Chapter 3: Materials, methods, techniques and equipment

Introduction

In order to present sound experimental results, one needs to be familiar with the experimental procedures. The purpose of this chapter is to briefly discuss the methods, techniques, equipment, reagents and materials that were used in this study.

The quinine sulfate tablet monographs are found in the pharmacopoeia - *Ph.Int*., USP, and the BP. A summary of the methods and tests prescribed by these monographs are given in Table 3-1.

Table 3-1: Summary of analytical QC tests for the quinine sulfate tablet monographs of the USP, BP and *Ph.Int.* (USP, 2013 ; BP, 2013 ; *Ph.Int.*, 2013)

Test	Technique	USP	BP	*Ph.Int.*
ID	TLC	Yes	Yes	Yes
	HPLC	Yes	No	Yes
	UV	No	No	Yes
	Fluorescence	Yes	No	No
	pH	No	Yes	Yes
	Sulfate precipitation	Yes	Yes	Yes
Drug release	Dissolution	Yes	Yes	Yes
	Disintegration	No	No	Yes
Assay	HPLC	Yes	No	No
	Non-aqueous Titration	No	Yes	Yes
Related substances (other cinchona alkaloids or chromatographic purity)	HPLC	No	Yes	Yes
	TLC	Yes	No	No
Uniformity of dosage units	Uniformity of content, weight variation or uniformity of mass as specified by pharmacopoeia	Yes	Yes	Yes

From Table 3-1 it can be seen that:

- General wet chemistry techniques are employed for qualitative testing (identification of sulfates, identification by fluorescence and identification by pH);
- TLC is utilised for qualitative (identification) and semi-quantitative testing (related substances testing);

- HPLC is used for qualitative (identification) and quantitative testing (related substances and assay testing);
- Non-aqueous titration is used for quantitative testing (assay) and
- UV-Vis spectrophotometry is used for qualitative (identification) and quantitative testing (analysis of dissolution samples).

A short description of these different tests and the principles upon which they are based are presented in sections 3.1 - 3.8. The list of equipment and materials relevant to each of these tests as employed in this study are listed in these respected sections where relevant.

3.1 Wet chemistry techniques/principles

3.1.1 Fluorescence

Some compounds have the ability to fluoresce. Such a compound absorbs light energy (photons) which results in some of its electrons shifting from a lower atomic orbital to a higher atomic orbital. In order to return to the ground/natural state, the molecule will need to emit energy as heat or as light which is referred to as fluorescence (Solomon *et al.,* 2002:176). The quinine sulfate tablet monograph of the USP specifies fluorescence as a means of identification. Quinine is natively fluorescent, and has high quantum efficiency for fluorescence (Willard *et al.,* 1981:106).

The identification test of the USP monograph describes the addition of sulphuric acid to quinine sulfate which delocalizes the *pi*-electrons of quinine, duly enhancing its ability to fluoresce. The result is a vivid blue fluorescent colour when radiated with UV light (Figure 3-1, right). Many compounds will however emit a blue fluorescence, which is why the process continues to describe the addition of hydrochloric acid as a means to increase the specificity (the ability to distinguish between similar compounds) of the test. The hydrochloric acid is added to the fluorescing solution which quenches quinine's ability to fluoresce, resulting in the blue colour to disappear (Figure 3-1, left).

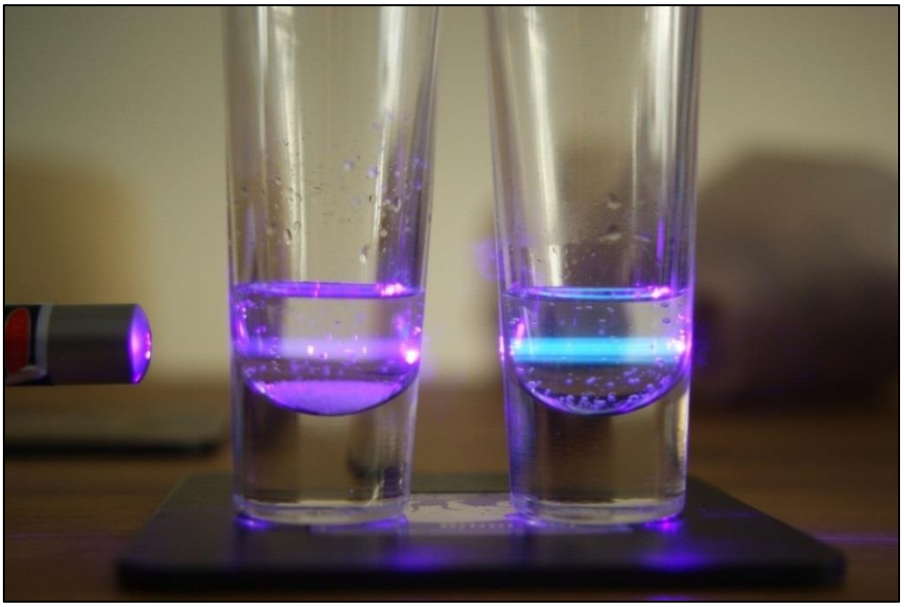

Figure 3-1: Visual presentation of the fluorescent properties of quinine sulfate (Willard *et al*., 1981:107). Quinine fluorescence can be seen on the right and when hydrochloric acid is added the fluorescence disappear as seen on the left.

A list of the equipment and reagents used for the fluorescence test is tabulated in Table 3-2 and Table 3-3.

Table 3-2: List of equipment used for the USP monograph fluorescence test

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Sartorius analytical balance, model number R200D	Labotec	Germany
CAMAG Reprostar UV Light	Camag (BSC)	Switzerland
Heidolph Magnetic stirrer	Labotec	Germany

Table 3-3: List of reagents used for the USP fluorescence test

Material	Batch number	Manufacturing company	Country of origin
Milli Q Water	n/a	RIIP®/CENQAM®	South Africa
Sulphuric acid	1035329	Merck Chemicals (Pty) Ltd	South Africa
Hydrochloric acid	1040952	Merck Chemicals (Pty) Ltd	South Africa

3.1.2 Precipitation reactions

Precipitation reactions are described as exchange reactions in which one of the resultant products is an insoluble compound (precipitate). Precipitation is based on solubility differences that exist between the particular compounds (Kotz *et al.,* 2003:152). The USP, BP and *Ph.Int*. employ similar procedures to test for the presence of sulfates (as seen in the three quinine sulfate tablet monographs). Barium chloride ($BaCl_2$) is used to produce insoluble barium sulfate ($BaSO_4$) which forms when reacting with the sulfate anions from quinine sulfate in solution (equation 3.1). Barium sulfate ($BaSO_4$) is practically insoluble in water and in acid (Skoog, 1997:94; Kotz *et al.,* 2003:155).

$$Ba^{2+}(aq) + SO_4^{2-}(aq) \rightarrow BaSO_4(s) \qquad \text{Equation 3.1}$$

The reaction described in equation 3.1 may not be specific enough to confirm the presence of sulfates on its own, as many other insoluble compounds may form an insoluble barium compound (for example thiosulfates and sulfites). For this reason, additional measures are employed to increase the specificity (the means to distinguish between similar compounds) of this test. As a secondary measure (in addition to the $BaSO_4$ test), the USP specifies that lead acetate ($Pb(CH_3COO)_2$) be added to the quinine sulfate to form a lead sulfate ($PbSO_4$) precipitate (equation 3.2), which is soluble in ammonia acetate.

$$Pb^{2+}(aq) + SO_4^{2-}(aq) \rightarrow PbSO_4(s) \qquad \text{Equation 3.2}$$

As a secondary measure (in addition to the $BaSO_4$ test), the BP describes the use of iodine (I^- (aq)) and stannous chloride to increase the sensitivity of the sulfate identification test ($BaSO_4$ precipitate). When iodine (I^- (aq)) is added to the barium sulfate ($BaSO_4$(s)) a yellow suspension is formed, as the iodine (I^-(aq)) displaces the sulfate from the barium sulfate ($BaSO_4$) to form barium iodide (BaI_2). Stannous chloride (tin chloride - $SnCl_2$(aq)) is thereafter added to the yellow suspension, which allows the unbound SO_4^{2-} anions to displace the chloride from stannous chloride ($SnCl_2$(aq)) to form a soluble tin sulfate ($Sn_2(SO_4)_4$) compound, which will then turn the yellow solution to a colourless solution.

A list of the equipment and reagents used for test for sulfates is tabulated in Table 3-4 and Table 3-5.

Table 3-4: List of equipment used for test for sulfates

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Shimadzu analytical balance, model number AUW220D	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number AUW620H	Shimadzu	Japan
Heidolph magnetic stirrer	Labotec	Germany

Table 3-5: List of reagents used for test for sulfates

Material	Batch number	Manufacturing company	Country of origin
Milli-Q water	n/a	RIIP®/CENQAM®	South Africa
Barium chloride	MK0M603122	Merck Chemicals (Pty) Ltd	South Africa
Hydrochloric acid	1040952	Merck Chemicals (Pty) Ltd	South Africa
Lead acetate	1038771	Merck Chemicals (Pty) Ltd	South Africa
Ammonium acetate	QF1Q610666	Merck Chemicals (Pty) Ltd	South Africa
Sodium hydroxide	QF1Q610446	Merck Chemicals (Pty) Ltd	South Africa
Nitric acid	1035275	Merck Chemicals (Pty) Ltd	South Africa
Iodine	1035421	Merck Chemicals (Pty) Ltd	South Africa
Stannous chloride	B0771214208	Merck (Pty) Ltd	Germany

3.1.3 Measuring pH for identification purposes

pH is a means to determine whether a solution is acidic (pH < 7), neutral (pH ± 7) or basic (pH > 7) - equation 3.3. The pH of a solution is determined by the hydronium-ion concentration (H_3O^+ or sometimes referred to as H^+) in solution (Watson, 2005:24 and Kotz *et al.,* 2003:181).

$$\mathrm{pH} = -\log[\mathrm{H}^+] \qquad \text{Equation 3.3}$$

The $\mathrm{K_a}$value, known as the dissociation constant (equation 3.5) describes to what extent a weak acid [HA] or base will dissociate in a solution (Watson, 2005:25):

$$\mathrm{HA} \leftrightarrow [\mathrm{H}^+] + [\mathrm{A}^-] \qquad \text{Equation 3.4}$$

$$\mathrm{K_a} = \frac{[\mathrm{A}^-][\mathrm{H}^+]}{\mathrm{AH}} \qquad \text{Equation 3.5}$$

The $\mathrm{pK_a}$value is derived from the $\mathrm{K_a}$ value (equation 3.6):

$$\mathrm{pK_a} = -\log \mathrm{K_a} \qquad \text{Equation 3.6}$$

Since the pKa of a compound is unique, pH may be used as a means of identification (Steenekamp, 2012:87-88). It is possible to calculate the pH of a solution when the concentration and pK_a value of the compound in solution are known. The pH of a 10 mg/ml quinine sulfate suspension in water ranges between 5.7 and 6.6. (Reynolds *et al.,* 1993:408). If the measured pH corresponds with the calculated/known pH it is considered as a positive identification.

The BP monograph specifies a pH of 5.7 – 6.6 for a 1% w/v (10 mg/ml) suspension whereas the *Ph.Int.* monograph specifies a pH of 5.5 – 7.0. The two monographs therefore allows for different ranges from the actual theoretical pH value (pH 6.15, average between 5.7 - 6.6 and pH 6.25, average between 5.5 - 7.0) to accommodate differences in experimental and environmental conditions, which will be discussed in the results section (Chapter 5).

A list of the equipment and reagents used for the pH identification test is tabulated in Table 3-6 and Table 3-7.

Table 3-6: List of equipment used for pH identification testing

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Crison Basic 20 pH meter	Lasec	Europe
Sonic Bath, model number PS-100	RoHS Celsius Scientific	China
Binder oven, model number ED23	Apollo Scientific	Germany
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Shimadzu analytical balance, model number AUW220,	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number UW620H,	Shimadzu	Japan
Millipore PVDF 0.45µm filters	Separations	Ireland
Heidolph magnetic stirrer	Labotec	Germany

Table 3-7: List of reagents used for pH identification testing

Material	Batch number	Manufacturing company	Country of origin
Milli-Q water	n/a	RIIP®/CENQAM®	South Africa
Chloroform	1040030	Merck Chemicals (Pty) Ltd	South Africa
Ethanol	1041234	Merck Chemicals (Pty) Ltd	South Africa
Ether	1039564	Merck Chemicals (Pty) Ltd	South Africa

3.1.4 Thin Layer Chromatography (TLC)

TLC is a chromatographic separation technique which is often used to identify a substance or to test for the presence of impurities (Watson, 2005:315). It employs a stationary phase (Figure 3-4) and mobile phase within an enclosed chamber (Figure 3-3). Examples of solvents used to prepare mobile phases are listed in Table 3-8, together with their polarity indices (Watson, 2005:315). The most common stationary phase for TLC is silica gel (refer to Figure 3-4).

A sample solution is applied to the stationary phase (spotted) at a specific predetermined origin. The mobile phase (driven by capillary force) moves up the stationary phase and allows for the elution of the different constituents of the sample solution on the stationary phase (Figure 3-2). The final position of the spot(s) depends on the affinity of the compound for the mobile phase and the stationary phase. The greater the affinity a compound has for the mobile phase, the more distance it will cover, and vice versa. The different constituents within a sample solution have different affinities for the stationary and mobile phase which allows for the separation of the different constituents onto the stationary phase at different positions (Watson, 2005:315).

In many instances, the spots from a TLC plate cannot be detected by mere visual inspection. Detection with ultraviolet light or spraying reagents are employed to make these spots visible (Figure 3-3) (Watson, 2005:323).

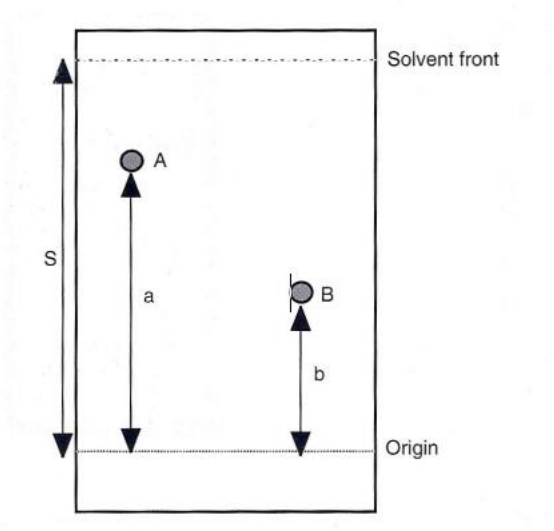

Figure 3-2: An example of a TLC plate, where S is the distance travelled by the mobile phase, 'A' is compound A with travel distance a, and 'B' is compound B with travel distance b (Watson, 2005:318).

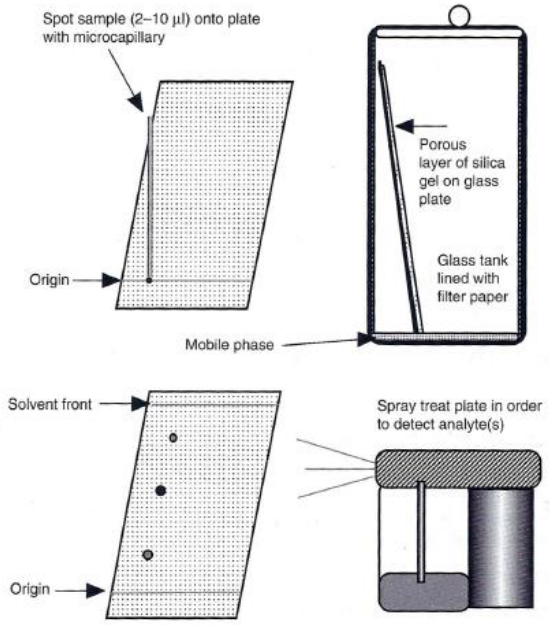

Figure 3-3: Chromatographic chamber and TLC sprayer (Watson, 2005:317).

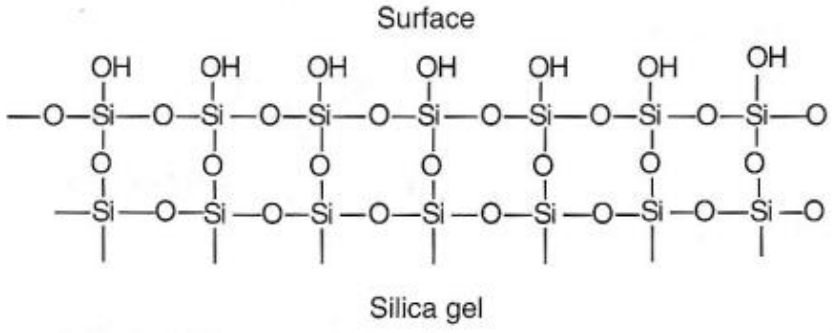

Figure 3-4: The surface of silica gel (Watson, 2005:318).

Table 3-8: A list of common solvents with increasing polarity (Watson, 2005:320)

Solvent	Polarity Index
Hexane (C6H14)	0
Toluene (C7H8)	2.4
Diethylether (C4H10O)	2.8
Dichloromethane (CH2Cl2)	3.1
Butanol (C4H9OH)	3.9
Chloroform (CHCl3)	4.1
Ethyl acetate (C2H5COOH3)	4.4
Acetone (CH3COCH3)	5.1
Methanol (CH3OH)	5.1

Solvent	Polarity Index
Ethanol (C2H5OH)	5.2
Acetonitrile (CH3CN)	5.8
Acetic acid (CH3COOH)	6.2
Water (H2O)	9.0

For identification purposes (as is the case for quinine sulfate tablets monographs of the *Ph.Int*., BP and USP), a reference standard of the compound of interest is prepared in the same theoretical concentration to a sample solution and developed concurrently. Should the appearance, intensity and position (R_f) of the spot in the sample correspond with that of the standard, the identification is positive. TLC is also used as a semi-quantitative technique for the chromatographic purity of quinine sulfate tablet monograph of the USP monograph. Similar as for identification purposes, reference standard solutions are prepared together with the sample solution. The intensity of the spots then serves as a means to semi-quantitatively evaluate whether the impurity spot is more intense or less intense than the required known concentration of the standard compound of interest.

A list of the equipment and reagents used for analysis by means of TLC is tabulated in Table 3-9 and Table 3-10.

Table 3-9: List of equipment used for analysis by means of TLC

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Chromatographic chamber	Camag	Switzerland
Brand micropipettes	Merck (Pty) Ltd	Germany
Macherey-Nagel Sil G-25 UV 254 (20 cm x 20 cm glass plates pre-coated with 0.25 mm silica gel with fluorescent indicator)	Separations	Germany
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Handhold SpectrolineLonglife™ Filter UV light, model ENF-240 C/F	Camag	U.S.A
Millipore PVDF 0.45µm filters	Separations	Ireland
Gamag TLC sprayer, model number CH-4132	Camag	Switzerland
Shimadzu analytical balance, model number AUW220D, Unibloc corporation	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number AUW620H, Unibloc corporation	Shimadzu	Japan
Heidolph Magnetic stirrer	Labotec	Germany

Table 3-10: List of reagents used for analysis by means of TLC

Material	Batch number	Manufacturing company	Country of origin
Milli Q water	n/a	RIIP®/CENQAM®	South Africa
Ether	1039564	Merck Chemicals (Pty) Ltd	South Africa
Diethylamine	SC1G600815	Merck Chemicals (Pty) Ltd	South Africa
Acetone	1036657	Merck Chemicals (Pty) Ltd	South Africa
Toluene	1035073	Merck Chemicals (Pty) Ltd	South Africa
Chloroform	1040030	Merck Chemicals (Pty) Ltd	South Africa
Ethanol	1041234	Merck Chemicals (Pty) Ltd	South Africa
Sulfuric acid	1035329	Merck Chemicals (Pty) Ltd	South Africa
Potassium iodobismuthate spray reagent	081M6064	Sigma	USA
Glacial acetic acid	K43568363222	Merck KGaA	Germany

3.1.5 Non-aqueous titration

Assay by means of titration is based on the stoichiometric chemical reaction between an analyte and a titrant. The stoichiometric ratio is the amount of titrant that will completely react with a certain amount of analyte (Kotz *et al.,* 2003:185). “A titration is performed by slowly adding a standard solution (the titrant) from a buret to a solution of the analyte which is continuously stirred until the reaction between the two is judged complete.” (Skoog *et al*., 1997:661-662). The end point of a titration is reached when the amount of added titrant (titre volume) is chemically equivalent to the amount of analyte of interest and is usually depicted by a change in colour (indicator) or a change in electric potential (potentiometry) (Skoog *et al.,* 1997:661-662).

In the case of the assay methods of the BP and the *Ph.Int*., the quinine sulfate reaction occurs in a ratio of one to three (1:3). One mol of quinine sulfate will react with three molar (M = mol/l) of the perchloric acid.

Duly, 1 M of perchloric acid will react with one third ($^1/_3$) of the molar mass of quinine sulfate.

3 M perchloric acid : 1 mol quinine sulfate (783 g/mol)

1 M perchloric acid : $^1/_3$ mol quinine sulfate (261.0 g)

0.1 M perchloric acid : 26.10 g quinine sulfate

1 ml of 0.1 M perchloric acid : 26.10 mg quinine sulfate

The final stoichiometric relationship of 1 ml of perchloric acid (0.1 M) reacted with 26.10 mg of the quinine sulfate salt corresponds with that stipulated under the assay procedures in the BP and *Ph.Int.* monographs.

A list of the equipment and reagents used for the assay analysis by means of non-aqueous titration is tabulated in Table 3-11 and Table 3-12.

Table 3-11: List of equipment used for assay analysis by means of non-aqueous titration

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Shimadzu analytical balance, model number AUW220D, Unibloc corporation	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number AUW620H, Unibloc corporation	Shimadzu	Japan
Heidolph Magnetic stirrer	Labotec	Germany
Calibrated Burette 20 ml	Merck (Pty) Ltd	Germany

Table 3-12: List of reagents used for assay analysis by means of non-aqueous titration

Material	Batch number	Manufacturing company	Country of origin
Milli-Q water	n/a	RIIP®/CENQAM®	South Africa
Acetic anhydride	27230	ACE	South Africa
Perchloric acid	B0610814	Merck KGaA	Germany
Potassium hydrogen phthalate	MH0M60246	Merck Chemicals (Pty) Ltd	South Africa
Glacial acetic acid	K43568363222	Merck KGaA	Germany
Crystal violet	1036321	Merck KGaA	Germany
Anhydrous acetic acid	K43568363222	Merck KGaA	Germany

3.2 Disintegration

Disintegration is the process in which a tablet breaks into smaller fragments and is defined by the USP as "that state in which any residue of the tablet, except fragments of insoluble coating, remaining on the screen of the test apparatus in the soft mass have no palpable firm core" (USP, 2013).

Disintegration testing entails agitating (up-and-downwards) a given number of tablets (usually six) in an aqueous medium and recording the time it takes for each tablet to disintegrate. Figure 3-5 illustrates the apparatus used for the disintegration testing. A disintegration apparatus consist usually of six chambers/tubes. These tubes are open at the top and closed by a screen/mesh on the lower end. A tablet is placed within each of the tubes, and agitation is achieved by moving the apparatus up and down within the aqueous medium until the tablets disintegrate. (Alderborn, 2007:462).

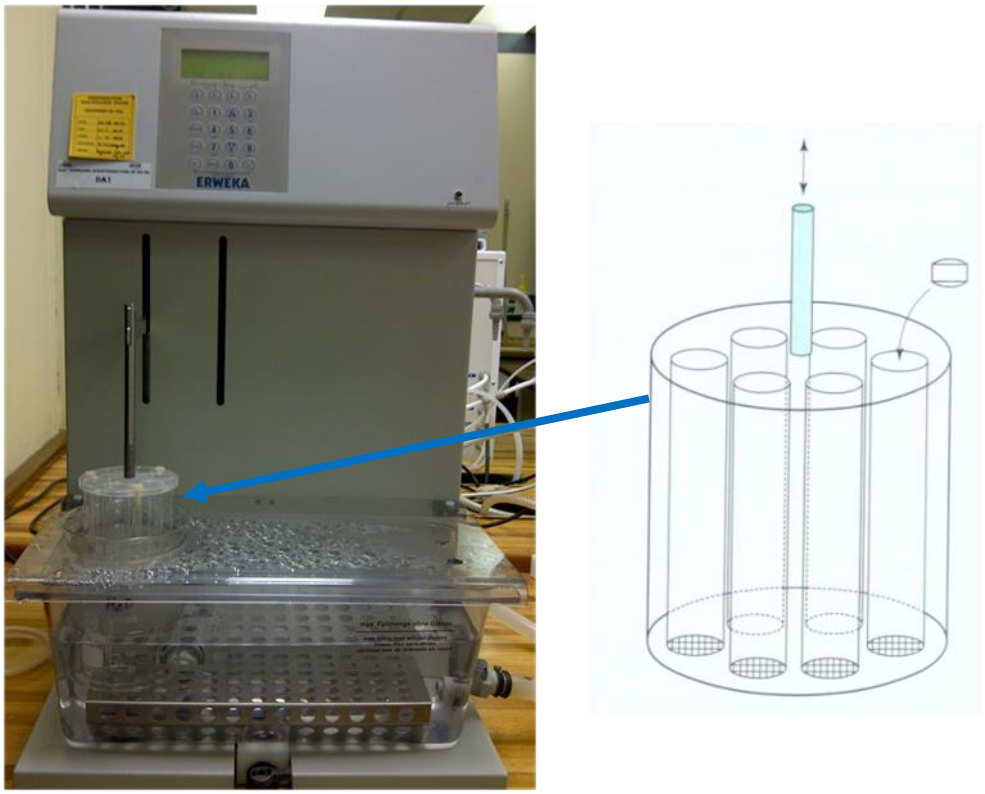

Figure 3-5: An example of a disintegration apparatus for the testing of tablet disintegration (Alderborn, 2007:462 ; RIIP®/CENQAM®).

Disintegration testing is not a requirement of the BP and USP quinine sulfate tablet monographs (dissolution tests are specified). The *Ph.Int.* monograph for quinine sulfate tablets however specifies a choice between a dissolution- (A) or disintegration test (B). The *Ph.Int.* monograph states that should the disintegration test fail, the dissolution test be performed.

A list of the equipment and reagents used for disintegration testing is tabulated in Table 3-13 and Table 3-14.

Table 3-13: List of equipment used for disintegration testing

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Erweka D-63150 GmbH disintegration system	Apollo	Germany

Table 3-14: List of reagents used for disintegration testing

Material	Batch number	Manufacturing company	Country of origin
Milli-Q water	n/a	RIIP®/CENQAM®	South Africa

3.3 Dissolution

Dissolution tests are currently one of the most important tests (critical quality attribute) concerning the quality control of solid oral dosage forms (Shargel *et al.*, 2005:421). Dissolution testing is intended to mimic the physiological environment in which the API should be released from the dosage form (Shargel *et al.*, 2005:415; Azarmi *et al.,* 2007:13). A dissolution test measures the amount of API released in a specific medium as a function of time.

For a dissolution test, a tablet or capsule is placed into a known volume of medium, agitated at a specific rate and sampled over time. The withdrawn sample solutions are assayed (usually by HPLC or UV-Vis spectrophotometry) to determine the amount of API dissolved at that specific time point (Vaghela *et al.,* 2011:50). If a dissolution test is well developed and validated, it may provide valuable information about the API release capabilities of a FPP, it's *in vivo* performance as well as batch-to-batch consistency and possible manufacturing deviations (Vaghela *et al.,* 2011:50; Azarmi *et al*., 2007:13).

3.3.1 Dissolution of the API in the dissolution medium

The rate of dissolution is described by the Noyes-Whitney equation (equation 3.7), which is illustrated in Figure 3-9.

$$\frac{dC}{dt} = \frac{DA}{h(C_S - C)}$$ Equation 3.7

Where:

$\frac{dC}{dt}$ = rate of dissolution at time *t*

D = diffusion rate constant

A = surface area of the API particle

C_S = concentration of the API (equal to the solubility of API) in the stagnant layer

C = concentration of the API in the bulk solvent

h = thickness of the stagnant layer

The Noyes-Whitney equation gives a clear mathematical indication of the factors influencing the rate of dissolution ($\frac{dC}{dt}$) (Shargel *et al.*, 2005:414):

- An increase in temperature will result in an increase in the kinetic energy of the molecules and then increase the value of the diffusion constant, *D,* improving the rate of dissolution.

- When increasing the surface area of the particle (*A)*, the dissolution rate will increase. The total surface area is inversely proportional to particle size. This means that the smaller particle size, the larger the surface area and the faster the rate/extent of dissolution will be (Shargel *et al.*, 2005:417). If particles form coherent masses (agglomerates) in the dissolution medium, it reduces the surface area of the sample and thus reduces the dissolution rate (Aulton, 2007:20).

- An increase in agitation (stirring speed) of the surrounding solvent medium, reduces the thickness of the stagnant layer (*h*) and thus allows for more rapid API dissolution (Shargel *et al.*, 2005:415).

- The concentration of the API (in both the stagnant layer and the bulk solvent) is dependent on its solubility in the dissolution medium. The solubility of the API in the medium is dependent on the temperature of the medium, the type of dissolution medium and the pH of the dissolution medium (Aulton, 2007:20). The rate of dissolution may therefore be impaired in media where low solubility is achieved.

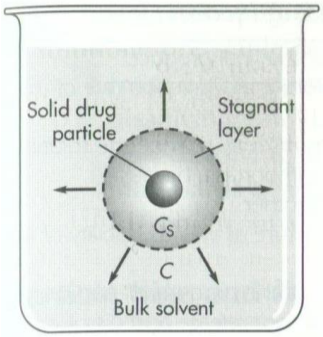

Figure 3-6: Dissolution of a solid particle in a solvent (Shargel *et al*., 2005:415).

3.3.2 Dissolution Apparatus

There are seven different types of dissolution apparatus. The first two types (Apparatus 1 and 2) are applicable to this study and therefore discussed below. The other types are the reciprocating cylinder method (Apparatus 3), flow-through cell method (Apparatus 4), paddle-over-disk method (Apparatus 5), the cylinder method (Apparatus 6) and the reciprocating disk method (Apparatus 7) (Shargel *et al.*, 2005:425-427). The basket (Apparatus 1) and the paddle (Apparatus 2) methods are most commonly employed for solid oral dosage form dissolution tests.

A typical dissolution bath consists of a control panel, a motor with at least six shafts (which are moved by the motor and onto which the paddles/baskets are affixed), an outer water bath, a heating pump, at least six vessels and vessel covers (Figure 3-7).

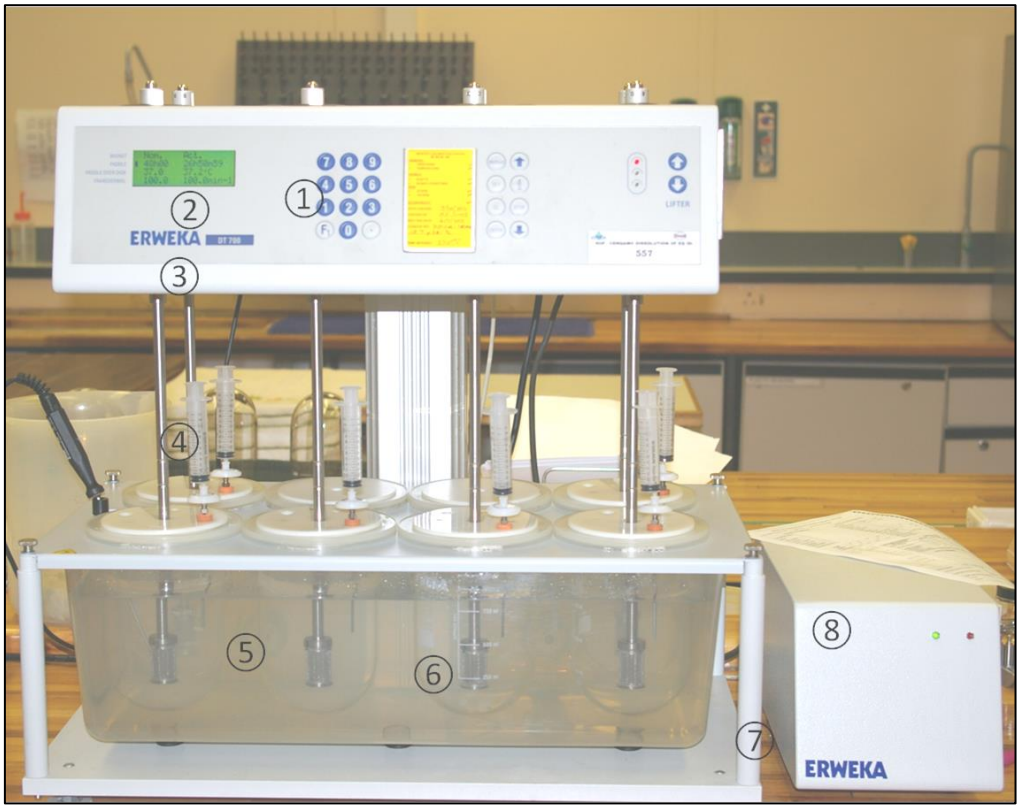

Figure 3-7: **An example of a dissolution apparatus with: (1) Control panel, (2) Parameter readout screen, (3) Drive motor, (4) Stainless steel shaft, (5) Water bath, (6) Glass dissolution vessel, (7) Waterbath and Vessel Support framework, (8) Free-standing heater.**

3.3.2.1 Rotating Basket Method (Apparatus 1)

When Apparatus 1 is specified by a monograph, it refers to the basket assembly. Using this method, the sample is placed inside the basket and then attached to the shaft, which is then lowered into the dissolution medium and rotated at a specific speed. The basket method is generally used when samples tend to float e.g. capsules or samples that disintegrate slowly (Shargel *et al.*, 2005:425). For the rotating basket method the most common rotation rate is 100 rotations per minute (rpm). The USP and BP technical requirements for the basket assembly are depicted in Figure 3-8.

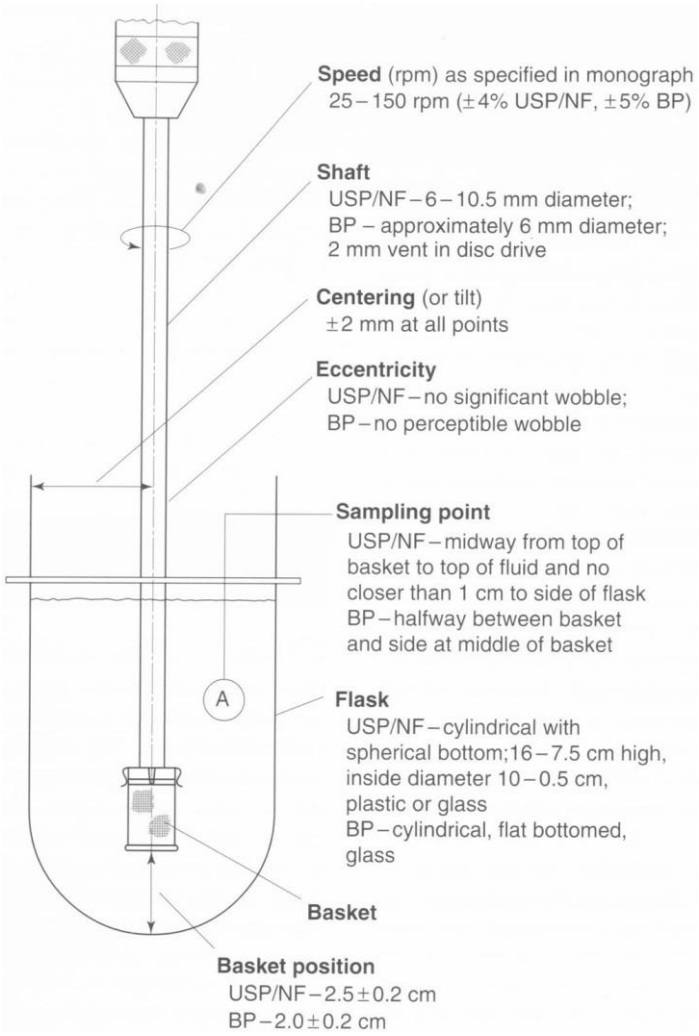

Figure 3-8: A graphical illustration of the rotating basket assembly and the technical requirements as required by the BP and USP (Alderborn, 2007:464).

3.3.2.2 Paddle Method (Apparatus 2)

When Apparatus 2 is specified, it refers to the paddle assembly. Using this method, the sample is dropped directly into the dissolution vessel and the motor (which rotates the paddles) initiated when the sample reaches the bottom of the vessel. Sinkers in the form of a stainless steel or glass helix may be used if the possibility exists for a film-coated tablet to stick to vessel walls or if the dosage form is prone to floating (Shargel *et al.*, 2005:426). For the paddle method a speed ranging between 50 - 100 rpm is used (Shargel *et al.*, 2005:426). The USP technical requirements for the paddle assembly is portrayed in Figure 3-9.

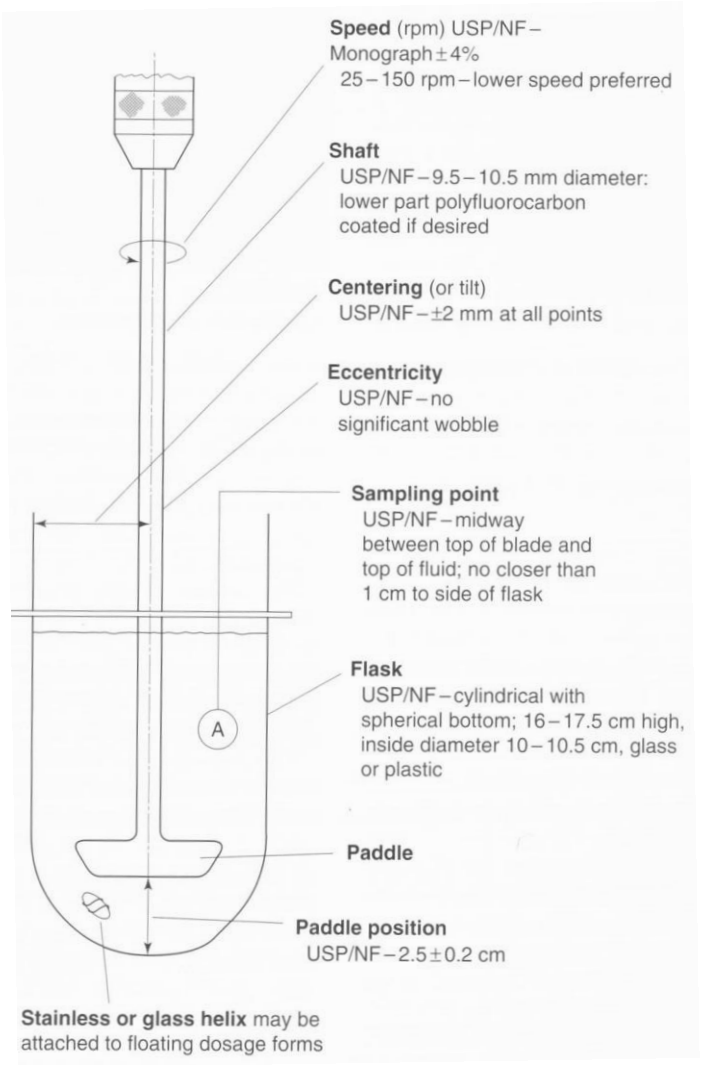

Figure 3-9: A graphical illustration of the rotating paddle assembly and the technical requirement thereof as required by the USP (Alderborn, 2007:465).

Pharmacopoeia may differ in their approach to dissolution testing of the same product. For instance, the BP and USP have similar requirements for the dissolution testing of quinine sulfate tablets, which include the use of basket apparatus, 900 ml of diluted hydrochloric acid (in different concentrations) as dissolution medium and a final withdrawal time at 45 minutes, whereas the *Ph.Int.* monograph specifies the use of the paddle apparatus, 500 ml of phosphate buffer, pH 6.8 as dissolution medium, and a final withdrawal time at 30 minutes. Each monograph has its own specific acceptance criterion to which conformance is measured. The quantification of quinine sulfate samples are however by the same technique (UV) allowing for certain differences (such as wavelength) which will be explained in the section on UV-Vis spectrophotometry to follow (section 3.4). A list of the equipment and reagents used for dissolution testing is tabulated in Table 3-15 and Table 3-16.

Table 3-15: List of equipment used for dissolution testing

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Erweka dissolution apparatus, paddles or baskets, model number DT 700HH	Apollo	Germany
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Crison Basic 20 pH meter	LAsec	Europe
Millipore PVDF 0.45µm filters for in-line filtering	Separations	Ireland
Roth stopwatch	Separations	PRC
Crison TM 65 digital thermometer	Lasec	Europe
Shimadzu analytical balance, model number AUW220D, Unibloc corporation	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number AUW620H, Unibloc corporation	Shimadzu	Japan
Heidolph magnetic stirrer	Labotec	Germany

Table 3-16: List of reagents used for dissolution testing

Material	Batch number	Manufacturing company	Country of origin
Milli-Q water	n/a	RIIP®/CENQAM®	South Africa
Sodium dihydrogen phosphate	SAAR5822650EM	Merck Chemicals (Pty) Ltd	South Africa
Sodium hydroxide	QF1Q610446	Merck Chemicals (Pty) Ltd	South Africa
Hydrochloric acid	1040952	Merck Chemicals (Pty) Ltd	South Africa
Glacial acetic acid	K43568363222	Merck KGaA	Germany
Sodium acetate trihydrate	SAAR582101EM	Merck Chemicals (Pty) Ltd	South Africa

3.4 Ultraviolet-Visible (UV-Vis) Spectrophotometry

The ability of organic molecules to absorb UV light is an important characteristic that enables their qualitative and quantitative analysis (Skoog, *et al*. 1997:557). When UV light is passed through a solution, a portion of the light will be absorbed by the components of the solution (if possible) and the remaining light will pass through the solution (transmission) (Watson, 2005:91).

Absorption of wavelength energy is concentration dependent, as the intensity of absorption is a function of the analyte concentration. The Beer-Lambert law dictates the quantification of absorbance – equation 3.8 (Watson, 2005:91):

$$A = \varepsilon bc \qquad \text{Equation 3.8}$$

Where:

A = Absorbance

ε = Molecular absorbtivity (constant for a specific compound)

b = The pathlength of the cell in cm

c = The concentration of the analyte

The Beer-Lambert law state that absorbance of a solution is linearly related to the concentration of the compound in solution as well as the path length it travels through (Watson, 2005:91). Thus, by comparing the amount of absorbance of a test sample with that of a known standard concentration, the sample's concentration can be calculated.

UV-Vis spectrophotometry is often used as a pharmacopoeial identification test (Figure 3-10) and is a popular and cost effective means of quantitative analysis (Watson, 2005:87). API's consist of functional groups with specific bonds and electron distributions which absorbs UV and visible light (also called chromophores). Chromophores are unique and specific for each compound (Watson, 2005:89). The absorption spectrum is unique for each compound because of these unique and specific chromophores present in its molecular structure (example in Figure 3-10).

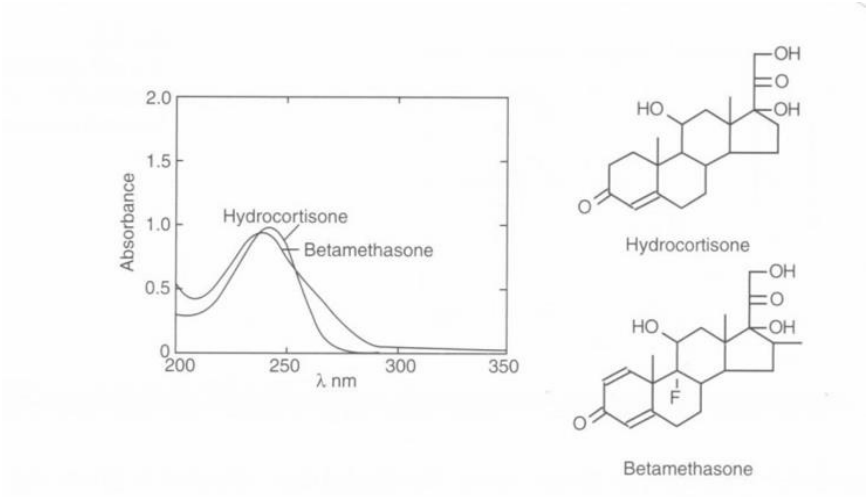

Figure 3-10: An example of absorbance spectra of hydrocortisone and betamethasone (Watson, 2005:89).

$A_{1cm}^{1\%}$is a constant value which is defined as the measured absorbance of a 1% (w/v) (1 g/100 ml) solution through a path length of 1 cm. The BP monographs occasionally provide an $A_{1cm}^{1\%}$ value for a compound which is to be quantified (Watson, 2005:91) – for instance an $A_{1cm}^{1\%}$ of 136 is reported for quinine sulfate in the BP monograph dissolution method (BP, 2013)

A blank solution (the solvent) is used to zero the spectrophotometer. This compensates for light that is absorbed by the solvent rather than the compound of interest (Skoog *et al*., 1997:510-511).

UV-Vis spectrophotometry is utilised by the USP, BP and *Ph.Int*. for quantitative (dissolution) and qualitative (identification-) testing of quinine sulfate tablets. The assay of quinine sulfate dissolution samples are by direct comparison with a reference standard of known concentration or by an $A_{1cm}^{1\%}$. The identification of quinine sulfate by UV-Vis spectrophotometry (*Ph.Int*.) specifies a maximum absorbance at about 347 nm, which is characteristic to quinine sulfate, based on its molecular structure and light absorbing functional groups, in that specific medium.

A list of the equipment and reagents that were used for UV-Vis Spectrophotometry is tabulated in Table 3-17 and Table 3-18.

Table 3-17: List of equipment used for tests requiring UV-Visible Spectrophotometry

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Millipore PVDF 0.45µm filters	Separations	Ireland
Shimadzu analytical balance, model number AUW220D, Unibloc corporation	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number AUW620H, Unibloc corporation	Shimadzu	Japan
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Cary 50 UV-Visible spectrophotometer with a xenon source	Chemtrix	Australia
Agilent Technologies Open-Top UV quarts cell, 10 mm	Chemetrix	Germany

Table 3-18: List of reagents used for tests requiring UV-Visible Spectrophotometry

Material	Batch number	Manufacturing company	Country of origin
Milli-Q Water	n/a	RIIP®/CENQAM®	South Africa
Hydrochloric acid	1040952	Merck Chemicals (Pty) Ltd	South Africa

3.5 High Performance Liquid Chromatography (HPLC)

Chromatography is the most frequently used analytical technique in pharmaceutical analysis and is used for qualitative and quantitative purposes (Watson, 2005:221). HPLC is a separation technique, based on the same principal as TLC, although the components and conditions differ. A HPLC system consists of a column packed with solid stationary phase and a liquid mobile phase flowing through it. Different columns (stationary phase) and mobile phases generally used when performing HPLC testing is listed in Table 3-19. The sample and standard solutions together with the mobile phase are pumped under high pressure through the column. Separation of the constituents in the solutions occurs according to the relative lengths of time spent by its components on the stationary phase (Watson, 2005:268). The time each compound spends on the column (stationary phase) before eluting, will depend on its affinity for the mobile phase or stationary phase (similar to the TLC - section 3.1.4). Figure 3-11 illustrates that those components that are more retained by the stationary phase will move slower through the column than those that are less retained.

Different columns and different mobile phases are employed to obtain suitable chromatographic conditions, depending on the nature of the analyte of interest (Watson, 2005:270).

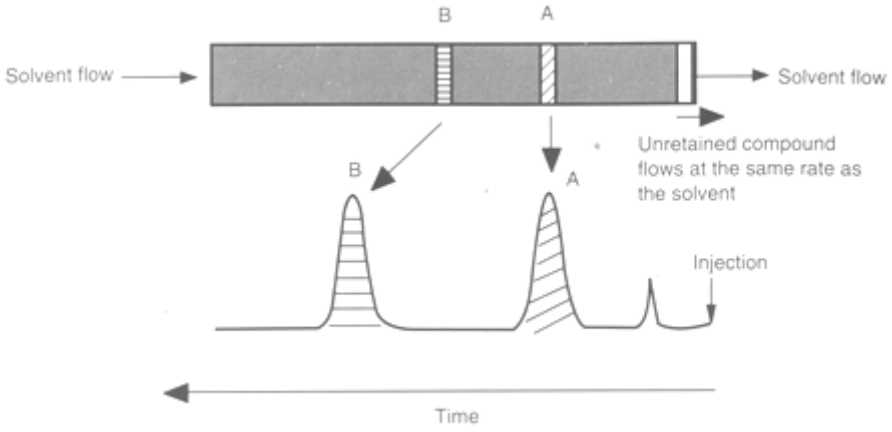

Figure 3-11: Example how different compounds are retained during HPLC analysis (Watson, 2005:222).

After the samples have eluted from the column, it moves through a detector (usually UV). The detector responds linearly to the concentration of the analyte (similar to UV, section 3.4). The signal is plotted as a function of time and this is called a chromatogram (Figure 3-12). The positions of the peaks on the time axis of the chromatogram can be used to identify the components of the sample and the areas under the peaks provide a quantitative measure of the amount of each species (Skoog *et al*., 1997:661-662).

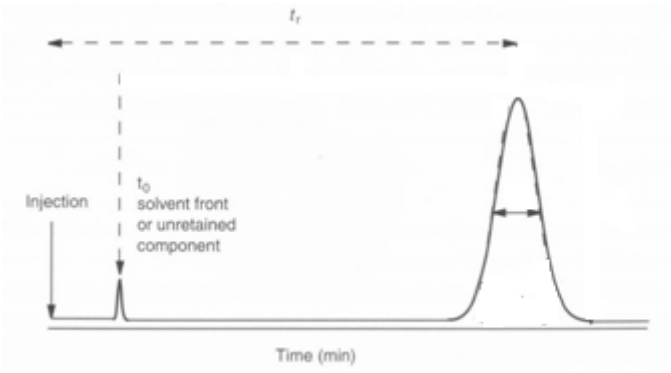

Figure 3-12: Example of a chromatogram where the retention time (t_r) is used for identification purposes and the area under the peak for qualitative analysis (Watson, 2005:223).

Table 3-19: Classification of high performance liquid chromatographic techniques (Watson, 2005:270)

Chromatography	Stationary phase	Mobile phase	Elution
Normal Phase	Hydrophilic packing e.g. silica, cyano and amino columns	Lipophilic e.g. methylene chloride, chloroform or diethylether	The least polar component will be eluted first
Reverse phase	Lipophilic packing e.g. C18, C8 or phenyl columns	Hydrophilic e.g. methanol, water, acetonitrile and tetrahydrofuran	Most polar component eluted first

Combining HPLC with a UV/visible detector provides an accurate, precise and robust means for quantitative analysis of pharmaceutical products. It is also useful in monitoring the integrity of an API with the ability to detect and quantify degradation or impurities in very small concentrations (Watson, 2005:268).

For the quinine sulfate tablet monograph testing, HPLC is used for:

- the identification of quinine sulfate (USP and *Ph.Int.),*
- the assay analysis of quinine sulfate (USP) and
- quantifying the related substances of quinine sulfate (*Ph.Int*. and BP).

A list of the equipment and reagents for assay, related cinchona alkaloids and identification testing by means of HPLC analysisis tabulated in Table 3-20 and Table 3-21.

Table 3-20: List of equipment used for assay, related cinchona alkaloids and identification testing by means of HPLC analysis

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
La Pha Pak 1.5ml amber glass vials	Separations	Germany
Millipore PVDF 0.45µm filters	Separations	Ireland
Agilent 1200 HPLC system with DAD and binary pump	Chemetrix	Germany
Luna C 18 Phenomene x reverse phase column 150 mm x 4.6 mm, 5 µm	Separations	USA
µBondapak C 18 reverse phase column 300 mm x 3.9 mm, 10 µm	Waters	Ireland
Crison Basic 20 pH meter	Lasec	Europe

Equipment	Supplier	Country of origin
Shimadzu analytical balance, model number AUW220D, Unibloc corporation	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number AUW620H, Unibloc corporation	Shimadzu	Japan
Heidolph magnetic stirrer	Labotec	Germany

Table 3-21: List of reagents used for assay, related cinchona alkaloids and identification testing by means of HPLC analysis

Material	Batch number	Manufacturing company	Country of origin
Milli-Q Water	n/a	RIIP®/CENQAM®	South Africa
Sulphuric acid	1035329	Merck Chemicals (Pty) Ltd	South Africa
Potassium dihydrogenphosphate	1040257	Merck Chemicals (Pty) Ltd	South Africa
Hexylamine	S6546626308	Merck KGaA	Germany
Phosphoric acid	1039538	Merck Chemicals (Pty) Ltd	South Africa
Acetonitrile	I682130316	Merck KGaA	Germany
Methanesulfonic acid	S6428222	Merck Chemicals (Pty) Ltd	South Africa
Diethylamine	SC1G600815	Merck Chemicals (Pty) Ltd	South Africa
Methanol	I682707317	Merck KGaA	Germany
Glacial acetic acid	K43568363222	Merck KGaA	Germany
Hydrochloric acid	1040952	Merck Chemicals (Pty) Ltd	South Africa

3.6 Uniformity of dosage units

To ensure the consistency of dosage units (a constant dose of API between individual tablets), each tablet should have an API content within a narrow range around the label claim (USP, 2013). The pharmacopoeia specify the assessment of the consistency of dosage units by either uniformity of weight/mass (weight variation) or uniformity of dosage units. A summary on dosage uniformity test interpretation is given in Figure 3-13.

Uniformity of weight/mass (required by *Ph.Int*.) entails that 20 individual tablets be weighed and the average mass be determined. Not more than 2 of the individual masses should deviate from the average mass by a percentage specified by the pharmacopoeia and none by twice that percentage specified (*Ph.Int*., 2013).

Evaluation of uniformity of dosage units (required by USP and BP) are subdivided into content uniformity and weight variation (Figure 3-13). Content uniformity is evaluated by means of assaying 10 individual units and then using these 10 individual assay values to calculate an acceptance value.

Weight variation entails the weighing of 10 individual units and calculating theoretical percentage assay values. This is calculated using the weight of the individual tablet and the average value of the assay. As with content uniformity, the 10 values calculated are then used to determine an acceptance value. The acceptance value (AV) is calculated by means of equation 3.9 (USP, 2013).

$$AV = |M - \overline{X}| + ks \qquad \text{Equation 3.9}$$

Where:

$\overline{X}$ = mean of the individual contents ($(X_1, X_2, X_3 \ldots\ldots, X_n)$ expressed as percentage of the label claim where *n* represents the number of the units tested

k = acceptability constant (If *n* = 10 then k = 2.4, if *n* = 30, then k = 2.0)

s = sample standard deviation

M (when T ≤ 101.5) = $\overline{X}$ if 98.5% ≥ $\overline{X}$ ≥ 101.5% and AV = ks

M (when T ≤ 101.5) = 98.5% if $\overline{X}$ < 98.5% and AV = $98.5 - \overline{X} + ks$

M (when T ≤ 101.5) = 101.5% if $\overline{X}$ > 101.5% and AV = $\overline{X} - 101.5 + ks$

T = target content per dosage unit at the time of manufacture, expressed as percentage of the label claim. Unless otherwise stated T = 100%

When T > 101.5 other conditions apply for the reference value, M.

If an API forms the greater part of the tablet mass, then it is understandable that a variation in weight may indicate a variation in the content of active ingredient. Therefore, pharmacopoeia specify that if the API consists more than a specific percentage of the average tablet mass, that uniformity of weight or weight variation be performed (Alderborn, 2007:461). In the instance where the tablet contains a very small percentage of API (in relation to the total tablet weight) it is understandable that a variation in weight would not necessarily provide an accurate indication of the degree of the API uniformity in the product. Such products will be subjected to uniformity of content testing (Alderborn, 2007:461).

Should a product or dosage form monograph not provide a specific uniformity of dosage units procedure, then general pharmacopoeial requirements of that specific dosage form must still be met. If the product specific monograph do however specify a specific test procedure for content uniformity/dosage uniformity, then this test must be performed. The results from this specific

test can then be subjected to acceptance value calculation or calculated on a different principal if specified otherwise.

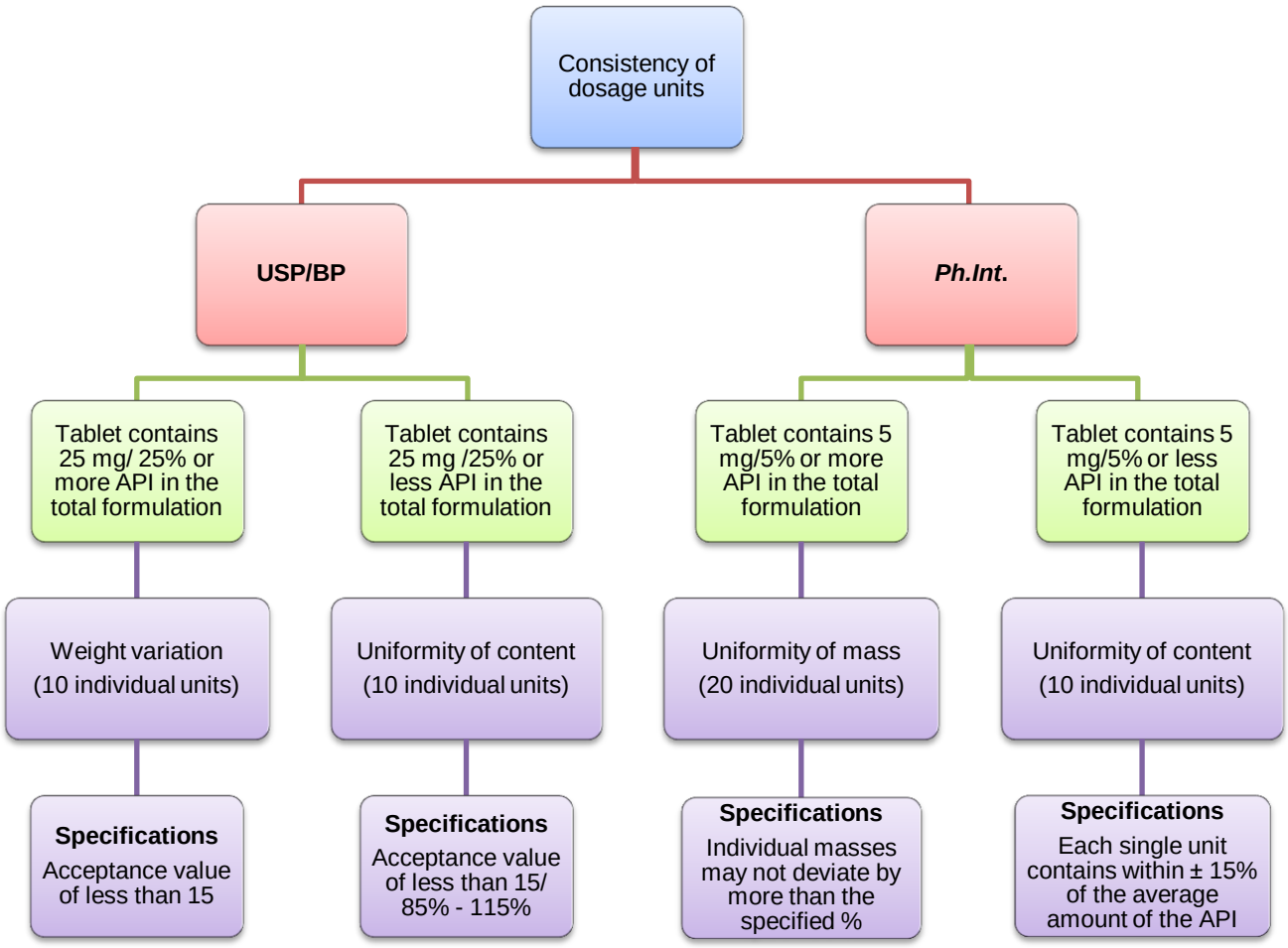

Figure 3-13: Summary of the evaluation of consistency of dosage units as specified by USP, BP and *Ph.Int.*

A list of the equipment used for uniformity of dosage units is tabulated in Table 3-22.

Table 3-22: List of equipment used for uniformity of mass/weight variation

Equipment	Supplier	Country of origin
Sartorius analytical balance, model number R200D	Labotec	Germany

3.7 Non-compendial procedures

As described in section 3.3.1, the dissolution rate of an API is dependent on the solubility thereof in the dissolution medium. Since the available literature depicted controversy surrounding the solubility of quinine sulfate (Chapter 2, section 2.1.6), it may be possible that the dissolution methods of the quinine sulfate monographs be justified on erroneous solubility results. Literature did not provide sufficient data for the solubility of quinine sulfate in the different dissolution media specified by the three monographs (0.1 M hydrochloric acid, 0.01 M hydrochloric acid and phosphate buffer, pH 6.8). Solubility studies were duly performed to address this shortcoming. In addition to the three media specified in the monographs, the solubility of quinine sulfate was also determined in acetate buffer, pH 4.5 to create a more comprehensive solubility profile in physiological pH range, pH 1.2 – 6.8.

3.7.1 Solubility experiments

Solubility studies were performed in a water bath equipped with a thermostat that maintained the temperature of the water bath at 37 ± 0.5°C. The solubility of quinine sulfate was determined in four media (0.1 M hydrochloric acid, 0.01 M hydrochloric acid, phosphate buffer, pH 6.8 and acetate buffer, pH 4.5). 5 ml of the respective media was transferred to each test tube (containing enough quinine sulfate to ensure a saturated suspension) and sealed with a cap. This was done in three fold. A rotator was used to rotate the test tubes at 50 rpm inside the thermally controlled water bath. After 24 hours the test tubes were removed from the apparatus and allowed to cool to room temperature, and then suitably filtered and diluted. The absorption of each of the final diluted samples was determined spectrophotometrically at 248 nm (0.01 M HCl), 348 nm (0.1M HCl), 330 nm (phosphate buffer) and 333 nm (acetate buffer). The concentration of each sample was determined by means of substitution into the standard linear calibration curves obtained and discussed in Chapter 4.

Quinine sulfate reference material (see Certificate of Analysis - Annexure A) was used for the solubility studies.

A list of the equipment and reagents used for solubility testing is tabulated in Table 3-23 and Table 3-24.

Table 3-23: List of equipment used for solubility testing

Equipment	Supplier	Country of origin
Elix 10 Milipore water system	Merck (Pty) Ltd	France

Equipment	Supplier	Country of origin
Millipore PVDF 0.45µm automation compatible filters	Separations	Ireland
Scott Duran A-grade analytical glassware	Merck (Pty) Ltd	Germany
Shimadzu analytical balance, model number AUW220D	Shimadzu	Philippines
Sartorius analytical balance, model number R200D	Labotec	Germany
Shimadzu top balance, model number AUW620H	Shimadzu	Japan
Water bath equipped with thermostat and rotator	NWU	South Africa

Table 3-24: List of reagents used for solubility testing

Material	Batch number	Manufacturing company	Country of origin
Milli-Q water	n/a	RIIP®/CENQAM®	South Africa
Sodium hydroxide	QF1Q610446	Merck Chemicals (Pty) Ltd	South Africa
Sodium dihydrogen phosphate	SAAR5822650EM	Merck Chemicals (Pty) Ltd	South Africa
Hydrochloric acid	1040952	Merck Chemicals (Pty) Ltd	South Africa
Glacial acetic acid	K43568363222	Merck KGaA	Germany
Sodium acetate trihydrate	SAAR582101EM	Merck Chemicals (Pty) Ltd	South Africa

3.8 Quinine sulfate tablet samples and reference standards

The details of the commercial products that were used for analysis are tabulated in Table 3-25**.** The Micro labs and Remedica products were sponsored by the WHO (Switzerland). The Aspen Pharmacare product was procured from a local pharmacy.

The products were assigned random sample numbers to maintain the confidentiality of outcomes in the result sections (Chapters 5 and 6).

The certificates of analysis of all the reference standards that were used for the analysis presented in Annexures A - D.

Table 3-25: Description of the quinine sulfate tablet products used in this study

Label claim	Manufacturer	Distributor	Batch no	Packaging
300mg/tablet	Micro labs Ltd., India	Medical Export group BV, The Netherlands	QSIH0099	Blister 10's
300mg/tablet	Micro labs Ltd., India	IDA foundation, The Netherlands	QSPH0059	Securitainer 1000's
300mg/tablet	Remedica, Europe	Not available	45734	Securitainer 500's
300mg/tablet	Aspen Pharmacare, South Africa	Not available	A803214	Securitainer 100's

Conclusion

In order to ensure sound experimental results, the purposes of this chapter were set out to:

- Obtain an understanding of the analytical principles/techniques required for this study,
- ensure that the correct grade of materials were sourced for testing, and
- ensure that the equipment was maintained in accordance with GMP requirements.

From the discussion presented in this chapter it is clear that all experimental proceedings that are about to follow, are in accordance with good laboratory practice (GLP) and good manufacturing practice (GMP).