliminate any organic interference (*Ph. Int.*, 2011). The following procedure was executed to evaluate the effect of organic material eradication (by means of incineration) on the colour development using a zinc gluconate API test solution spiked to contain 10 µg of lead ions:

Place the quantity (1.0 g) *of substance specified in the monograph in a suitable crucible, preferably made of silica, mix it well with about 0.5 g of magnesium oxide R and incinerate until a homogeneous white mass is obtained. If after 15 minutes of incineration the residue is still coloured, let the crucible cool, mix the contents well with a glass rod and resume heating. Next, dissolve the residue in hydrochloric acid (~70 g/l) TS, add, drop by drop, a solution of ammonia (~100 g/l) PbTS, until the pH of the solution is between 8 and 8.5, then add, also drop by drop, hydrochloric acid (~70 g/l) TS, to adjust the pH to 3 - 4, filter, dilute with water to 40 ml, and mix.*

To 40 ml of the liquid contained in the comparison tube add 10 ml of freshly prepared hydrogen sulfide TS, mix and allow to stand for 5 minutes.

In another comparison tube place a volume of solution of dilute lead PbTS, containing the lead equivalent of the heavy metals limit specified in the monograph,

dilute with water, adjust the pH with ammonia (~100 g/l) PbTS and hydrochloric acid (~70 g/l) TS to 3 - 4; dilute with water or the solvent used to 40 ml, mix, add 10 ml of freshly prepared hydrogen sulfide TS, mix and allow to stand for 5 minutes.

Compare the colours by viewing down the vertical axis of the tube in diffused light against a white background, or by another suitable method. The colour of the test solution is not darker than that of the lead standard.

The zinc gluconate API test solution spiked to contain 10 µg of lead ions produced a brown colour, comparable to the colour produced by the lead reference solution (Figure 11.4).

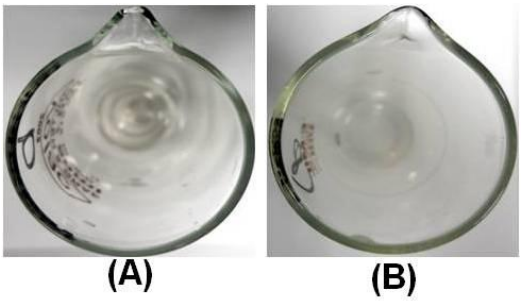


Figure 11.4 Photograph of the heavy metal limit test executed on zinc gluconate API utilising the recommended procedure (Procedure 4 - without the use of acetic acid); (A) reference solution, and (B) zinc gluconate API test solution spiked to contain 10 µg of lead ions.

11.3.1 Proposed method to test for the presence of heavy metals in zinc gluconate APIs

Based on the information presented in section 11.3, the following method (adapted from *The Ph. Int.*) is proposed to test for the presence of heavy metals in zinc gluconate API.

11.3.1.1 Heavy metal limit test for zinc gluconate API

Heavy metals. Use 1.0 g for the preparation of the test solution as described under 2.2.3 Limit test for heavy metals, Procedure 4. Note: substitute acetic acid (~60 g/l) PbTS with hydrochloric acid (~70 g/l) TS in all cases. Not more than 10 µg/g.

The sample mass was determined by taking into consideration the limit of the test (Table 11.2) and the concentration of the reference solution (10 µg lead in 1 ml); and calculated as 1 g (similarly calculated as discussed in section 10.3.1 – Chapter 10).

11.3.2 Materials and equipment

The information of the materials used in the heavy metals test for zinc gluconate API can be seen in Table 11.3. A Sartorius R200D+ balance (Labotec, SA) and a Sartorius ED623S+ balance (IMP, SA) were used during this test.

Table 11.3 Materials used in the heavy metals test for zinc gluconate API

Material	Batch number	Manufacturing company	Country of origin
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Magnesium oxide	TA1664666 045	Merck KGaA	Germany
Hydrochloric acid	K42075217 111	Merck KGaA	Germany
Nitric acid	1033014	Merck Chemicals (Pty) Ltd.	South Africa
Lead nitrate	1030452	Merck Chemicals (Pty) Ltd.	South Africa
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Iron (II) sulfide	03806HJ	Sigma-Aldrich	Germany
Ammonia	1035758	Merck Chemicals (Pty) Ltd.	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

11.3.3 Validation of the heavy metals test for zinc gluconate API

A method validation was performed to verify the validity and suitability of the amended method. The validation parameters (according to Table 3.4 – Chapter 3) which were considered, for an impurity limit test, are specificity and limit of detection.

To investigate the specificity of the method three solutions were utilised:

- Solution A: A blank solution that did not contain any heavy metal ions.
- Solution B: A reference solution that contained 10 µg of lead ions.

- Solution C: A test solution (as described in section 11.3.1.1), which was spiked to contain 10 µg of lead ions.

To investigate a relative limit of detection three test solutions (solutions D - F) were prepared (as described in section 11.3.1.1). The three test solutions were spiked with 2 µg, 5 µg and 10 µg lead ions, which is equivalent to the zinc gluconate API containing 2 µg/g, 5 µg/g and 10 µg/g lead.

11.3.3.1 Procedure

Prepare the following solutions:

A) Place 0.5 g of magnesium oxide R in a suitable crucible and incinerate until a homogeneous white mass is obtained. Dissolve the residue in hydrochloric acid (~70 g/l) TS, add, drop by drop, a solution of ammonia (~100 g/l) PbTS, until the pH of the solution is between 8 and 8.5, then add, also drop by drop, hydrochloric acid (~70 g/l) TS, to adjust the pH to 3 - 4, filter, dilute with water R to 40 ml, and mix.

B) Use 1 ml of dilute lead PbTS, adjust the pH with ammonia (~100 g/l) PbTS and hydrochloric acid (~70 g/l) TS to 3 - 4; dilute with water R to 40 ml, mix, add 10 ml of freshly prepared hydrogen sulfide TS, mix and allow to stand for 5 minutes.

C) Place 1.0 g of zinc gluconate in a suitable crucible and add 1 ml of dilute lead PbTS, mix it well with about 0.5 g of magnesium oxide R and incinerate until a homogeneous white mass is obtained. Dissolve the residue in hydrochloric acid (~70 g/l) TS, add, drop by drop, a solution of ammonia (~100 g/l) PbTS, until the pH of the solution is between 8 and 8.5, then add, also drop by drop, hydrochloric acid (~70 g/l) TS, to adjust the pH to 3 - 4, filter, dilute with water R to 40 ml, and mix.

D) Place 1.0 g of zinc gluconate in a suitable crucible and add 1 ml of dilute lead PbTS, mix it well with about 0.5 g of magnesium oxide R and incinerate until a homogeneous white mass is obtained. Dissolve the residue in hydrochloric acid (~70 g/l) TS, add, drop by drop, a solution of ammonia (~100 g/l) PbTS,

until the pH of the solution is between 8 and 8.5, then add, also drop by drop, hydrochloric acid (~70 g/l) TS, to adjust the pH to 3 - 4, filter, dilute with water R to 40 ml, and mix.

E) Place 1.0 g of zinc gluconate in a suitable crucible and add 0.5 ml of dilute lead PbTS, mix it well with about 0.5 g of magnesium oxide R and incinerate until a homogeneous white mass is obtained. Dissolve the residue in hydrochloric acid (~70 g/l) TS, add, drop by drop, a solution of ammonia (~100 g/l) PbTS, until the pH of the solution is between 8 and 8.5, then add, also drop by drop, hydrochloric acid (~70 g/l) TS, to adjust the pH to 3 - 4, filter, dilute with water R to 40 ml, and mix.

F) Place 1.0 g of zinc gluconate in a suitable crucible and add 0.2 ml of dilute lead PbTS, mix it well with about 0.5 g of magnesium oxide R and incinerate until a homogeneous white mass is obtained. Dissolve the residue in hydrochloric acid (~70 g/l) TS, add, drop by drop, a solution of ammonia (~100 g/l) PbTS, until the pH of the solution is between 8 and 8.5, then add, also drop by drop, hydrochloric acid (~70 g/l) TS, to adjust the pH to 3 - 4, filter, dilute with water R to 40 ml, and mix.

Compare the colours by viewing down the vertical axis of the tube in diffused light against a white background, or by another suitable method.

11.3.3.2 Acceptance criteria

i. A sufficient level of specificity is illustrated when solution A does not produce any colour (remains clear); and solution C produces a colour of the same intensity than that of the lead standard solution (solution B).
ii. A relative detection limit can be assigned for the lowest concentration of heavy metals, where the colour produced by solutions D - F can be readily distinguished from solution A. The relative detection limit is at or below the threshold of the limit test.

11.3.3.3 Results and discussion

When the procedure for specificity was executed solution A remained clear, and the colour produced by solution C was comparable to the colour produced by solution B; see Figure 11.5 for a photograph of the results. The colour of the solutions was more prominent during the visual inspection than the colour depicted in the photographs.

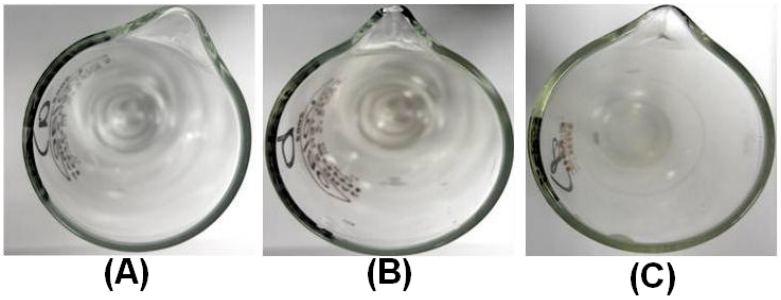


Figure 11.5 Photograph of specificity results: (A) solution A, (B) solution B (lead standard solution), (C) solution C (test solution spiked to contain 10 µg lead ions).

The colour of all three lead-spiked zinc gluconate test solutions (solutions D - F) could readily be distinguished from solution A. The relative detection limit could thus be considered being lower than 2 µg/g, which is below the threshold of the limit test. Photographs of the results can be seen in Figure 11.6.

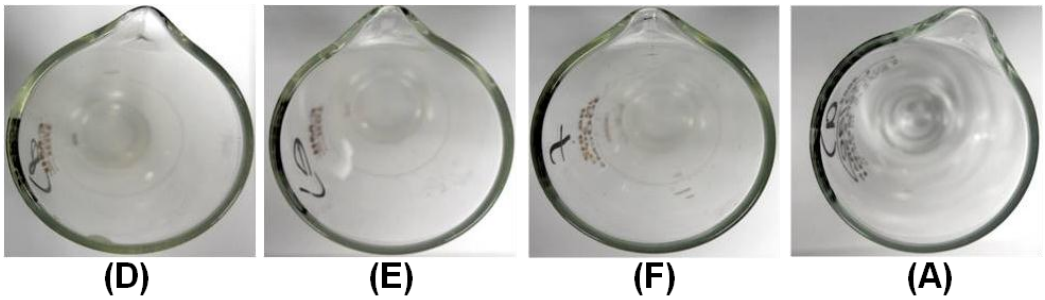


Figure 11.6 Photographs of the relative detection limit results: (D) zinc gluconate solution spiked to contain 10 µg/g lead ions, (E) zinc gluconate solution spiked to contain 5 µg/g lead ions, (F) zinc gluconate solution spiked to contain 2 µg/g lead ions; and (A) solution A.

11.3.3.4 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The lead limit in the spiked zinc gluconate samples was detected in a quantity of up to 2 µg/g.

It can thus be concluded that this limit test is fit for the limiting of heavy metals in zinc gluconate API.

11.3.4 Heavy metal limit test executed on commercially available zinc gluconate API using the proposed method

The proposed method for zinc gluconate (section 11.3.1.1) was successfully validated. The method is thus deemed suitable, and was utilised to test for the presence of heavy metals in commercially available zinc gluconate API.

11.3.4.1 Procedure

Execute the procedure as described in section 11.3.1.1. The test complies if the colour produced is not greater than the colour of the similarly prepared lead standard solution when viewed down the vertical axis of the tube in diffused light against a white background, or by another suitable method.

11.3.4.2 Results and discussion

The colour produced by the zinc gluconate test solution was not darker than that of the lead standard solution, when viewed down the vertical axis of the tube in diffused light against a white background (Figure 11.7).

Figure 11.7 Results of the heavy metal test for commercially available zinc gluconate API: (A) lead standard solution; (B) zinc acetate API test solution.

11.3.4.3 Conclusion

The colour of the test solution was not darker than the colour of the lead standard, which indicated that the API did not contain more than 10 µg/g of heavy metal ions. It can thus be concluded that the commercially available zinc gluconate API sample complied with the limit test for heavy metals.

11.4 Development of methods for Group III specific metallic impurities

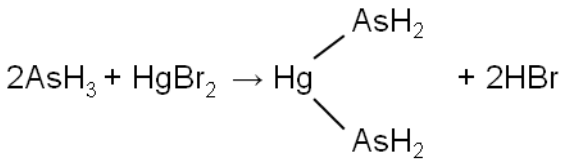
To test for the presence of arsenic (As) in zinc acetate dihydrate API the general test in *The Ph. Int.* was applied; and to test for the presence of the other metal impurities, namely aluminium (Al), iron (Fe), cadmium (Cd), copper (Cu) and lead (Pb), atomic absorption spectrometry was applied.

11.4.1 Development of methods for Group III specific metallic impurities – arsenic

The established arsenic limit test procedure in section 2.2.5 Limit test for arsenic of *The Ph. Int.* was utilised to test for the presence of arsenic in zinc acetate dihydrate API.

This test is based on a development of the Gutzeit Test, wherein all the arsenic present is converted into arsine gas, by subjecting the arsenic to a reduction reaction with zinc and hydrochloric acid. The arsine must come into contact with dry paper permeated with mercuric bromide to produce a yellow strain, of which the intensity is directly proportional to the quantity of arsenic present (Beckett & Stenlake, 1975:27).

The reaction that occurs between the arsine and mercuric bromide may be represented by the following reaction (Beckett & Stenlake, 1975:28):



A comparison is made between the stain of the test solution and a standard stain (stannated hydrochloric acid (~250 g/l) arsenic test solution / stannated hydrochloric acid (~250 g/l) AsTS), to establish whether the arsenic content of the test solution exceeds the limit reported in the specific monograph in terms of micrograms of arsenic ions per gram of the substance being tested. The standard stain against which the comparison is made contains 10 µg of As (*Ph. Int.*, 2011). The limit set for the arsenic test is 2 µg/g, as specified by the Ph.Eur. / BP (Table 11.1). The experimental setup and procedure for this limit test is described in *The Ph. Int*. as follows:

"***Apparatus***

A suitable type of apparatus is described below, though other acceptable constructions are available.

A wide-mouthed bottle of about 120 ml capacity, is fitted with a rubber bung through which passes a glass tube. The latter, made from ordinary glass tubing, has a total length of 200 mm and an internal diameter of exactly 6.5 mm (external diameter about 8 mm), is drawn out at one end to a diameter of about 1 mm, and has a hole not less than 2 mm in diameter blown in the side of the tube, near the constricted part. The tube is passed through the bung fitting the bottle so that, when inserted in the bottle containing 70 ml of liquid, the constricted end of the tube is above the surface of the liquid and the hole in the side is below the bottom of the bung. The upper end of the tube is cut off square, and is either slightly rounded off or ground smooth.

Two rubber bungs (about 25 mm × 25 mm), each with a hole bored centrally and true and exactly 6.5 mm in diameter, are fitted with a rubber band or spring clip for

holding them tightly together. Alternatively, the two bungs may be replaced by any suitable construction satisfying the conditions of the test, as described below." (*Ph. Int.*, 2011) (Figure 11.8).

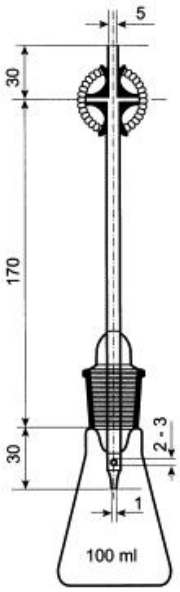


Figure 11.8 Illustration (dimensions in mm) of the apparatus proposed for the arsenic limit test described in the BP (BP, 2011).

"Recommended procedure

Pack the glass tube lightly with cotton-wool, previously moistened with lead acetate (80 g/l) TS and dried, so that the upper surface of the cotton-wool is not less than 25 mm below the top of the tube.

Insert the upper end of the tube into the narrow end of one of the pair of rubber bungs, either (1) to a depth of about 10 mm in the case of the tube with the rounded-off end or (2) so that the ground end of the tube is flush with the larger end of the bung. Place a piece of mercuric bromide paper AsR flat on the top of the bung, and place the other bung over it. Secure the assembly by means of a rubber band or spring clip, in such a manner that the borings of the two bungs (or the boring of the upper bung and the glass tube) meet to form a true tube 6.5 mm in diameter interrupted by a diaphragm of mercuric bromide paper AsR.

Instead of this method of attaching the mercuric bromide paper AsR, any other method may be used provided (1) that the whole of the evolved gas passes through

the paper, (2) that the portion of the paper in contact with the gas is a circle 6.5 mm in diameter, and (3) that the paper is protected from sunlight during the test.

Place the solution, prepared as specified in the monograph, in the wide-mouthed bottle, add 1 g of potassium iodide AsR and 10 g of granulated zinc AsR, and place the prepared glass tube assembly quickly into position. Allow the reaction to proceed for 40 minutes. Compare any yellow stain that is produced on the mercuric bromide paper AsR, with a standard stain, produced in a similar manner with a known quantity of dilute arsenic AsTS. Make the comparison in daylight and immediately after simultaneous preparation of the test and standard stains; the stains fade on keeping.

The most suitable temperature for carrying out the test is generally about 40 °C but, as the rate of evolution of the gas varies somewhat with different batches of granulated zinc AsR, the temperature may be adjusted to obtain a regular, but not too violent, evolution of gas. The reaction may be accelerated by placing the apparatus on a warm surface, care being taken to ensure that the mercuric bromide paper AsR remains quite dry throughout the test.

Between successive tests, the tube must be washed with hydrochloric acid (~250 g/l) AsTS, rinsed with water, and dried.

Standard stain

Prepare a solution by adding 10 ml of stannated hydrochloric acid (~250 g/l) AsTS and 1 ml of dilute arsenic AsTS, to 50 ml of water. The resulting solution, when treated as described in the general test, yields a stain on the mercuric bromide paper AsR, referred to as the standard stain." (Ph. Int., 2011*).*

11.4.1.1 Proposed method to test for the presence of arsenic in zinc acetate dihydrate API

The following method (derived from *The Ph. Int.*) is proposed to test for the presence of arsenic in zinc acetate dihydrate APIs.

The sample masses / sizes were determined by taking into consideration the limit of the test (Table 11.1) and the concentration of the standard solution 10 µg arsenic). A similar approach was followed to calculate the required mass as that in section 10.3.1 (Chapter 10).

11.4.1.1.1 Arsenic limit test for zinc acetate dihydrate API

Arsenic. Use a solution of 5.0 g in 50 ml of water R, add 10 ml stannous hydrochloric acid (~250 g/l) AsTS, and proceed as described under 2.2.5 Limit test for arsenic; not more than 2 µg/g.

11.4.1.2 Materials and equipment

The information of the materials used in the limit test for arsenic in zinc acetate dihydrate can be seen in Table 11.4. A Sartorius ED623S+ balance (IMP, SA) was used during this test.

Table 11.4 Materials used in the limit test for arsenic in zinc acetate dihydrate API

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Mercury bromide	E422463/IV	Sigma-Aldrich	Switzerland
Acetic acid	K42028063107	Merck KGaA	Germany
Whatman filter paper	J11535490	Whatman International Ltd.	England
Potassium iodide	1036559	Merck Chemicals (Pty) Ltd.	South Africa
Zinc powder	K40857189 011	Merck KGaA	Germany
Sodium hydroxide	MBOM600313	Merck Chemicals (Pty) Ltd.	South Africa
Arsenic trioxide	BCBD1149V	Sigma-Aldrich Chemie GmbH	Germany
Tin	A0149906108	Merck KGaA	Germany
Hydrochloric acid	K42075217 111	Merck KGaA	Germany
Ethanol	1036688	Merck Chemicals (Pty) Ltd.	South Africa
Water R	N/A	RIIP®/CENQAM®	South Africa

A photograph of the apparatus used during for limit test for arsenic is illustrated in Figure 11.9.

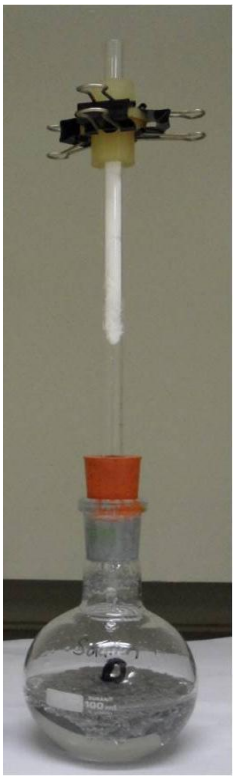


Figure 11.9 Photograph of the apparatus used for the arsenic limit test in zinc acetate dihydrate API.

11.4.1.3 Method verification of the arsenic limit test for zinc acetate dihydrate API

To ensure this method's fitness for purpose, specificity and a relative detection limit of the method were investigated.

To investigate the specificity of the method three solutions were utilised:

- Solution A: A blank solution (water R) that did not contain any arsenic ions.
- Solution B: A standard stain solution that contained 10 µg of arsenic ions (dilute arsenic AsTS).
- Solution C: A test solution (as described in section 11.4.1.1.1), which was spiked to contain 10 µg of arsenic ions.

To investigate the relative limit of detection three test solutions (solutions D - F) were prepared (as described in section 11.4.1.1.1). The three test solutions were spiked with 2 µg, 5 µg and 10 µg arsenic, which is equivalent to the zinc acetate dihydrate API containing 0.4 µg/g, 1 µg/g and 2 µg/g arsenic.

11.4.1.3.1 Procedure

Prepare the following solutions:

A) To 50 ml of water R add 10 ml of stannated hydrochloric acid (~250 g/l) AsTS.

B) To 50 ml of water R add 10 ml of stannated hydrochloric acid (~250 g/l) AsTS and 1 ml of dilute arsenic AsTS.

C) Dissolve 5.0 g of zinc acetate dihydrate in 50 ml of water R; add 1.0 ml dilute arsenic AsTS and 10 ml of stannated hydrochloric acid (~250 g/l) AsTS.

D) Dissolve 5.0 g of zinc acetate dihydrate in 50 ml of water R; add 1.0 ml dilute arsenic AsTS and 10 ml of stannated hydrochloric acid (~250 g/l) AsTS.

E) Dissolve 5.0 g of zinc acetate dihydrate in 50 ml of water R; add 0.5 ml dilute arsenic AsTS and 10 ml of stannated hydrochloric acid (~250 g/l) AsTS.

F) Dissolve 5.0 g of zinc acetate dihydrate in 50 ml of water R; add 0.2 ml dilute arsenic AsTS and 10 ml of stannated hydrochloric acid (~250 g/l) AsTS.

Respectively place the abovementioned solutions into the glass container, add 1 g potassium iodide AsR and 10 g of granulated zinc. Place the prepared glass tube quickly into position. Allow the reaction to proceed for 40 minutes, and inspect the stains produced.

11.4.1.3.2 Acceptance criteria

i. A sufficient level of specificity is illustrated when solution A does not produce a stain; and solution C produces a stain equal to the stain produced by solution B.
ii. A relative detection limit can be assigned for the lowest concentration of arsenic, where a stain is produced by solutions D - F. The relative detection limit is at or below the threshold of the limit test.

11.4.1.3.3 Results and discussion

When the procedure for specificity was executed solution A did not produce a stain, and the stain produced by solution C was comparable to the stain produced by solution B; see Figure 11.10 for photographs of the results.

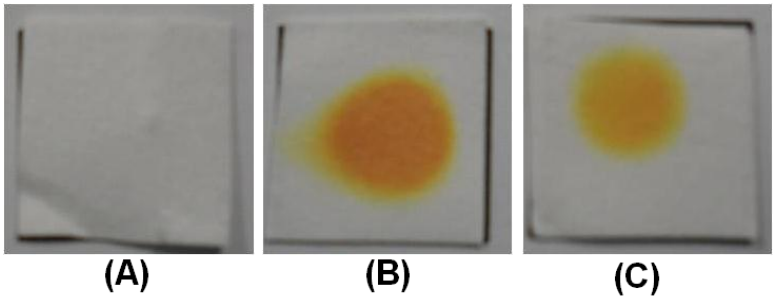


Figure 11.10 Photograph of specificity results: (A) solution A (water R), (B) solution B (dilute arsenic AsTS), (C) solution C (test solution spiked to contain 10 µg arsenic ions).

A stain was produced by all three arsenic-spiked zinc acetate dihydrate API test solutions (solutions D - F). The relative detection limit could thus be considered being lower than 0.4 µg/g, which is below the threshold of the limit test. Photographs of the results can be seen in Figure 11.11.

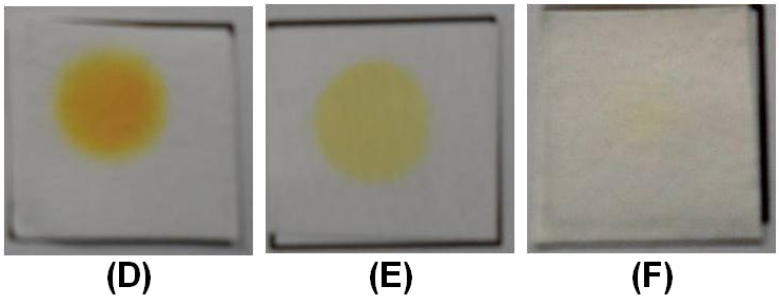


Figure 11.11 Photographs of the relative detection limit results: (D) zinc acetate dihydrate solution spiked to contain 2 µg/g arsenic ions, (E) zinc acetate dihydrate solution spiked to contain 1 µg/g arsenic ions, (F) zinc acetate dihydrate solution spiked to contain 0.4 µg/g arsenic ions.

11.4.1.3.4 Conclusion

A sufficient level of specificity was confirmed for the proposed method. The arsenic limit in the spiked zinc acetate dihydrate samples was detected in a quantity of up to 0.4 µg/g.

It can thus be concluded that this limit test is fit for the detection and limit of arsenic in zinc acetate dihydrate API.

11.4.1.4 Arsenic limit test executed on commercially available zinc acetate dihydrate API using the proposed method

The proposed method for the limit test of arsenic in zinc acetate dihydrate (section 11.4.1.1.1) was successfully verified. The method is thus deemed suitable, and was utilised to detect arsenic in commercially available zinc acetate dihydrate API.

11.4.1.4.1 Procedure

Execute the procedure as described in section 11.4.1.1.1; the test complies if the stain produced is not more intense than the similarly prepared standard stain.

11.4.1.4.2 Results and discussion

The zinc acetate dihydrate test solution did not produce a stain, and was therefore not more intense than the similarly prepared standard stain. Photographs of the stains can be seen in Figure 11.12.

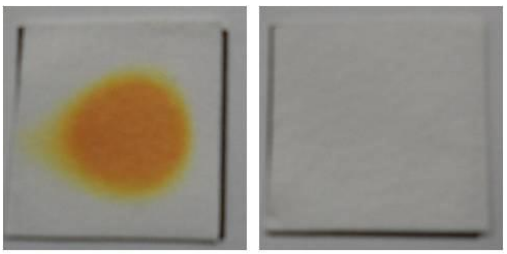


Figure 11.12 Photographs of the stains produced during limit test for arsenic in commercially available zinc acetate dihydrate: (A) standard stain; (B) zinc acetate dihydrate API test solution stain.

11.4.1.4.3 Conclusion

The commercially available zinc acetate dihydrate API complied with the limit test for arsenic (not more than 2 µg/g) due to the absence of any stain on the mercury bromide paper.

11.4.2 Development of methods for Group III specific metallic impurities – aluminium, cadmium, copper, iron and lead

Atomic absorption spectrometry (AAS) can determine the presence of metals in samples at low concentrations (typically in the low µg/ml range). Metal ions that can be analysed include Fe, Cu, Al, Pb, Ca, Zn, Cd and many more. AAS methods can be suitably validated to ensure compliance with current GMP and Good Laboratory Practices (GLP) which is required for the quality assurance of medicines (Taylor & Schulman, 2002:418).

AAS uses the absorption of light to measure the concentration of gas-phase atoms. Since samples are usually liquids or solids, the analyte atoms or ions must be vaporised in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The analyte concentration is determined from the amount of absorption. Concentration measurements are usually determined from a working curve / line after calibrating the instrument with standards of known concentration (Tissue, 2000).

11.4.2.1 Proposed methods to test for the presence of specific metallic impurities in zinc acetate dihydrate and zinc gluconate APIs

The procedures and the limits for the determination of Al, Cd, Cu, Fe and Pb in zinc acetate dihydrate and for Cd in zinc gluconate were derived from the applicable monographs from the Ph. Eur. / BP (BP, 2011; Ph. Eur., 2011). Table 11.5 summarises the experimental parameters for each of the analyses. The limits for the specific metallic impurities reported in Table 11.5 were obtained by converting the limits reported in Table 9.1 (expressed as ppm) to µg/g as required by *The Ph. Int.*

Table 11.5 Experimental parameters for the AAS determination of selected metallic impurities in zinc acetate dihydrate and zinc gluconate APIs (BP, 2011)

Parameter	Zinc acetate dihydrate					Zinc gluconate
Element / Impurity	Al	Cd	Cu	Fe	Pb	Cd
Sample mass (g) dissolved in 25 ml diluent	2.5	2.5	1.25	1.25	5.0	1.25
Method	I – Direct calibration					II - Standard addition
Diluent	200 g/l cadmium & lead free nitric acid R					Water R
Standard solution range (µg/ml)	0.4 - 5.0	0.1 - 3.0	1.0 - 5.0	1.0 - 5.0	1.0 - 10.0	0.05 - 1.0
Source (hollow cathode lamp)	Al	Cd	Cu	Fe	Pb	Cd
Wavelength (nm)	309.3	228.8	324.8	248.3	283.3	228.8
Slit width	0.5	0.5	0.5	0.2	0.5	0.5
Atomisation device	Acetylene-Nitrous oxide flame	Air-Acetylene flame				
Limit NMT (µg/g)	5	2	50	50	10	2

For these methods to be adopted by *The Ph. Int*. the following reagents and test solution need to be defined and included into the "*Reagents, test solutions and volumetric solutions*" section of *The Ph. Int*. The following text is recommended for inclusion:

Cadmium R.

Cd

A commercially available reagent of suitable grade.

Cadmium standard (1000 µg Cd/ml) TS.

Procedure. Dissolve 0.100 g of cadmium R in sufficient amount of equal volumes of hydrochloric acid (~330 g/l) TS and water R and dilute to 100 ml with a 1 per cent V/V solution of hydrochloric acid (~330 g/l) TS.

Note. For the preparation of this test solution commercially available cadmium standard solution 1000 µg Cd/ml can also be used.

Nitric acid (~1000 g/l), cadmium-free and lead-free, TS.
[nitric acid, cadmium-free and lead-free (70 per cent.) R].

Nitric acid (~200 g/l), cadmium-free and lead-free, TS.
Nitric acid (~1000 g/l), cadmium-free and lead-free, TS, diluted with water R to contain 200 g/l of HNO_3.

There are two general methods which are routinely used for AAS analysis by pharmacopoeias namely: external standard method and standard addition method (BP, 2011; *Ph. Int.,* 2011). The general procedures for the external standard method and standard addition method described by *The Ph. Int.* (*Ph. Int.*, 2011) were adopted for the AAS procedures for the metal impurity limit tests.

11.4.2.1.1 Procedure for Method 1: External standard method

Prepare the solution of the substance to be tested (i.e. test solution) as specified in the method (refer to Table 11.5 for the applicable experimental parameters). Prepare concurrently, adding any reagents in the same concentration as for the test solution, not fewer than three reference solutions (five reference solutions were used for this study based on literature recommendations {Ermer & Miller 2005:312}) of the metallic impurity to be determined that cover the expected concentration range of the test solution (refer to Table 11.5). Similarly, prepare a blank solution.

After calibration / optimisation of the instrument, introduce each reference solution into the instrument three times, recording instrument response (absorbance) obtained. Wash the apparatus after each introduction with the blank solution to ensure that the reading returns to its initial setting, seeing that the atomisation device specified for these analyses were either an acetylene-nitrous oxide or an air-acetylene flame. Prepare a calibration curve by plotting the mean of each group of three readings obtained for the reference solutions against the concentration (Figure 11.13).

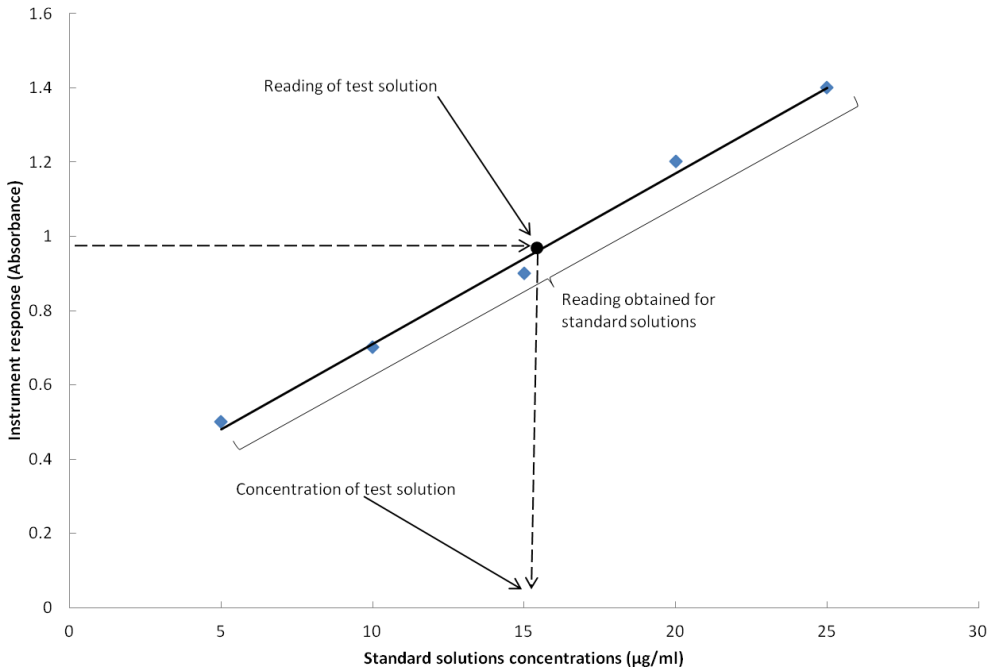


Figure 11.13 Theoretical calibration curve constructed for illustration purposes of the external standard method of *The Ph. Int.*

Introduce the test solution into the instrument three times, and record the absorbance readings. Determine the concentration of the metallic impurity in the test solution (µg/ml) using the mean of the readings and interpolating from the calibration curve.

Calculate the amount of the specific metallic impurity in the test sample (i.e. API) (µg/g) utilising the following equation:

$$\text{Impurity concentration (µg/g)} = \frac{\text{impurity concentration in test solution (µg/ml)} \times \text{final volume of test solution (ml)}}{\text{sample mass (g)}}$$

11.4.2.1.2 Procedure for Method 2: Standard addition method

The standard addition method is used to exclude the interference of the components that may be present in a sample matrix, and is commonly applied in metal impurity limit testing using AAS (Skoog *et. al.,* 1997:572). Organic interferences in the heavy metals limit test for zinc gluconate API were observed (section 11.3), justifying the use of method 2 (standard addition method) for the cadmium limit test.

Bruce & Gill (1999:805) described the basic principles of the standard addition method as follows:

Equal volumes, V_x, of a test solution which contains an unknown concentration of the specific metallic impurity, C_x, are added to a series of volumetric flasks of final volume, V_t. A standard analyte solution with a known concentration of the specific impurity, C_s, is used to spike the volumetric flasks using a different volume, V_s, in each case. Each volumetric flask is then filled to the calibration mark with the appropriate solvent. The concentration of the impurity in each of the volumetric flasks can be expressed in the following equation:

$$C_{Impurity} = \frac{C_s V_s}{V_t} + \frac{C_x V_x}{V_t}$$

Within the linear calibration range it is assumed that the response signal, R, of the instrument is directly proportional to the impurity concentration; that is, $R = k\ C_{Impurity}$. Thus for each of the volumetric flasks the following equation is applicable:

$$R = k\left(\frac{C_s V_s}{V_t} + \frac{C_x V_x}{V_t}\right) = k\left[\frac{V_x}{V_t}\left(\frac{C_s V_s}{V_x}\right) + \frac{C_x V_x}{V_t}\right]$$

When c' is defined as the increase in the impurity concentration in the original test solution volume (V_x) due to the added spikes, the following equation is applicable:

$$c' = \frac{C_s V_s}{V_x}$$

The correlation between the response signal, R, of the instrument used and the impurity concentration can thus also be expressed as:

$$R = mc' + b$$

Where: the slope, m, is defined as:

$$m = k\frac{V_x}{V_t}$$

And the intercept, *b*:

$$b = k\frac{C_x V_x}{V_t}$$

The x-axis intercept, C'_0, of the extrapolated calibration curve (Figure 11.14) occurs at $R = 0$ and $b = -mC'_0$, which can be written as $b/m = -C'_0$. On substituting the abovementioned equations for the slope and the intercept, this simplifies to give $C_x = b/m = -C'_0$ (Bruce & Gill, 1999:805).

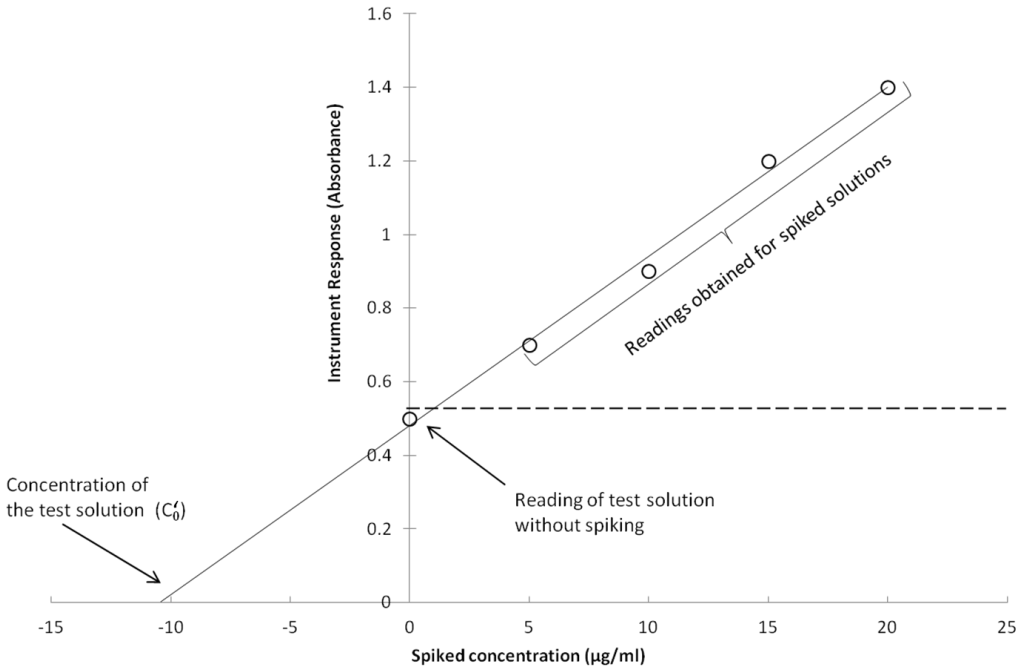


Figure 11.14 Theoretical calibration curve constructed for illustration purposes of the method of standard additions of *The Ph. Int.*

The following general procedure of *The Ph. Int.* (2011) for the method of addition was used during this study:

Prepare in at least three similar volumetric flasks a series of solutions (four solutions were used for this study based on literature recommendations {Ermer & Miller 2005:312}) containing equal quantities of the substance to be tested as specified in the monograph (refer to Table 11.5 for the applicable experimental parameters) and increasing volumes of the reference solution containing known concentrations of the metallic impurity to be determined. The concentrations selected (refer to Table 11.5)

should be expected to produce responses in the linear part of the calibration curve. One of the solutions of the substance to be tested should contain no added reference solution.

Once the AAS instrument has been calibrated / optimised, introduce each solution into the instrument three times, recording the steady reading obtained. Wash the apparatus after each introduction with blank solution to ensure that the reading returns to its initial setting, seeing that the atomisation device specified for this analysis was an air-acetylene flame.

Prepare a calibration curve by plotting the mean of each group of three readings obtained for the four solutions against the spiked concentration of the specific metallic impurity in the solutions. Extrapolate the straight line joining the points on the graph to an extended concentration axis. Calculate the x-axis intercept of the extrapolated curve, which represents the impurity concentration in the test solution (µg/ml). Calculate the impurity concentration present in the API (µg/g) using the following equation:

$$\text{Impurity concentration (µg/g)} = \frac{\text{impurity concentration in test solution (µg/ml)} \times \text{final volume of test solution (ml)}}{\text{sample mass (g)}}$$

11.4.2.2 Materials and equipment

The information of the materials used in the metallic impurity determination by means of AAS can be seen in Table 11.6, and the information of equipment used in Table 11.7.

Table 11.6 Materials used in the metallic impurity determination by means of AAS in zinc acetate dihydrate and zinc gluconate APIs

Material	Batch number	Manufacturing company	Country of origin
Zinc acetate dihydrate	A892302 020	Merck KGaA	Germany
Zinc gluconate	K38073079 025	Merck KGaA	Germany
Nitric acid (chemically pure)	1035275	Merck Chemicals (Pty) Ltd.	South Africa
1000 µg/ml Aluminium standard	B2-AL04072	Teknolab AB	Sweden
1000 µg/ml Cadmium standard	C2-CD02021	Teknolab AB	Sweden
1000 µg/ml Copper standard	C2-CU02116	Teknolab AB	Sweden
1000 µg/ml Iron standard	D2-FE03128	Teknolab AB	Sweden
1000 µg/ml Lead standard	D2-PB03020	Teknolab AB	Sweden
Water R	N/A	RIIP®/CENQAM®	South Africa

Table 11.7 Equipment used in the metallic impurity determination by means of AAS in zinc acetate dihydrate and zinc gluconate APIs

Equipment	Supplier	Country of origin
Sartorius ED623S+ balance	IMP	South Africa
AAFS240 Varian fast sequential AA	SMM	South Africa
Aluminium hollow cathode lamp	Varian	Australia
Cadmium hollow cathode lamp	Varian	Australia
Copper hollow cathode lamp	Varian	Australia
Cobalt/Chromium/Copper/Iron/ Manganese/Nickel hollow cathode lamp	Varian	Australia
Lead hollow cathode lamp	Varian	Australia

11.4.2.3 Method verification of the specific metallic impurity limit test methods for zinc acetate dihydrate APIs

To ensure the fitness of purpose for these limit test methods, specificity and limit of detection were investigated (Table 3.4 – Chapter 3).

The technique of AAS is specific since the atom emits or absorbs radiation at discrete spectral lines when the appropriate source and wavelength, for the element

to be determined, are used. However, interferences, such as chemical, physical, ionisation and spectral, may be encountered due to optical and / or chemical effects (Ermer & Miller, 2005:312).

In order to avoid or reduce chemical interferences only chemically pure solvents were used. Physical interferences were eliminated by matrix matching and dilution of the samples. Spectral interferences were avoided by using source background correction; and, as far as possible, single-element hollow-cathode lamps were used. Background absorption was corrected by using a blank solution and the instrumental parameters have been optimised to avoid further interferences (Ermer & Miller, 2005:312). Therefore, only detection limits should be established to ensure the success of the method verifications.

11.4.2.3.1 Procedure

Execute the procedure in section 11.4.2.1.1 for the metallic impurities Al, Cd, Cu, Pb and Fe, using the experimental parameters described in Table 11.5.

Plot the absorbance values as a function of the standard solution concentrations (µg/ml) of the specific metallic impurity. Perform a linear regression analysis to determine the correlation coefficient, y-intercept and slope of the regression line, using a 95 % confidence interval.

Determine the absorbance value in triplicate for each blank solution by means of AAS utilising the procedure as described in section 11.4.2.1.1. Calculate the standard deviation (σ) of the three absorbancies obtained for each blank solution at the prescribed experimental conditions with the following equation:

$$SD\,(\sigma) = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Where: *x* is the analytical background response values; $\bar{x}$ represents the average of the triplicate values; and *n* is the sample size (n=3).

Calculate the detection limit utilising the slope obtained from the regression analyses and the standard deviations of the blank solution absorbance values:

$$Detection\ limit\ (DL) = \frac{3.3\sigma}{S}$$

Where: σ = the standard deviation of the blank solution absorbance values; and S = the slope of the calibration curve.

Calculate the detection limit criterion (DLC) for each metallic impurity (i.e Al, Cd, Cu, Pb and Fe). The DLC could be defined as the concentration of the impurity present in the test solution when it is assumed that the test substance under investigation contains the maximum allowable limit of the specified impurity (Table 11.5). For example, zinc acetate dihydrate may contain up to 5 µg/g aluminium, thus the test solution (specified in Table 11.5) may contain a maximum aluminium concentration of: (2.5 g x 5 µg/g) ÷ 25 ml = 0.5 µg/ml, which is referred to as the DLC.

11.4.2.3.2 Acceptance criteria

i. A linear relationship should exist between the standard solution concentrations (µg/ml) and absorbance values obtained; with a linear correlation coefficient (r^2) not less than 0.99 ($r^2 \geq 0.99$).
ii. The determined DL for Al, Cd, Cu, Pb and Fe in zinc acetate dihydrate API is lower or equal to the DLC set for that specific metallic impurity (DLC ≥ DL).

11.4.2.3.3 Results and discussion

The concentrations of the standard solutions were determined based on the theoretical final concentrations of the impurity in the test solutions (DLC) and the concentration range that would be in the linear part of the curve (Varian, 1989:4). These concentrations are tabulated in Table 11.8.

Table 11.8 Concentration of the standard solutions prepared based on the optimum linear working range of the AAS and the expected concentration range of the test solution concentrations (DLC)

Impurity	Optimum linear working range of AAS (µg/ml)*	DLC (µg/ml)	Concentration of the standard solutions prepared (µg/ml)
Al	0.3 - 100.0	0.5	Solution 1: 0.4 Solution 2: 0.8 Solution 3: 1.6 Solution 4: 3.2 Solution 5: 5.0
Cd	0.02 - 3.0	0.2	Solution 1: 0.1 Solution 2: 0.2 Solution 3: 1.0 Solution 4: 2.0 Solution 5: 3.0
Cu	0.03 - 5.0	2.5	Solution 1: 1.0 Solution 2: 2.0 Solution 3: 3.0 Solution 4: 4.0 Solution 5: 5.0
Fe	0.06 - 5.0	2.5	Solution 1: 1.0 Solution 2: 2.0 Solution 3: 3.0 Solution 4: 4.0 Solution 5: 5.0
Pb	0.5 - 15.0	2.0	Solution 1: 1.0 Solution 2: 2.0 Solution 3: 3.0 Solution 4: 5.0 Solution 5: 10.0

*Optimum linear working range for AAFS240 Varian fast sequential AA (Varian, 1989:6; 15; 19; 23 & 46).

The calibration curves for the metallic impurities in zinc acetate dihydrate were constructed using STATISTICA 10 (2011) software (StatSoft, Inc., USA); the graphs can be seen in Figures 11.15 – 11.19 for Al, Cd, Cu, Pb and Fe respectively.

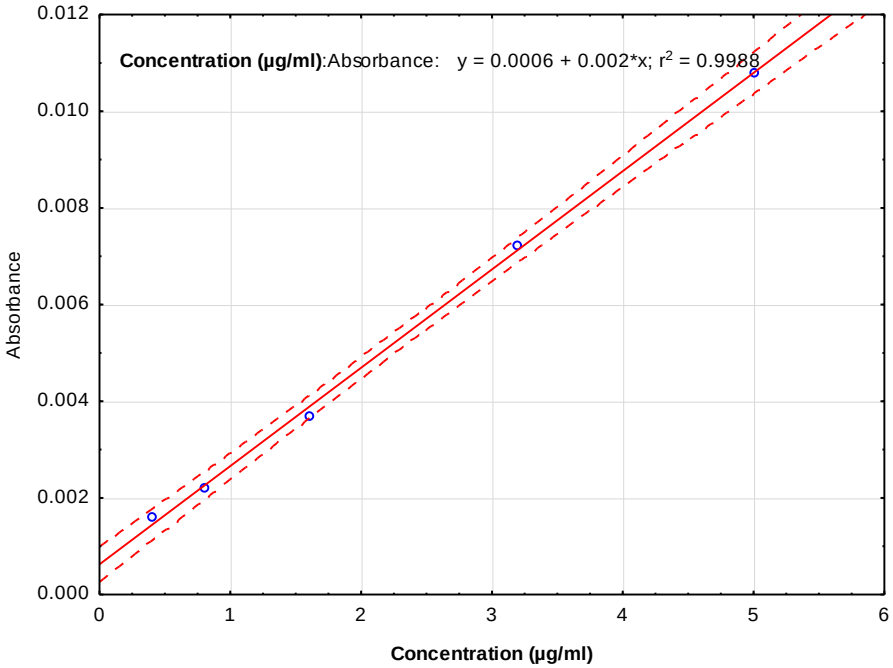


Figure 11.15 Graph of absorbance versus concentration of aluminium standard solutions as obtained by means of AAS. The dashed line illustrates the 95 % prediction interval for the linear regression line.

The linear regression analysis reported r^2 = 0.9988, therefore a linear relationship existed between the aluminium standard solution concentration and the absorbance values in the range 0.4 – 5.0 µg/ml. The intercept of the regression line in Figure 11.15 was calculated as 0.0006 (± 0.0001), which did not differ significantly from zero.

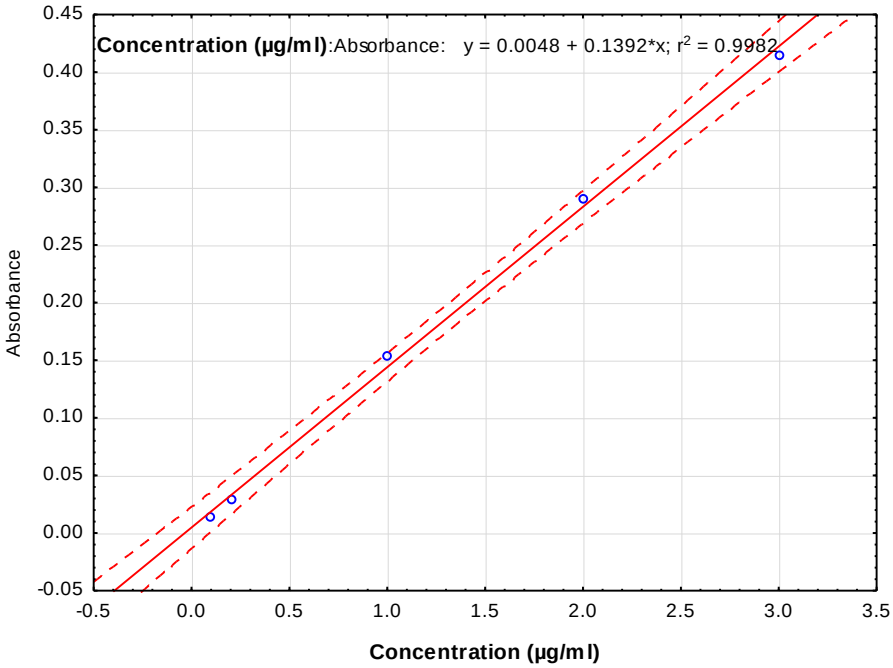


Figure 11.16 Graph of absorbance versus concentration of cadmium standard solutions as obtained by means of AAS. The dashed line illustrates the 95 % prediction interval for the linear regression line.

The linear regression analysis reported r^2 = 0.9982, therefore a linear relationship existed between the cadmium standard solution concentration and the absorbance values in the range 0.1 - 3.0 µg/ml. The intercept of the regression line in Figure 11.16 was calculated as 0.0048 (± 0.0057), which did not differ significantly from zero.

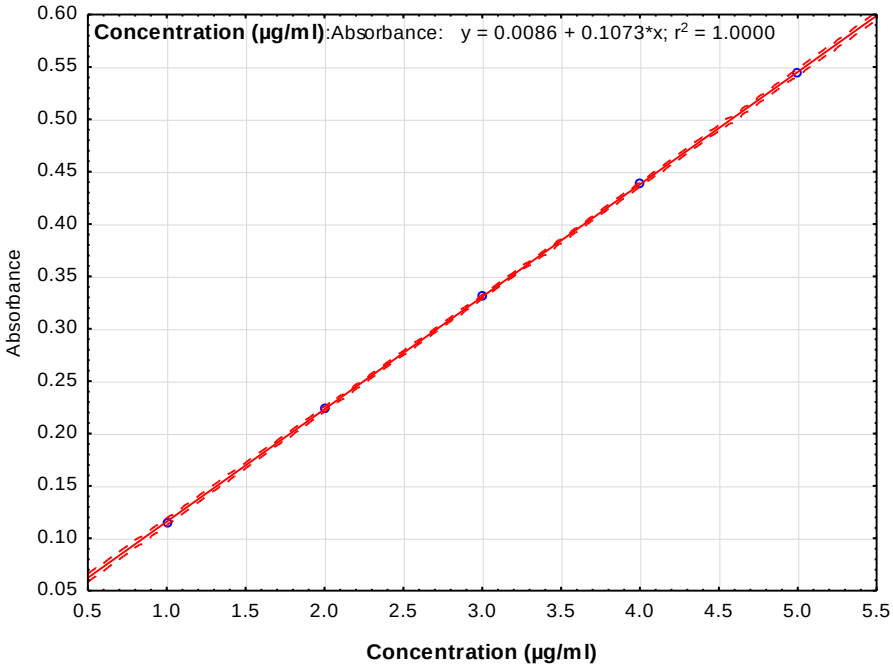


Figure 11.17 Graph of absorbance versus concentration of copper standard solutions as obtained by means of AAS. The dashed line illustrates the 95 % prediction interval for the linear regression line.

The linear regression analysis reported r^2 = 1.0000, therefore a linear relationship existed between the copper standard solution concentration and the absorbance values in the range 1.0 - 5.0 µg/ml. The intercept of the regression line in Figure 11.17 was calculated as 0.0086 (± 0.0014), which did not differ significantly from zero.

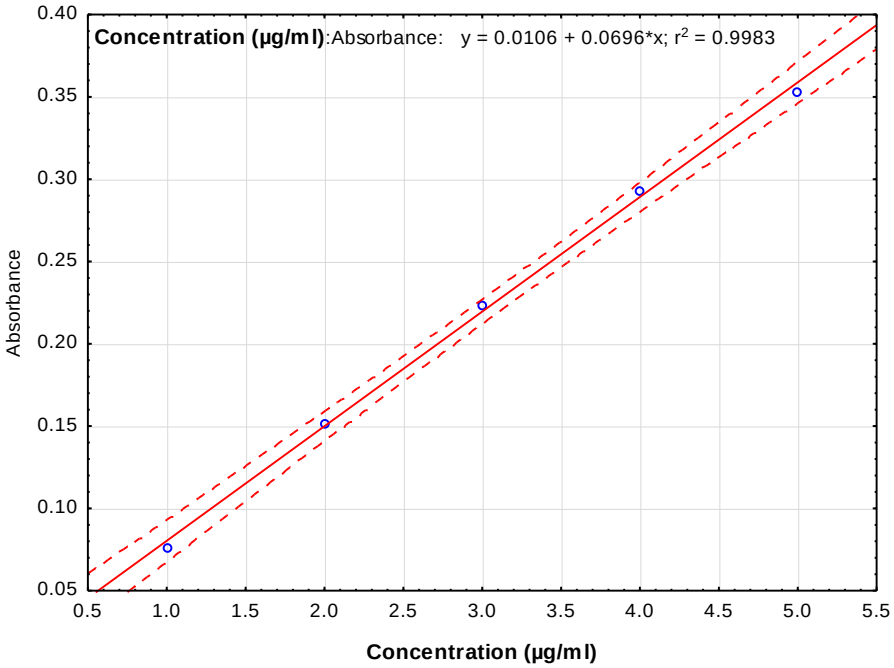


Figure 11.18 Graph of absorbance versus concentration of iron standard solutions as obtained by means of AAS. The dashed line illustrates the 95 % prediction interval for the linear regression line.

The linear regression analysis reported r^2 = 0.9983, therefore a linear relationship existed between the iron standard solution concentration and the absorbance values in the range 1.0 - 5.0 µg/ml. The intercept of the regression line in Figure 11.18 was calculated as 0.0106 (± 0.0054), which did not differ significantly from zero.

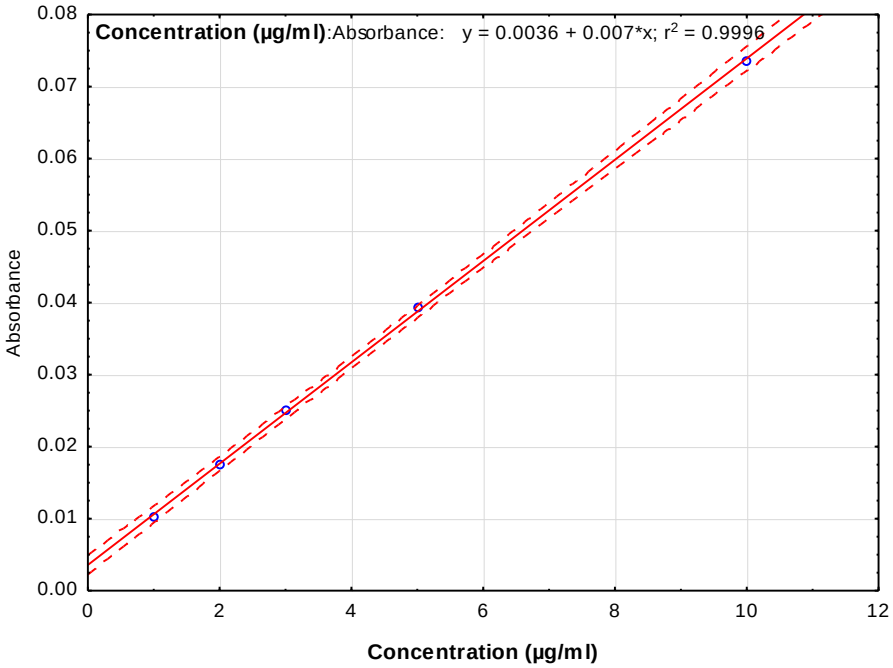


Figure 11.19 Graph of absorbance versus concentration of lead standard solutions as obtained by means of AAS. The dashed line illustrates the 95 % prediction interval for the linear regression line.

The linear regression analysis reported r^2 = 0.9996, therefore a linear relationship existed between the lead standard solution concentration and the absorbance values in the range 1.0 - 10.0 µg/ml. The intercept of the regression line in Figure 11.19 was calculated as 0.0036 (± 0.0004), which did not differ significantly from zero.

The standard deviation (σ) of the three absorbancies obtained for each blank solution at the prescribed experimental conditions, the DL and the DLC values were calculated as described in section 11.4.2.3.1 and are tabulated in Table 11.9.

Table 11.9 Detection limit results for the metallic impurities in zinc acetate dihydrate API by means of AAS

Test substance	Impurity	σ of blank	Slope	DL (µg/ml)	DLC (µg/ml)
Zinc acetate dihydrate	Al	0.000286	0.0020	0.47	0.50
	Cd	0.001132	0.1392	0.03	0.20
	Cu	0.000207	0.1073	0.01	2.50
	Fe	0.000553	0.0696	0.03	2.50
	Pb	0.000442	0.0070	0.21	2.00

The calculated DL for each of the elements (Al, Cd, Cu, Pb and Fe) was found to be lower than the DLC for the specific metallic impurity, which rendered the proposed limit method suitable to control the specific metallic impurities in zinc acetate dihydrate API.

The BP offers a choice of two atomisation devices, namely air-acetylene or acetylene-nitrous oxide flame. When the air-acetylene flame (2100 - 2400 °C) was utilised the instrument could not be optimised for quantitative analyses due to the inability of the flame to ionise the aluminium (Skoog et al., 1997:165). The instrument response increased when the hotter flame of acetylene-nitrous oxide (2600 - 2800 °C) was used. However, even with the hotter flame a definite lower instrument response to Al was observed, when compared to the instrument response obtained with the other metallic impurities tested (Figures 11.15 – 11.19). This may be attributed to the fact that acetylene-nitrous oxide flame only partially ionises aluminium (Varian, 1989:6), which results in a lower instrument response (absorbance) and thus a higher DL (Table 11.9).

11.4.2.3.4 Conclusion

AAS shows a suitable specificity for the detection of Al, Cd, Cu, Pb and Fe in zinc acetate dihydrate API.

An acceptable limit of detection was established for each of the metallic impurities (Al, Cd, Cu, Pb and Fe) in zinc acetate dihydrate API, where none of the DLs exceeded the specified DLCs of the specific impurities.

11.4.2.4 Method verification of the specific metallic impurity limit test method for zinc gluconate API

To ensure the fitness of purpose for the cadmium limit test, specificity and limit of detection were investigated (Table 3.4 – Chapter 3). The specificity of AAS for the metallic impurities was discussed in section 11.4.2.3. In section 11.4.2.4.1 the limit of detection for cadmium with the proposed method will be determined.

11.4.2.4.1 Procedure

Execute the procedure in section 11.4.2.1.2 for the Cd impurity, using the experimental parameters described in Table 11.5.

Prepare a calibration curve by plotting the mean of each group of three readings obtained for the four solutions against the spiked concentration of the Cd impurity in the solutions. Perform a linear regression analysis to determine the correlation coefficient, y-intercept and slope of the regression line, using a 95 % confidence interval. Extrapolate the regression line to illustrate the x-axis intercept thereof.

Determine the absorbance value in triplicate for the blank solution by means of AAS utilising the procedure as described in section 11.4.2.1.2. Calculate the standard deviation (σ) of the three absorbancies obtained for the blank solution at the prescribed experimental conditions with the following equation:

$$SD\,(\sigma) = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Where: *x* is the analytical background response values; $\bar{x}$ represents the average of the triplicate values; and *n* is the sample size (n=3).

Calculate the DL utilising the slope obtained from the regression analyses and the standard deviation of the blank solution absorbance values:

$$Detection\ limit\ (DL) = \frac{3.3\sigma}{S}$$

Where: σ = the standard deviation of the blank solution absorbance values; and S = the slope of the calibration curve.

Calculate the DLC for the Cd impurity in zinc gluconate API (refer to section 11.4.2.3.1).

11.4.2.4.2 Acceptance criteria

i. A linear relationship should exist between the spiked concentrations of the Cd impurity (µg/ml) and absorbance values obtained; with a linear correlation coefficient (r^2) not less than 0.99 ($r^2 \geq 0.99$).
ii. The determined DL for Cd in zinc gluconate API is lower or equal to the DLC set for the Cd impurity (DLC ≥ DL).

11.4.2.4.3 Results and discussion

The spiked concentration of the Cd impurity in the test solutions were determined based on the theoretical final concentration of the impurity in the test solution (DLC) and the concentration range that would be in the linear part of the curve (Varian, 1989:4). These concentrations are tabulated in Table 11.10.

Table 11.10 Concentration of the standard solutions prepared based on the optimum linear working range of the AAS and the expected concentration range of the test solution concentrations

Impurity	Optimum linear working range of AAS (µg/ml)*	DLC (µg/ml)	Spiked concentrations of the test solutions prepared (µg/ml)
Cd	0.02 - 3.0	0.1	Solution 1: 0.0 Solution 2: 0.05 Solution 3: 0.1 Solution 4: 1.0

* Optimum linear working range for AAFS240 Varian fast sequential AA (Varian, 1989:15).

For zinc gluconate only four test solutions were prepared. This is due to the fact that the zinc gluconate solutions are very viscous and tends to clog the burner, reducing the quality of the flame. However, it still complied with the number of test solutions required by *The Ph. Int.* (*Ph. Int.,* 2011) and literature (Ermer & Miller, 2005:312).

The calibration curve for the Cd impurity in zinc gluconate API was constructed using STATISTICA 10 (2011) software (StatSoft, Inc., USA); the graph can be seen in Figure 11.20.

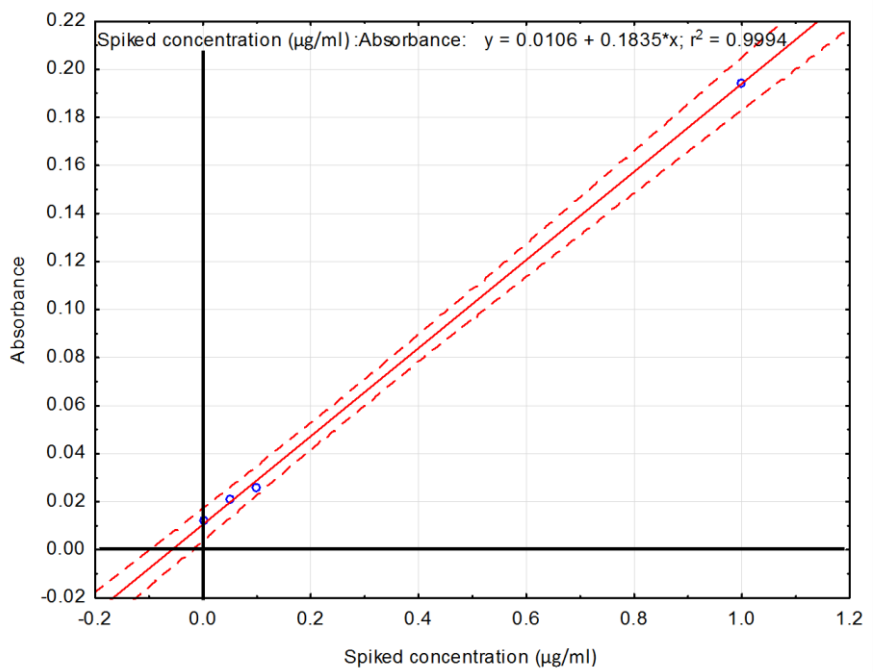


Figure 11.20 Graph of absorbance versus concentration of cadmium spiked sample solutions as obtained by means of AAS for zinc gluconate API. The dashed line illustrates the 95 % prediction interval for the linear regression line.

The standard deviation of the three absorbancies obtained for the blank solution at the prescribed experimental conditions, the DL and the DLC were calculated as described in section 11.4.2.4.1 and are tabulated in Table 11.11.

Table 11.11 Detection limit results for the Cd metallic impurity in zinc gluconate API by means of AAS

Test substance	Impurity	σ of blank	Slope	DL (µg/ml)	DLC (µg/ml)
Zinc gluconate	Cd	0.002176	0.1835	0.04	0.1

The calculated DL was found to be lower than the DLC for the Cd impurity, which rendered the proposed limit method suitable to control Cd impurity in zinc gluconate API.

11.4.2.4.4 Conclusion

AAS shows a suitable specificity for the detection of Cd in zinc gluconate API.

An acceptable limit of detection was established for the Cd impurity in zinc gluconate API, where the DLC exceeded the calculated DL of the Cd impurity.

11.4.2.5 Specific metallic impurity tests for commercially available zinc acetate dihydrate and zinc gluconate APIs using the proposed methods

The proposed methods (described in section 11.4.2.1) were applied to test commercially available samples of zinc acetate dihydrate and zinc gluconate APIs for the presence of specific metallic impurities. The product information is summarised in Table 11.6.

11.4.2.5.1 Procedure

Execute the procedures as described in section 11.4.2.1 for the zinc acetate dihydrate and zinc gluconate APIs to establish compliance with the proposed metallic impurity limit tests.

11.4.2.5.2 Results and discussion

The results for the metallic impurities in zinc acetate dihydrate and zinc gluconate samples by means of AAS are summarised in Table 11.12 and Table 11.13 respectively.

Table 11.12 Results of the metallic impurity limit tests in zinc acetate dihydrate API samples by means of AAS

Impurity	Sample mass (g)	Mean absorbance value of test solution	Regression equation	Concentration of impurity in the test solution (µg/ml)	Impurity concentration present in the API (µg/g)
Al	2.507	0.0012	y = 0.0006 + 0.002x	0.3	2.99
Cd	2.502	0.0014*	y = 0.0048 + 0.1392x	Not detected	Not detected
Cu	1.246	0.0000*	y = 0.0086 + 0.1073x	Not detected	Not detected
Fe	1.252	0.0017*	y = 0.0106 + 0.0696x	Not detected	Not detected
Pb	5.006	0.0001*	y = 0.0036 + 0.007x	Not detected	Not detected

* Below instrument detection limit (absorbance): Cd = 0.0090; Cu = 0.0097; Fe = 0.0127 & Pb = 0.0051

Only aluminium impurity was detected in zinc acetate dihydrate, and was found to be not more than 5 µg/g.

Table 11.13 Result for the Cd metallic impurity in a zinc gluconate API sample by means of AAS

Impurity	Mass of sample (g)	Regression equation	Concentration of impurity in the test solution (µg/ml)	Impurity concentration present in the API (µg/g)
Cd	5.006	y = 0.0106 + 01835x	0.0106	0.05

The calculated Cd concentration in zinc gluconate API was found to be not more than 2 µg/g.

11.4.2.5.3 Conclusion

The commercially available sample of zinc acetate dihydrate and zinc gluconate APIs complied with the specification of the proposed metallic impurity limit tests.

11.5 Chapter conclusion

Metallic impurities in APIs need to be controlled due their inherent toxicity, formation of insoluble metal complexes, and oxidative and hydrolytic catalytic activity.

Six specific metallic impurity limit tests (for: arsenic, aluminium, cadmium, copper, iron and lead) were established for zinc acetate dihydrate API; and two metallic impurity limit tests for zinc gluconate API, i.e. heavy metals limit test and cadmium limit test. Criteria were established for the mentioned limit tests based on the information available in the literature.

The general heavy metal limit test and the arsenic limit test of *The Ph. Int.* were used to control the heavy metal and arsenic content in zinc gluconate and zinc acetate dihydrate APIs respectively. Atomic absorption spectrometric methods (presented in the Ph. Eur. / BP) were utilised to limit Al, Cd, Cu, Fe and Pb impurities in zinc acetate dihydrate using the external standard method described in *The Ph. Int.* The

Cd content in zinc gluconate API was limited by means of the standard addition method.

To illustrate the fitness for purpose of the limit tests, method verifications were performed. All the verification parameters were met, deeming the methods suitable for use. Minor adjustments were made (and validated) to the general heavy metal limit test published in *The Ph. Int.* to ensure the specificity of the test.

Commercially available zinc acetate dihydrate and zinc gluconate APIs were tested using the proposed methods and it was found that both APIs complied with the proposed criteria.

The proposed limit tests are recommended for inclusion in the zinc acetate dihydrate and zinc gluconate API monographs of *The Ph. Int*.

CHAPTER 12

CONCLUSION

12.1 Introduction

Acute diarrhoea remains a leading cause of childhood deaths, with approximately 1.3 million annual deaths of children under the age of five years (Chapter 1).

A new formulation for ORS, with a reduced osmolarity, and added zinc supplementation have been proposed by the WHO and several other institutions in order to reduce the severity and duration of diarrhoea. Zinc supplementation suitable for the treatment of diarrhoea include: zinc sulfate, zinc acetate dihydrate and zinc gluconate (Chapter 1).

With no monographs available in *The Ph. Int*. for either zinc acetate dihydrate API, or zinc gluconate API, the development of these monographs has become a priority to the WHO. The WHO requested the RIIP® incorporating CENQAM® to assist in this challenge.

To pursue the set study objectives, a systematic research approach was followed. The reaction path for chemical reactions and the activation energy required to obtain the final product, as described in 1889 by the Swedish scientist Svante Arrhenius (Kotz & Treichel, 1999:715), could be used as a metaphor to describe the monograph development process (Figure 12.1) in this study.

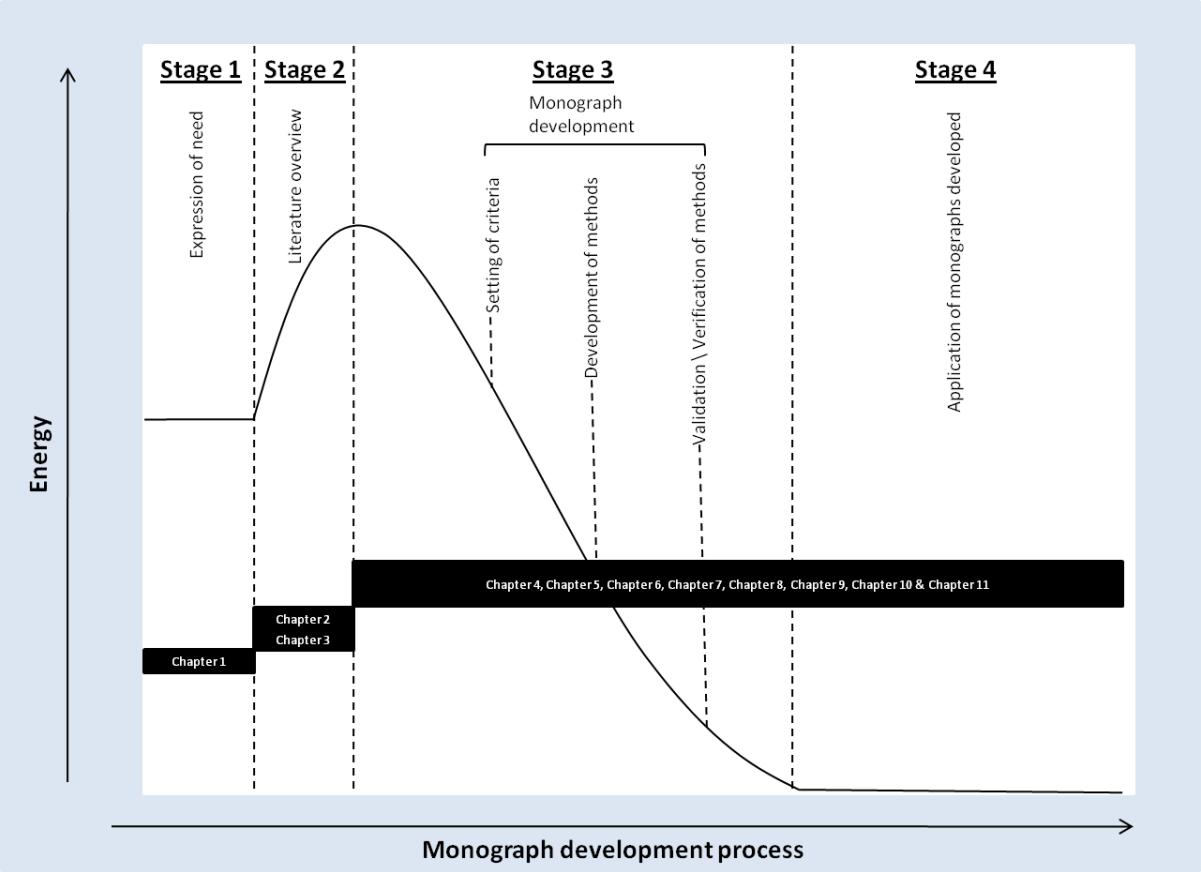


Figure 12.1 The reaction path as a metaphor to describe the monograph development process in this study.

"Enthusiasm is the yeast that makes your hopes shine to the stars. Enthusiasm is the sparkle in your eyes, the swing in your gait. The grip of your hand, the irresistible surge of will and ***energy*** *to* ***execute your ideas****."*

Henry Ford

(Source: http://www.brainyquote.com/quotes/keywords/energy.html)

The development of the monographs in this study could be divided into four stages:

- Stage 1: Expression of need.
- Stage 2: Literature review.
- Stage 3: Monograph development.
- Stage 4: Application of monographs developed.

12.2 Expression of need

The need expressed by the WHO to draft compendial monographs for zinc acetate dihydrate and zinc gluconate APIs, and the literature available which illustrated the efficacy of the zinc salts to potentially reduce the alarming high mortality rate in children, fuelled this study. Monographs are already available in the Ph. Eur. / BP and USP. However, these monographs are not available free of charge, which is especially important to developing countries to ensure the quality of zinc salts to be utilised in the treatment of paediatric diarrhoea.

Chapter 1 provided a concise overview of the pathogenesis, complications and treatment of diarrhoea. From the information presented in this chapter it was clear that diarrhoea is a current and real-time problem in our modern day society, especially in malnourished populations, emphasising the importance of this study.

12.3 Literature review

Chapter 2 provided an overview of the pharmaceutical and pharmacological properties of zinc acetate dihydrate API and zinc gluconate API, which were considered during the development of the mentioned monographs. Valuable information was obtained to draft the general information which appears in the API monographs such as: chemical formula, relative molecular mass, chemical name of API, description, solubility, category, storage, labelling and other applicable information.

Chapter 3 highlighted the important role that monographs play in the QC-testing of APIs and pharmaceutical dosage forms, to ensure the quality, safety and efficacy thereof. This chapter also summarised the steps and general requirements for the development of monographs to be published in *The Ph. Int.* These steps were

followed for the development of the zinc acetate dihydrate and zinc gluconate API monographs.

To conclude, Chapters 2 and 3 provided the necessary information and tools ("activation energy") which were required to initiate the monograph development process.

12.4 Monograph development

The basic structure of an API monograph which is to be published in *The Ph. Int.* - presented in Chapter 3 (Figure 3.2) - was used to determine which information and requirements should be presented in the zinc acetate dihydrate and zinc gluconate API monographs.

After a comprehensive study of all available literature and official compendial monographs, the information and the applicable requirements were identified and set. Appropriate methods were identified or developed for all the requirements. Figure 12.2 and Figure 12.3 provide a schematic presentation of the requirements set for the monographs of the two APIs, as well as the relevant methods proposed. The monographs compiled for *The Ph. Int.*, with their relevant criteria, are presented in Annexure A and Annexure B.

These two monographs have been presented to the WHO and the peer-review panel for review. The final monographs will be presented to the WHO Expert Committee on Specifications for Pharmaceutical Preparations for formal adoption and publication in October 2012.

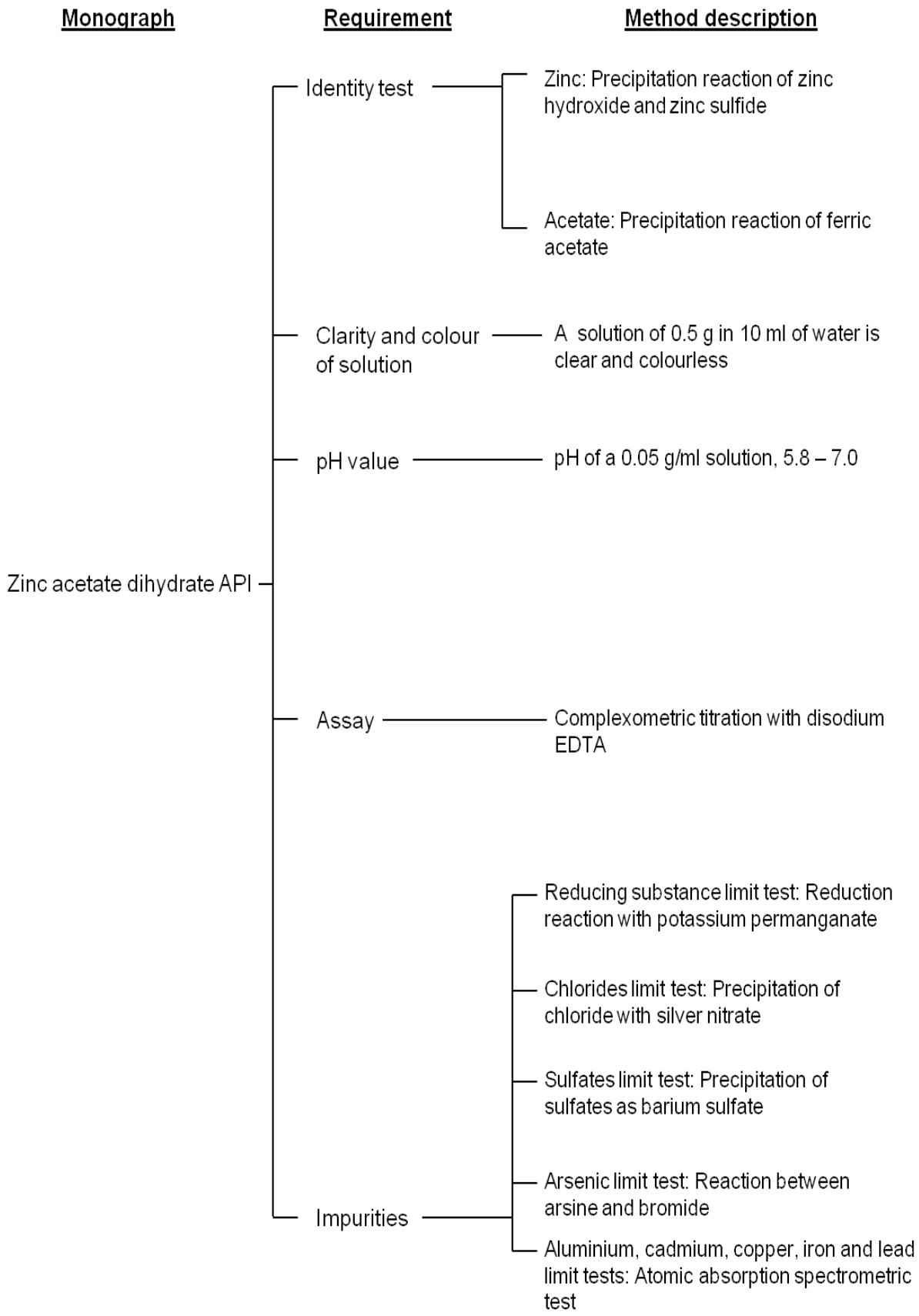


Figure 12.2 Schematic presentation of the requirements set for the zinc acetate dihydrate API monograph.

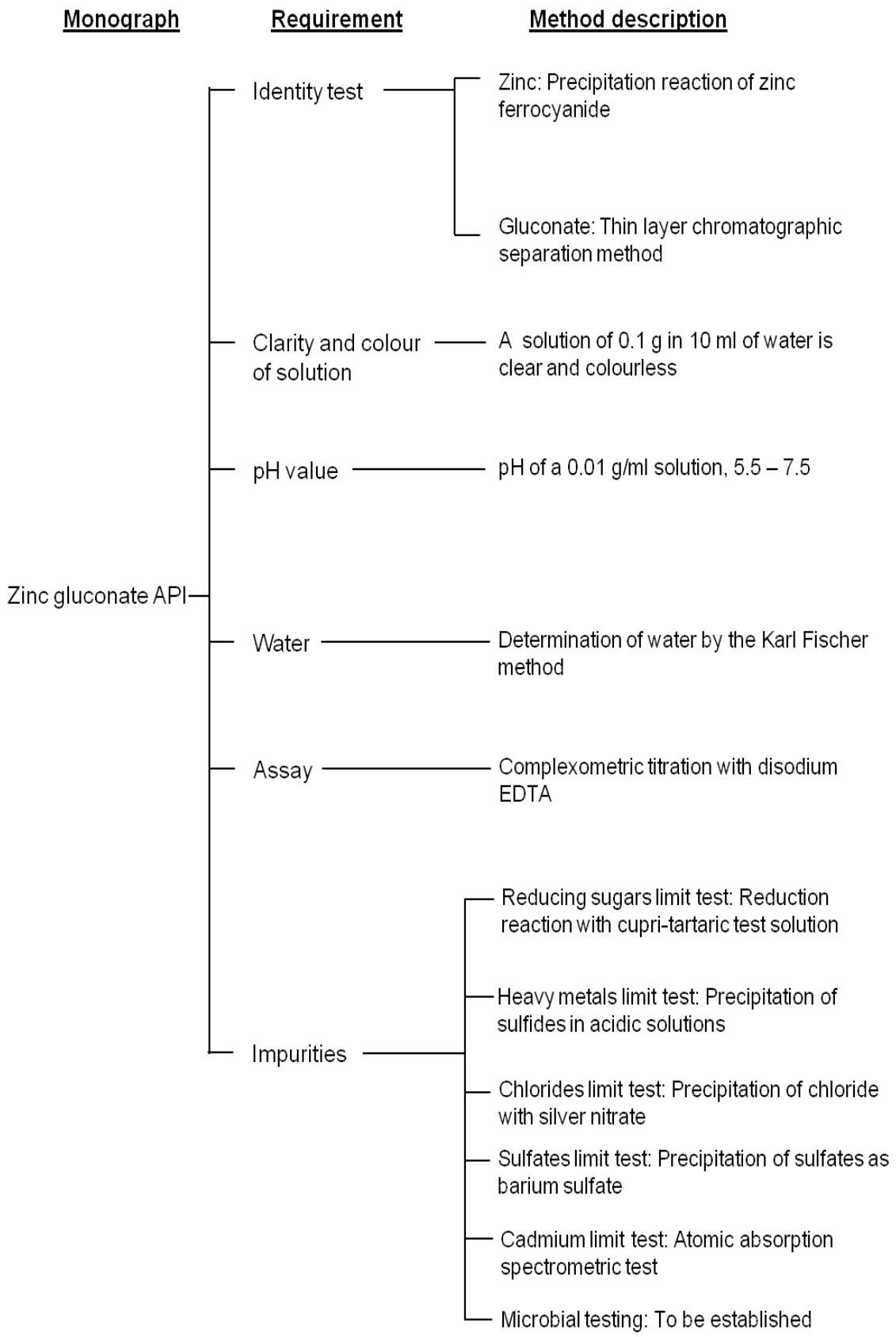


Figure 12.3 Schematic presentation of the requirements set for the zinc gluconate API monograph.

12.4.1 Identity requirements

Both zinc salts investigated in this study consist of a metal cation and an ionic salt, thus identification tests for the individual ions are required. For the identification of zinc in zinc acetate dihydrate and zinc gluconate APIs general identification tests by means of zinc hydroxide & zinc sulfide, and zinc ferrocyanide precipitation reactions were recommended respectively (Chapter 4).

Acetate was identified using a ferric acetate precipitation reaction. For the identification of gluconate a TLC method was developed. This method was developed using the Ph. Eur. / BP TLC method as point of reference. Poor chromatography was obtained when the Ph. Eur. / BP TLC method was executed, which necessitated adjustments to the composition of the mobile phase. The amended method revealed significantly better chromatography and was suitably validated to illustrate the suitability (fitness for use) of this identification method (Chapter 4).

12.4.2 Clarity and colour of solution requirements

There are a number of miscellaneous characteristics included in official compendia to ascertain the purity, authenticity and identification of an API. Clarity and colour is included under these. A clarity and colour test is recommend to be included for both zinc acetate dihydrate and zinc gluconate APIs, where a solution of the APIs in water is compared to water R as the standard solution (Chapter 5).

Water R proved to be a cost- and time-effective reference solution and is also utilised in the BP for clarity and colour testing (Chapter 5).

12.4.3 pH requirements

The acidity or alkalinity of an aqueous solution is defined by the pH value of the solution. In Chapter 6 it was illustrated that this characteristic can potentially be used to distinguish between different solutions or between the salt forms of the same active moiety. From the data presented in Chapter 6 it is clear that zinc gluconate

solution was slightly more acidic (pH ≈ 6.2) when compared to the zinc acetate solution (pH ≈ 6.4).

12.4.4 Water content requirements

The quality of pharmaceutical products may be influenced by the water content thereof. Determination of water content is thus important in cases where the API is known to be hygroscopic or hydrolysed by water (Chapter 7).

From the literature presented in Chapter 7 it was clear that zinc acetate dihydrate is chemically stable and that the dihydrate crystal lattice is also stable at ambient conditions. The assay specification thereof is expressed with reference to the dihydrate, thus the moisture determination of this API was not required for inclusion in the zinc acetate dihydrate API monograph.

Zinc gluconate, on the other hand, is a known hygroscopic API, thus the assay specification is expressed with reference to the dried substance which necessitated the inclusion of a water content requirement.

From the results presented in Chapter 7 it was clear that loss on drying was not a suitable method to determine the moisture content of zinc gluconate API due to the hygroscopic nature of the API. The hygroscopic behaviour of zinc gluconate prevented the samples to be dried to constant mass, thus the results could not be utilised with absolute confidence.

Karl Fischer moisture analysis on a 250 mg sample was found to be a suitable technique for the determination of the moisture content of zinc gluconate API and is recommended to be included in zinc gluconate API monograph.

12.4.5 Assay requirements

Disodium EDTA complexometric titration is considered a well established, relatively simple and accurate method for the quantitative determination of zinc (Chapter 8). Complexometric titration methods were developed for the assay of zinc acetate dihydrate and zinc gluconate APIs. These two methods were based on the current zinc-EDTA complexometric titration method in the general chapter of *The Ph. Int*.

As mentioned in section 12.4.4, the assay specification for zinc acetate dihydrate API is expressed with reference to the hydrated form, whereas the assay specification for zinc gluconate API is expressed with reference to the dried / anhydrous base.

12.4.6 Impurity requirements

Chapters 9, 10 and 11 focused on the development of suitable criteria and methods to limit the presence of impurities in zinc acetate dihydrate and zinc gluconate APIs.

The routes of synthesis reported in Chapter 9 for zinc gluconate API indicated the presence of a fermentation step, thus microbial contamination testing should be included in the monograph for zinc gluconate API. Unfortunately microbial testing is not performed at the RIIP® incorporating CENQAM®, thus it was not covered in this dissertation.

Nine potential impurities were identified for zinc acetate dihydrate API and five potential impurities for zinc gluconate API (Figure 12.2 and Figure 12.3) which need to be controlled by means of suitable limit tests. The limits / specifications set for the respective limit tests were adopted from the available literature.

To limit organic impurities, the reducing substances limit test (for zinc acetate dihydrate API) and the reducing sugars limit test (for zinc gluconate API) were adopted from the Ph. Eur. / BP and are recommended for inclusion in the respective monographs (Chapter 9).

To limit the acid radical impurities (i.e. chlorides and sulfates), the limit test for chlorides and limit test for sulfates of *The Ph. Int.* were utilised to develop suitable methods for both zinc acetate dihydrate and zinc gluconate APIs (Chapter 10).

Metallic impurities present in APIs need to be controlled due their inherent toxicity, formation of insoluble metal complexes, and oxidative and hydrolytic catalytic activity (Chapter 11).

Six specific metallic impurity limit tests (for: arsenic, aluminium, cadmium, copper, iron and lead) were established for zinc acetate dihydrate API (Figure 12.2); and two

metallic impurity limit tests for zinc gluconate API (Figure 12.3), i.e. heavy metals limit test and cadmium limit test.

The heavy metal limit test for zinc gluconate API published in the current Ph. Eur. / BP monographs was considered as a potential method. When the aforementioned method was executed, the “system suitability” failed. The reference solution did not show a slight brown colour, which indicated that there was an interference with the colour production / precipitation of lead sulfide.

Minor adjustments were made (and validated) to the general heavy metal limit test published in *The Ph. Int.* to ensure the required specificity of the test. This modified test is therefore recommended for inclusion in the monographs.

The established arsenic limit test procedure in the general chapter of *The Ph. Int.* is proposed to test for the presence of arsenic in zinc acetate dihydrate API. This test is based on a development of the Gutzeit Test, wherein all the arsenic present is converted into arsine gas by subjecting the arsenic to a reduction reaction with zinc and hydrochloric acid. The arsine must come into contact with dry paper permeated with mercuric bromide to produce a yellow stain, of which the intensity is directly proportional to the quantity of arsenic present (Chapter 11).

The AAS limit tests for aluminium, cadmium, copper, iron and lead described in the zinc acetate dihydrate and zinc gluconate APIs monographs of the Ph. Eur. / BP were adopted and are recommended for inclusion in *The Ph. Int.* monographs. Organic interferences in the heavy metals limit test for zinc gluconate API was observed (Chapter 11 - section 11.3), which justified the use of method 2 (standard addition method) for the cadmium limit test for zinc gluconate API.

12.5 Method validation or verification

To ensure the quality and reliability of the results which are to be produced using the proposed monographs, method validations or method verifications were performed. Method validation studies were performed for newly developed methods, or in the event where significant changes were made to existing / established methods. Method verifications were performed on existing / established methods to illustrate

the suitability (fitness for use) of these methods under actual conditions in the laboratory.

Figures 12.4 and 12.5 summarise the validation / verification approaches which were followed and successfully executed for all of the proposed requirements / methods.

The clarity & colour and pH tests provide information with regards to intrinsic properties of the API under investigation. For the clarity and colour test a specified experimental setup and reference solution (water R) are required and for the pH determination a calibrated pH meter. Thus no specific method validation or verification was required.

Method	Origin of method	Method validation									Method verification								Method suitable for use?	
		Accuracy	Robustness	Repeatability	Intermediate precision	Specificity	Detection limit	Quantitation limit	Linearity	Range	Accuracy	Repeatability	Intermediate precision	Specificity	Detection limit	Quantitation limit	Linearity	Range	Yes	No
1. Identification																				
A) Identification of zinc	Adopted from Ph. Eur. / BP													•					•	
B) Identification of acetate	Adopted from USP													•					•	
2. Clarity & colour test	*Ph. Int.* method with BP Reference solution	N/A									N/A								•	
3. pH	Adopted from Ph. Eur. / BP	N/A									N/A								•	
4. Assay	Method developted based on *Ph. Int.* general method	•	•	•	•	•			•	•									•	
5. Impurity testing																				
A) Reducing substances limit test	Adopted from Ph. Eur. / BP													•	•				•	
B) Chlorides limit test	General *Ph. Int.* method													•	•				•	
C) Sulfates limit test	General *Ph. Int.* method													•	•				•	
D) Arsenic limit test	General *Ph. Int.* method													•	•				•	
E) Aluminium limit test	Adopted from Ph. Eur. / BP													•	•				•	
F) Cadmium limit test	Adopted from Ph. Eur. / BP													•	•				•	
G) Copper limit test	Adopted from Ph. Eur. / BP													•	•				•	
H) Iron limit test	Adopted from Ph. Eur. / BP													•	•				•	
I) Lead limit test	Adopted from Ph. Eur. / BP													•	•				•	

Figure 12.4 Summary of the validation / verification approaches which were followed for all of the proposed requirements / methods in the zinc acetate dihydrate API monograph.

Method	Origin of method	Method validation									Method verification									Method suitable for use?	
		Accuracy	Robustness	Repeatability	Intermediate precision	Specificity	Detection limit	Quantitation limit	Linearity	Range	Accuracy	Robustness	Repeatability	Intermediate precision	Specificity	Detection limit	Quantitation limit	Linearity	Range	Yes	No
1. Identification																					
A) Identification of zinc	Adopted from Ph. Eur. / BP														•					•	
B) Identification of gluconate	Adapted Ph. Eur. / BP method		•		•	•														•	
2. Clarity & colour test	*Ph. Int.* method with BP Reference solution	N/A									N/A									•	
3. pH	Adopted from Ph. Eur. / BP	N/A									N/A									•	
4. Water content	General *Ph. Int.* method												•		•	•				•	
5. Assay	Method developted based on *Ph. Int.* general method	•	•	•	•	•			•	•										•	
6. Impurity testing																					
A) Reducing sugars limit test	Adopted from Ph. Eur. / BP														•	•				•	
B) Chlorides limit test	General *Ph. Int.* method														•	•				•	
C) Sulfates limit test	General *Ph. Int.* method														•	•				•	
D) Heavy metals limit test	Adapted general *Ph. Int.* method					•	•													•	
E) Cadmium limit test	Adopted from Ph. Eur. / BP														•	•				•	

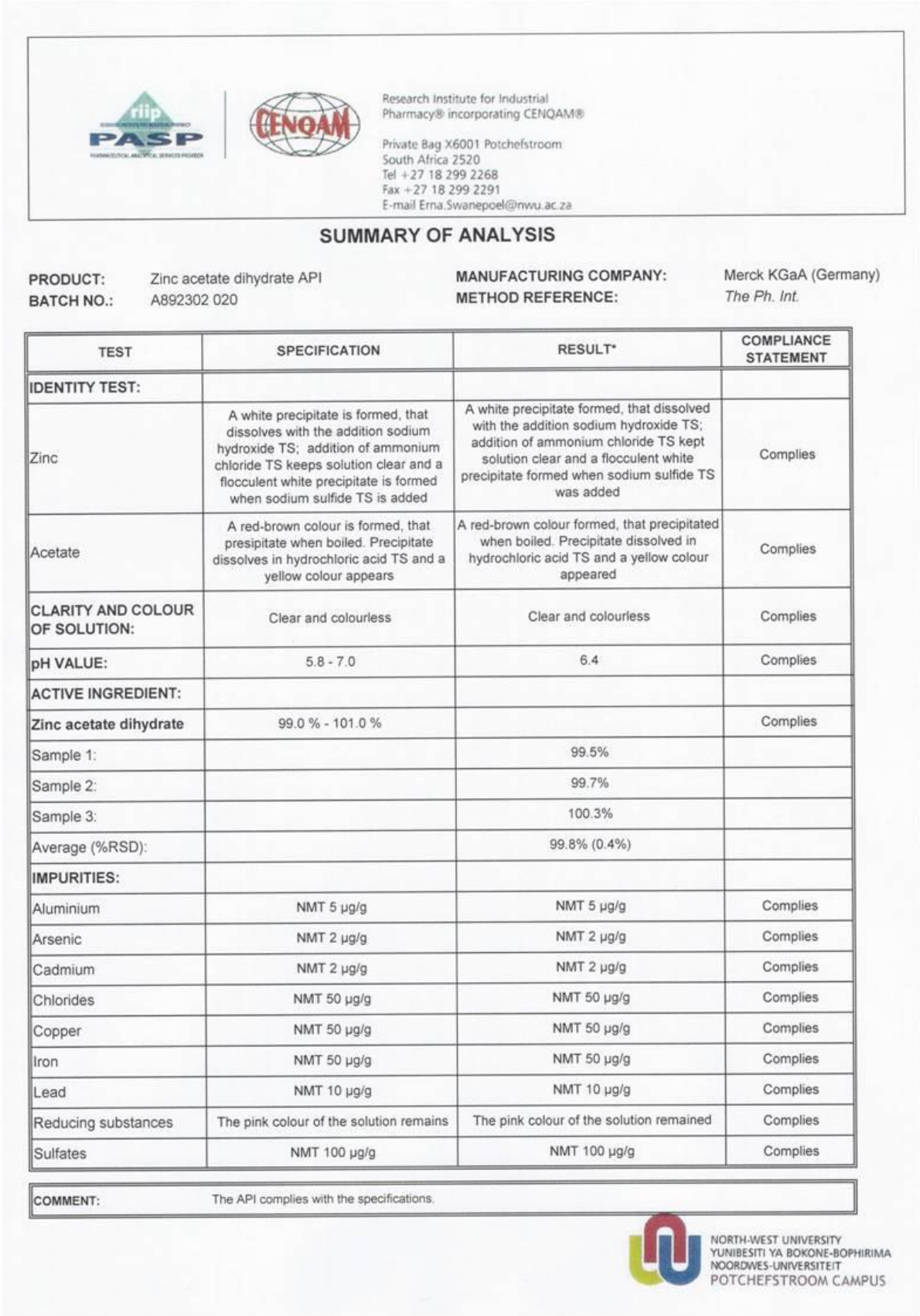
Figure 12.5 Summary of the validation / verification approaches which were followed for all of the proposed requirements / methods in the zinc gluconate API monograph.

12.6 Application of monographs developed

A market surveillance study was performed to identify commercial suppliers of pharmaceutical grade zinc acetate dihydrate and zinc gluconate APIs. Unfortunately, only APIs from one supplier (Merck KGaA, Germany) was commercially available in South Africa, which were used to evaluate the newly developed monographs.

Compliance of the commercially available samples with the newly developed monographs was evaluated. It was found that both samples complied with the relevant monograph's specifications, which supported the implementation of the recommended API monographs (Figure 12.6 & Figure 12.7).

The Ph. Int. is internationally available free-of-charge via the internet (http://apps.who.int/phint/en/p/about/) to all countries. The global availability of these two monographs will provide a quality gauge to ensure the availability of safe and efficious zinc acetate dihydrate and zinc gluconate APIs, for the treatment of paediatric diarrhoea.



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SUMMARY OF ANALYSIS

PRODUCT: Zinc acetate dihydrate API
BATCH NO.: A892302 020
MANUFACTURING COMPANY: Merck KGaA (Germany)
METHOD REFERENCE: *The Ph. Int.*

TEST	SPECIFICATION	RESULT*	COMPLIANCE STATEMENT
IDENTITY TEST:			
Zinc	A white precipitate is formed, that dissolves with the addition sodium hydroxide TS; addition of ammonium chloride TS keeps solution clear and a flocculent white precipitate is formed when sodium sulfide TS is added	A white precipitate formed, that dissolved with the addition sodium hydroxide TS; addition of ammonium chloride TS kept solution clear and a flocculent white precipitate formed when sodium sulfide TS was added	Complies
Acetate	A red-brown colour is formed, that presipitate when boiled. Precipitate dissolves in hydrochloric acid TS and a yellow colour appears	A red-brown colour formed, that precipitated when boiled. Precipitate dissolved in hydrochloric acid TS and a yellow colour appeared	Complies
CLARITY AND COLOUR OF SOLUTION:	Clear and colourless	Clear and colourless	Complies
pH VALUE:	5.8 - 7.0	6.4	Complies
ACTIVE INGREDIENT:			
Zinc acetate dihydrate	99.0 % - 101.0 %		Complies
Sample 1:		99.5%	
Sample 2:		99.7%	
Sample 3:		100.3%	
Average (%RSD):		99.8% (0.4%)	
IMPURITIES:			
Aluminium	NMT 5 µg/g	NMT 5 µg/g	Complies
Arsenic	NMT 2 µg/g	NMT 2 µg/g	Complies
Cadmium	NMT 2 µg/g	NMT 2 µg/g	Complies
Chlorides	NMT 50 µg/g	NMT 50 µg/g	Complies
Copper	NMT 50 µg/g	NMT 50 µg/g	Complies
Iron	NMT 50 µg/g	NMT 50 µg/g	Complies
Lead	NMT 10 µg/g	NMT 10 µg/g	Complies
Reducing substances	The pink colour of the solution remains	The pink colour of the solution remained	Complies
Sulfates	NMT 100 µg/g	NMT 100 µg/g	Complies

COMMENT: The API complies with the specifications.

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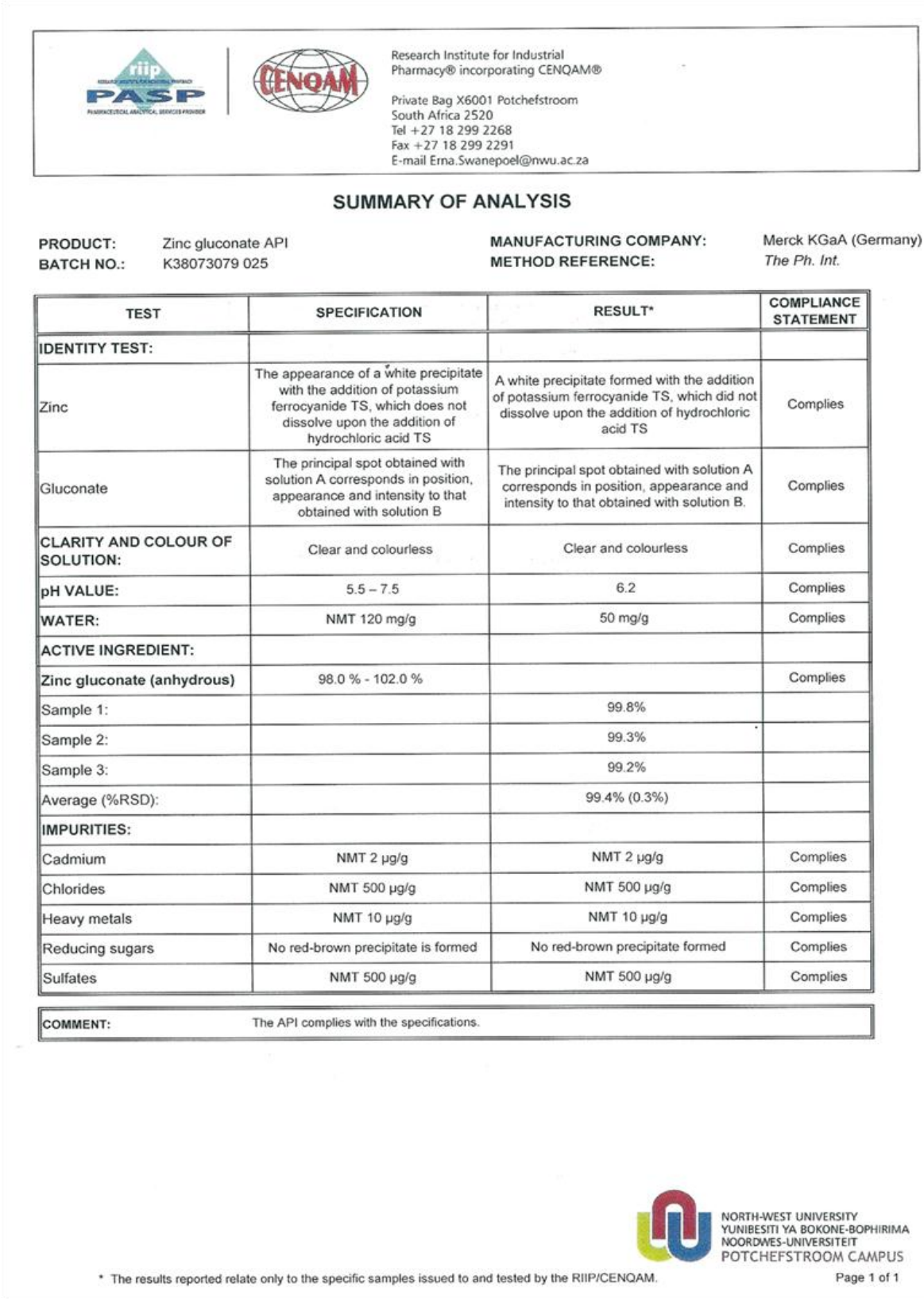
Figure 12.6 Summary of analysis for zinc acetate dihydrate API tested according to the proposed *Ph. Int*. monograph.



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SUMMARY OF ANALYSIS

PRODUCT: Zinc gluconate API
BATCH NO.: K38073079 025
MANUFACTURING COMPANY: Merck KGaA (Germany)
METHOD REFERENCE: *The Ph. Int.*

TEST	SPECIFICATION	RESULT*	COMPLIANCE STATEMENT
IDENTITY TEST:			
Zinc	The appearance of a white precipitate with the addition of potassium ferrocyanide TS, which does not dissolve upon the addition of hydrochloric acid TS	A white precipitate formed with the addition of potassium ferrocyanide TS, which did not dissolve upon the addition of hydrochloric acid TS	Complies
Gluconate	The principal spot obtained with solution A corresponds in position, appearance and intensity to that obtained with solution B	The principal spot obtained with solution A corresponds in position, appearance and intensity to that obtained with solution B.	Complies
CLARITY AND COLOUR OF SOLUTION:	Clear and colourless	Clear and colourless	Complies
pH VALUE:	5.5 – 7.5	6.2	Complies
WATER:	NMT 120 mg/g	50 mg/g	Complies
ACTIVE INGREDIENT:			
Zinc gluconate (anhydrous)	98.0 % - 102.0 %		Complies
Sample 1:		99.8%	
Sample 2:		99.3%	
Sample 3:		99.2%	
Average (%RSD):		99.4% (0.3%)	
IMPURITIES:			
Cadmium	NMT 2 µg/g	NMT 2 µg/g	Complies
Chlorides	NMT 500 µg/g	NMT 500 µg/g	Complies
Heavy metals	NMT 10 µg/g	NMT 10 µg/g	Complies
Reducing sugars	No red-brown precipitate is formed	No red-brown precipitate formed	Complies
Sulfates	NMT 500 µg/g	NMT 500 µg/g	Complies

COMMENT: The API complies with the specifications.

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* The results reported relate only to the specific samples issued to and tested by the RIIP/CENQAM.

Page 1 of 1

Figure 12.7 Summary of analysis for zinc gluconate API tested according to the proposed *Ph. Int*. monograph.

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Annexure A:

Zinc acetate dihydrate API monograph

Working document QAS/12.489
May 2012
RESTRICTED

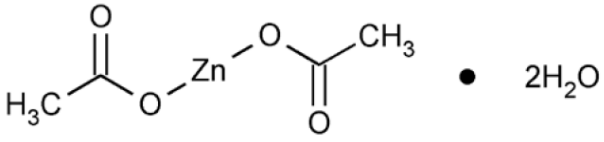
World Health Organization

DRAFT PROPOSAL OF MONOGRAPH IN THE 4TH EDITION OF

The International Pharmacopoeia

ZINC ACETATE
(May 2012)

Zinci Acetas
Zinc Acetate



$C_6H_6O_4Zn,2H_2O$

Relative molecular mass. 219.5.

Chemical name. Zinc acetate dihydrate; CAS Reg. No. 5970-45-6.

Description. A white or almost white crystalline powder or flakes.

Solubility. Freely soluble in water; soluble in ethanol (~750 g/l) TS.

Category. Adjunct to oral rehydration salts in (prevention and) treatment of dehydration due to diarrhoea; astringent.

Storage. Zinc acetate should be kept in a well-closed, non-metallic container.

Labelling. The designation on the container should state that the substance is in the dihydrate form and indicate the quantity in terms of the equivalent amount of elemental zinc.

Requirements

Definition. Zinc acetate dihydrate contains not less than **99.0%** and not more than **101.0%** of $C_6H_6O_4Zn,2H_2O$.

Identity tests

A. Dissolve 0.1 g in 5 ml of water R and add 0.2 ml of sodium hydroxide (~400 g/l) TS. A white precipitate is formed. Add a further 2 ml of sodium hydroxide (~400 g/l) TS. The precipitate dissolves. Add 10 ml of ammonium

chloride (~100 g/l) TS. The solution remains clear. Add 0.1 ml of sodium sulfide TS. A flocculent white precipitate is formed.

B. Dissolve 0.2 g in 4 ml of water R and add 4 ml of ferric chloride (~65 g/l) TS. A red-brown colour is formed. Boil the solution; a red-brown precipitate is produced. Add drop wise sufficient hydrochloric acid (~250 g/l) TS to dissolve the precipitate; a yellow colour appears.

Clarity and colour of solution. A solution of 1 g in 10 ml of water R is clear and colourless.

pH value (1.13). pH of a 0.05 g/ml solution in carbon-dioxide-free water R, 5.8 – 7.0.

Aluminum. Determine by atomic absorption spectrophotometry 1.8 Atomic spectrometry: emission and absorption, Method 1, at a wavelength of 309.3 nm using an aluminum hollow cathode lamp, an acetylene-nitrous oxide flame, and a slit width of 0.5 nm. Dissolve 2.5 g in 25 ml of cadmium-free and lead-free nitric acid (~200 g/l) TS. As a reference solution use aluminum standard (10 µg Al/ml) TS; not more than 5 µg of Al per g.

Arsenic. Use a solution of 5.0 g in 50 ml of water R, add 10 ml of stannated hydrochloric acid (~250 g/l) AsTS, and proceed as described under 2.2.5 Limit test for arsenic; not more than 2 µg As per g.

Cadmium. Determine by atomic absorption spectrophotometry 1.8 Atomic spectrometry: emission and absorption, Method 1, at a wavelength of 228.8 nm using a cadmium hollow cathode lamp, an air-acetylene flame, and a slit width of 0.5 nm. Dissolve 2.5 g in 25 ml of cadmium-free and lead-free nitric acid (~200 g/l) TS. As a reference solution use cadmium standard (1000 µg Cd/ml) TS; not more than 2 µg of Cd per g.

Copper. Determine by atomic absorption spectrophotometry 1.8 Atomic spectrometry: emission and absorption, Method 1, at a wavelength of 324.8 nm using a copper hollow cathode lamp, an air-acetylene flame, and a slit width of 0.5 nm. Dissolve 1.25 g in 25 ml of cadmium-free and lead-free nitric acid (~200 g/l) TS. As a reference solution use copper standard (10 µg Cu/ml) TS; not more than 50 µg of Cu per g.

Chlorides. Dissolve 5.0 g in 25 ml of water R, and proceed as described under 2.2.1 Limit test for chlorides; the chloride content is not more than 50 µg/g.

Iron. Determine by atomic absorption spectrophotometry 1.8 Atomic spectrometry: emission and absorption, Method 1, at a wavelength of 248.3 nm using an iron hollow cathode lamp, an air-acetylene flame, and a slit width of 0.2 nm. Dissolve 1.25 g in 25 ml of cadmium-free and lead-free nitric acid (~200 g/l) TS. As a reference solution use iron standard (20 µg Fe/ml) FeTS; not more than 50 µg of Fe per g.

Lead. Determine by atomic absorption spectrophotometry 1.8 Atomic spectrometry: emission and absorption, Method 1, at a wavelength of 283.3 nm using a lead hollow cathode lamp, an air-acetylene flame, and a slit width of 0.5 nm. Dissolve 5.0 g in 25 ml of cadmium-free and lead-free nitric acid (~200 g/l) TS. As a reference solution use strong lead PbTS; not more than 10 µg of Pb per g.

Reducing substances. Dissolve 1 g in 10 ml of water R. Boil with 90 ml of water R, add 5 ml of sulfuric acid (~100 g/l) TS and 1.5 ml of potassium permanganate (~0.3 g/l) TS; the pink colour of the solution remains.

Sulfates. Dissolve 4.8 g in 25 ml of water R, and proceed as described under 2.2.2 Limit test for sulfates; the sulfate content is not more than 100 µg/g.

Assay

Dissolve about 100 mg, accurately weighed, in 50 ml of acetic acid (~10 g/l) TS and proceed with the titration as described under 2.5 Complexometric titrations for zinc. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 10.98 mg of $C_4H_6O_4Zn,2H_2O$.

Reagents and test solutions to be added

Cadmium R.

Cd

A commercially available reagent of suitable grade.

Cadmium standard (1000 µg Cd/ml) TS.

Procedure. Dissolve 0.100 g of cadmium R in sufficient amount of equal volumes of hydrochloric acid (~330 g/l) TS and water R and dilute to 100 ml with a 1 per cent V/V solution of hydrochloric acid (~330 g/l) TS.

Note. For the preparation of this test solution commercially available cadmium standard solution 1000 µg Cd/ml can also be used.

Nitric acid (~1000 g/l), cadmium-free and lead-free, TS.

[nitric acid, cadmium-free and lead-free (70 per cent.) R].

Nitric acid (~200 g/l), cadmium-free and lead-free, TS.

Procedure. Dilute 200 ml cadmium-free and lead-free nitric acid (~1000 g/l) TS with water R to produce 1000 ml.

Potassium permanganate (~ 0.3 g/l) TS.

A solution of potassium permanganate R containing about 0.3 g of $KMnO_4$ per litre.

***

Annexure B:

Zinc gluconate API monograph

Working document QAS/12.490
May 2012
RESTRICTED

World Health Organization

DRAFT PROPOSAL OF MONOGRAPH IN THE 4TH EDITION OF

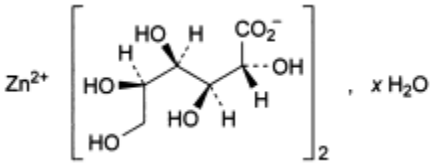
The International Pharmacopoeia

ZINC GLUCONATE
(May 2012)

[Note from the secretariat:

Comments are sought whether to include a specification on total aerobic microbial count (TAMC) and total combined yeasts/moulds count (TYMC).]

Zinci gluconas
Zinc gluconate



$C_{12}H_{22}ZnO_{14},xH_2O$

Relative molecular mass. 455.7 (anhydrous).

Chemical name. Zinc gluconate; CAS Reg. No. 4468-02-4.

Other names. Gluconic acid, zinc complex.

Description. White or almost white, hygroscopic, crystalline powder.

Solubility. Soluble in water; practically insoluble in anhydrous ethanol.

Category. Adjunct to oral rehydration salts in (prevention and) treatment of dehydration due to diarrhoea; astringent.

Storage. Zinc gluconate should be kept in a tightly closed container.

Additional information. Zinc gluconate is a hygroscopic material, and should be protected from atmospheric moisture.

Requirements

Definition. Zinc gluconate contains not less than **98.0%** and not more than **102.0%** of $C_{12}H_{22}ZnO_{14}$ calculated with reference to the anhydrous substance.

Identity tests

A. Dissolve 0.1 g in 5 ml of water R. Add 0.5 ml of potassium ferrocyanide (~53 g/l) TS. A white precipitate is formed that does not dissolve upon the addition of 5 ml of hydrochloric acid (~330 g/l) TS.

B. Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R5 as the coating substance and a mixture of 10 volumes of ethyl acetate R, 50 volumes of water R and 40 volumes of ethanol (~750 g/l) TS as the mobile phase. Apply separately to the plate 1 µl of each of 2 solutions in water R containing (A) 10 mg of the test substance per ml and (B) 10 mg of calcium gluconate R per ml. After removing the plate from the chromatographic chamber, heat the plate for 10 minutes at 105°C. Spray with ammonium molybdate / cerium sulfate / sulfuric acid TS. Heat the plate for 10 minutes at 105°C. Examine the chromatogram in daylight.

   The principal spot obtained with solution A corresponds in position, appearance and intensity to that obtained with solution B.

Clarity and colour of solution. A solution of 0.2 g in 10 ml of water R is clear and colourless.

pH value (1.13). pH of a 0.01 g/ml solution, 5.5 – 7.5.

Water. Determine as described under 2.8 Determination of water by the Karl Fischer method, Method A. Use 0.250 g of the test substance. The water content is not more than 120 mg/g.

Cadmium. Determine by atomic absorption spectrophotometry 1.8 Atomic spectrometry: emission and absorption, Method 2, at a wavelength of 228.8 nm using a cadmium hollow cathode lamp, an air-acetylene flame, and a slit width of 0.5 nm. Dissolve 1.25 g in 25 ml of water R. As a reference solution use cadmium standard (1000 µg Cd/ml) TS; not more than 2 µg of Cd per g.

Chlorides. Dissolve 0.5 g in 25 ml of water R, and proceed as described under 2.2.1 Limit test for chlorides; the chloride content is not more than 500 µg/g.

Heavy metals. Use 1.0 g for the preparation of the test solution as described under 2.2.3 Limit test for heavy metals, Procedure 4, not more than 10 µg/g, substituting acetic acid (~60 g/l) PbTS with hydrochloric acid (~70 g/l) TS in all cases.

Reducing sugars. Dissolve 0.5 g in a mixture of 2 ml of hydrochloric acid (~330 g/l) TS and 10 ml of water R. Boil for 5 minutes, allow to cool, add 10 ml of sodium carbonate (~10 g/l) TS and allow to stand for 10 minutes. Dilute to 25 ml with water R and filter. To 5 ml of the filtrate add 2 ml of cupri-tartaric TS and boil for 1 minute. Allow to stand for 2 minutes; no red-brown precipitate is formed.

Sulfates. Dissolve 0.96 g in 25 ml of water R, and proceed as described under 2.2.2 Limit test for sulfates; the sulfate content is not more than 500 µg/g.

Assay

Dissolve about 200 mg, accurately weighed, in 50 ml of acetic acid (~10 g/l) TS and proceed with the titration as described under 2.5 Complexometric titrations for zinc. Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 22.78 mg of $C_{12}H_{22}ZnO_{14}$.

Reagents and test solutions to be added

Ammonium molybdate / cerium sulfate / sulfuric acid TS.

Procedure. Dissolve 2,5 g ammonium molybdate R and 1,0 g cerium sulfate R in sulfuric acid (~100 g/l) to produce 100 ml.

Cadmium R.

Cd

A commercially available reagent of suitable grade.

Cadmium standard (1000 µg Cd/ml) TS.

Procedure. Dissolve 0.100 g of cadmium R in sufficient amount of equal volumes of hydrochloric acid (~330 g/l) TS and water R and dilute to 100 ml with a 1 per cent V/V solution of hydrochloric acid (~330 g/l) TS.

Note. For the preparation of this test solution commercially available cadmium standard solution 1000 µg Cd/ml can also be used.]

Calcium gluconate R.

$C_{12}H_{22}CaO_{14},H_2O$.

A commercially available reagent of suitable grade.

Cerium sulfate R.

CeO_8S_2.

A commercially available reagent of suitable grade.

Cupri-tartaric TS.

Procedure. Dissolve 34.6 g copper (II) sulfate R in sufficient water to produce 100 ml. Separately dissolve 173 g of potassium sodium tartrate R and 50 g sodium hydroxide in 400 ml water R; heat to boiling, allow to cool and dilute to 500 ml with water R. Shortly before use, mix together equal volumes of both solutions.]

Potassium ferrocyanide (~53 g/l) TS.

A solution of potassium ferrocyanide R containing about 53 g of $K_4Fe(CN)_6$ per litre.

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