

Investigation of solubility and permeability of sulfadoxine and pyrimethamine mixtures

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This thesis is dedicated to my Heavenly Father

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ABSTRACT

Sulfadoxine and pyrimethamine are commercially available in a fixed dose combination (FDC) product, which is used as prophylaxis against malaria. These drugs are still on the World Health Organization's (WHO) essential drug list, making research on sulfadoxine and pyrimethamine relevant. The Biopharmaceutics Classification System (BCS) categorize drugs according to their solubility and permeability properties. Some drugs, like sulfadoxine, have not yet been classified due to a lack of information regarding these properties.

In this study, the solubility, dissolution and membrane permeability of sulfadoxine and pyrimethamine (single compounds and various combination ratios) were investigated. Solid state investigations were employed to explain the results obtained for the solubility and dissolution in the combinations as compared to that of the single compounds.

Two sets of solubility studies were performed, for the first set; both single compounds and different combination ratios were tested in water. Increased solubility was obtained for both compounds in combination as compared to that of the single compounds. On average, sulfadoxine produced a two-fold increase in solubility when combined with pyrimethamine, while pyrimethamine produced a nine-fold increase in solubility when combined with sulfadoxine. From these results, it was decided to focus on the single compounds and the 1:1 mol and 1:1 weight ratio combinations. With each solubility study a different solvent was used. Though the mutual solubility enhancement effect was also present in different solvents (varying in pH value and polarity), it was not as pronounced as in water.

Powder dissolutions were performed on three combinations (the FDC, 1:1 mol and 1:1 weight combinations). In each case the single compounds were also tested in the same quantities as for each combination. The results displayed an increase in dissolution rate when these drugs were combined, which is in accordance with the solubility data.

Certain physico-chemical investigations (nuclear magnetic resonance spectroscopy, isothermal microcalorimetry and vapour sorption analysis) proved a possible intermolecular interaction occurs between sulfadoxine and pyrimethamine. However, this interaction only occurred in solution. No co-crystal formation was evident from any solid state test; however differential scanning calorimetry (DSC) showed a possible eutectic mixture.

The results obtained for the *in vitro* permeability studies (porcine intestinal tissue and Caco-2 monolayer studies) proved a relationship exists between the results obtained for the solubility studies and the permeability studies. An increase in permeation was observed for several of the combinations compared to the single compounds.

This study provided information regarding the solubility and permeability characteristics of sulfadoxine and pyrimethamine. As evident, an interaction occurs between these two drugs, the mechanism of which should be further investigated. Furthermore, the possibility of a eutectic mixture should be explored and then tested to investigate the influence it might have on the solubility and permeability of both drugs.

Keywords: Sulfadoxine, pyrimethamine, solubility, dissolution, permeability

UITTREKSEL

Sulfadoksien en pirimetamien is kommersieel beskikbaar in 'n vaste dosis kombinasie (VDK) produk, wat gebruik word as profilakse teen malaria. Hierdie geneesmiddels is nog steeds op die Wêreld Gesondheidsorganisasie (WGO) se essensiële medisynelyst wat navorsing op sulfadoksien en pirimetamien relevant maak. Die Biofarmaseutiese Klassifikasie Sisteem (BKS) kategoriseer geneesmiddels volgens hulle oplosbaarheids- en deurlaatbaarheidseienskappe. Sommige middels, soos sulfadoksien, is nog nie geklassifiseer nie as gevolg van 'n gebrek aan inligting rakende hierdie eienskappe.

In hierdie studie is die oplosbaarheid, dissolusie en membraandeurlaatbaarheid van sulfadoksien en pirimetamien (enkel komponente en verskeie kombinasie verhoudings) ondersoek. Vastetoestand ondersoeke is geloots om die resultate wat verkry is vir die oplosbaarheid en dissolusie in die kombinasies te verduidelik in vergelyking met dié van die enkel komponente.

Twee stelde oplosbaarheidstudies is uitgevoer, vir die eerste stel is beide enkel komponente en verskillende kombinasie verhoudings in water getoets. Verhoogde oplosbaarheid is vir beide middels in kombinasie verkry in vergelyking met dié van die enkel komponente. Sulfadoksien het 'n twee-voudige gemiddelde toename in oplosbaarheid getoon in kombinasie met pirimetamien; terwyl pirimetamien 'n nege-voudige gemiddelde toename in oplosbaarheid getoon het in kombinasie met sulfadoksien. Vanuit hierdie resultate, is daar besluit om op die enkelkomponente en die 1:1 mol en 1:1 massa verhouding kombinasies te fokus. Met elke oplosbaarheidstudie is 'n ander oplosmiddel gebruik. Hoewel die verbetering in oplosbaarheid van beide middels ook voorkom in verskillende oplosmiddels (wat wissel in pH-waarde en polariteit), is dit nie so beduidend soos in water nie.

Poeierdissolusies is op drie kombinasies (die VDK, 1:1 mol en 1:1 massa kombinasies) uitgevoer. In elke geval is die enkel komponente ook getoets in dieselfde hoeveelhede as vir elke kombinasie. Die resultate vertoon 'n toename in dissolusietempo wanneer hierdie middels gekombineer word, wat in ooreenstemming is met die oplosbaarheidsdata.

Sekere fisies-chemiese ondersoeke (kernmagnetiese resonansspektroskopie, isotermiese mikrokolorimetrie en damporspsie analise) bewys dat 'n moontlike intermolekulêre interaksie tussen sulfadoksien en pirimetamien plaasvind. Hierdie interaksie vind wel net plaas in oplossing. Dit was duidelik uit die vastetoestand ondersoeke dat geen mede-kristal vorming

plaasvind nie, hoewel differensiële skandeerkalorimetrie (DSK) op 'n moontlike eutektiese mengsel dui.

Die resultate wat verkry is vir die *in vitro* deurlaatbaarheidstudies (vark intestinale weefsel en Caco-2 monolaag studies) dui op 'n verwantskap tussen die resultate verkry vir die oplosbaarheidstudies en die deurlaatbaarheidstudies. 'n Toename in deurlaatbaarheid is waargeneem vir verskeie van die kombinasies in vergelyking met die enkel komponente.

Hierdie studie verskaf inligting rakende die oplosbaarheids- en deurlaatbaarheidseienskappe van sulfadoksien en pirimetamien. Dit is duidelik dat 'n interaksie plaasvind tussen die twee geneesmiddels, die meganisme daarvan moet verder ondersoek word. Die moontlikheid van 'n eutektiese mengsel moet verder verken en getoets word om die invloed wat dit moontlik op die oplosbaarheid en deurlaatbaarheid van beide middels mag hê te ondersoek.

Sleutelwoorde: Sulfadoksien, pirimetamien, oplosbaarheid, dissolusie, deurlaatbaarheid

Table of Contents

ABSTRACT	ii
UITTREKSEL	iv
CHAPTER 1 INTRODUCTION AND AIM	1
1.1 Introduction.....	1
1.1.1 Sulfadoxine and pyrimethamine	1
1.1.2 Drug solubility and bioavailability.....	2
1.2 Research Problem.....	3
1.3 Aim & Objectives	3
1.4 References	4
CHAPTER 2 LITERATURE OVERVIEW	6
2.1 Introduction.....	6
2.2 Expressions of solubility	7
2.3 The solubility process	8
2.4 Measurement and prediction of solubility	9
2.5 Dissolution.....	10
2.6 Factors and approaches influencing solubility and dissolution	11
2.6.1 Temperature	11
2.6.2 Particle-size	12
2.6.3 Crystal properties.....	13
2.6.4 Co-crystals.....	13
2.6.5 Solid dispersions.....	14
2.6.6 Co-solvents	15
2.6.7 pH.....	15
2.7 Factors influencing dissolution rate.....	16
2.7.1 Effective surface area (S).....	16
2.7.2 Solubility of the drug (C_s).....	17
2.7.3 Drug concentration in surrounding dissolution medium (C)	20
2.7.4 Diffusion coefficient and thickness of the diffusion layer (D/h)	20
2.8 Pharmaceutical importance of solubility	20
2.9 Membrane permeability	21
2.9.1 Factors influencing absorption.....	21
2.9.2 Permeability models.....	23
2.10 Antimalarial drugs.....	26
2.10.1 Pyrimethamine	27

2.10.2 Sulfadoxine	28
2.11 Summary.....	30
2.12 References	30
CHAPTER 3 METHODOLOGY	40
3.1 Introduction.....	40
3.2 Materials.....	40
3.3 Study design.....	40
3.4 Solubility studies.....	41
3.4.1 Solubility determination of various combinations	42
3.4.2 Solubility determination in different solvents.....	42
3.5 Dissolution.....	43
3.5.1 Powder dissolution	43
3.6 Solid state investigation	44
3.6.1 Differential Scanning Calorimetry (DSC)	45
3.6.2 X-Ray Powder Diffraction (XRPD).....	45
3.6.3 ¹ H Nuclear Magnetic Resonance (NMR) spectroscopy	45
3.6.4 Isothermal microcalorimetry	46
3.6.5 Vapour Sorption Analysis	46
3.7 Permeability studies	46
3.7.1 Excised pig intestinal tissue model	47
3.7.2 Caco-2 cell monolayer model.....	48
3.8 High-performance liquid chromatography (HPLC)	50
3.9 References	51
CHAPTER 4 RESULTS AND DISCUSSION	52
4.1 Introduction.....	52
4.2 Solubility studies.....	52
4.2.1 Solubility determination of various combinations	52
4.2.2 Solubility determination in different solvents.....	53
4.3 Dissolution.....	65
4.3.1 Powder dissolution	65
4.4 Solid state investigation	69
4.4.1 Differential Scanning Calorimetry (DSC)	70
4.4.2 X-Ray Powder Diffraction (XRPD).....	72
4.4.3 Nuclear Magnetic Resonance (NMR)	73
4.4.4 Isothermal microcalorimetry	74
4.4.5 Vapour Sorption Analysis	77
4.5 Permeability studies	81

4.5.1 Excised pig intestinal tissue studies	82
4.5.2 Caco-2 cell monolayer studies	84
4.6 Conclusion.....	85
4.7 References	86
CHAPTER 5 SUMMARY	87
5.1 Introduction.....	87
5.2 Final conclusions	87
5.3 Future recommendations	89
5.4 References	89
APPENDIX 1	91
APPENDIX 2	93

List of Figures

Figure 2.1:	Model to illustrate the solution process	9
Figure 2.2:	The solubility of lovastatin as a function of temperature in different solvents..	12
Figure 2.3:	Impact of chemical structure on the solubility demonstrated for lovastatin (compound 1) and simvastatin (compound 2) with only one extra methyl group	18
Figure 2.4:	Solubility curves of monotropic and enantiotropic polymorphs (Form I, II and III) as a function of temperature	19
Figure 2.5:	Higuchi's diffusion layer model.....	20
Figure 2.6:	The chemical structure of pyrimethamine.....	28
Figure 2.7:	The chemical structure of sulfadoxine.	29
Figure 4.1:	Graphical presentation of the concentrations obtained for sulfadoxine in water as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine.....	54
Figure 4.2:	Graphical presentation of concentrations obtained for pyrimethamine in water as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine.....	54
Figure 4.3:	Graphical presentation of the concentrations obtained for sulfadoxine in water as single compound and sulfadoxine in a 1:1 mol combination with pyrimethamine.....	55
Figure 4.4:	Graphical presentation of concentrations obtained for pyrimethamine in water as single compound and pyrimethamine in a 1:1 mol combination with sulfadoxine.....	55
Figure 4.5:	Graphical presentation of the concentrations obtained for sulfadoxine in water as single compound and sulfadoxine in a 500:25 weight combination with pyrimethamine.....	56
Figure 4.6:	Graphical presentation of concentrations obtained for pyrimethamine in water as single compound and pyrimethamine in a 500:25 weight combination with sulfadoxine.....	57
Figure 4.7:	Graphical presentation of the concentrations obtained for sulfadoxine in 0.1 N HCl as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine.....	58

Figure 4.8:	Graphical presentation of concentrations obtained for pyrimethamine in 0.1 N HCl as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine.....	58
Figure 4.9:	Graphical presentation of the concentrations obtained for sulfadoxine in 0.1 N NaOH as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine.....	59
Figure 4.10:	Graphical presentation of concentrations obtained for pyrimethamine in 0.1 N NaOH as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine	60
Figure 4.11:	Graphical presentation of the concentrations obtained for sulfadoxine in ethanol as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine.....	61
Figure 4.12:	Graphical presentation of concentrations obtained for pyrimethamine in ethanol as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine	61
Figure 4.13:	Graphical presentation of the concentrations obtained for sulfadoxine in methanol as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine	62
Figure 4.14:	Graphical presentation of concentrations obtained for pyrimethamine in methanol as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine	63
Figure 4.15:	Graphical presentation of the concentrations obtained for sulfadoxine in octanol as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine.....	64
Figure 4.16:	Graphical presentation of concentrations obtained for pyrimethamine in octanol as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine	65
Figure 4.17:	Dissolution profile of sulfadoxine as single compound and in a 500:25 weight combination with pyrimethamine.....	66
Figure 4.18:	Dissolution profile of pyrimethamine as single compound and in a 500:25 weight combination with sulfadoxine.....	66
Figure 4.19:	Dissolution profile of sulfadoxine as single compound and in a 1:1 weight combination with pyrimethamine.....	67
Figure 4.20:	Dissolution profile of pyrimethamine as single compound and in a 1:1 weight combination with sulfadoxine.....	67

Figure 4.21:	Dissolution profile of sulfadoxine as single compound and in a 1:1 mol combination with pyrimethamine.....	68
Figure 4.22:	Dissolution profile of pyrimethamine as single compound and in a 1:1 mol combination with sulfadoxine.....	69
Figure 4.23:	Differential scanning calorimetry thermogram for the 1:1 weight ratio combination (1) and the 1:1 mol ratio combination (2) from solubility residues	70
Figure 4.24:	Differential scanning calorimetry thermogram for pyrimethamine (1) and sulfadoxine (2), the 1:1 mol ratio combination (3) and the 1:1 weight ratio combination (4)	71
Figure 4.25:	X-ray powder diffraction pattern for pyrimethamine (1) and sulfadoxine (2) as single compounds, the predicted combined pattern (3) and the sulfadoxine-pyrimethamine 1:1 mol combination residues after solubility testing (4)	72
Figure 4.26:	¹ H-NMR spectra for sulfadoxine (a), pyrimethamine (b) and 1:1 mol combination (c)	73
Figure 4.27:	Heat flow data obtained with the sample in which sulfadoxine was combined with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.....	75
Figure 4.28:	Heat flow data obtained with pyrimethamine combined with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.....	75
Figure 4.29:	Heat flow data obtained with a 1:1 weight combination ratio of sulfadoxine and pyrimethamine with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.	76
Figure 4.30:	Heat flow data obtained with a 1:1 mol combination ratio of sulfadoxine and pyrimethamine with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.	77
Figure 4.31:	Moisture sorption isotherms obtained with sulfadoxine single compound at 25°C, without drying phase	78
Figure 4.32:	Moisture sorption isotherms obtained with pyrimethamine single compound at 25°C	79
Figure 4.33:	Moisture sorption isotherms obtained with the 1:1 weight combination ratio at 25°C.....	80
Figure 4.34:	Moisture sorption isotherms obtained with the 1:1 mol combination ratio at 25°C	81

Figure 4.35:	Graphical presentation of concentrations obtained for sulfadoxine in KRB as single compound and in various combinations (T = 0 min).....	82
Figure 4.36:	Cumulative concentration of sulfadoxine as single compound and in various combinations in the acceptor chamber after 120 min.....	83
Figure 4.37:	Graphical presentation of concentrations obtained for pyrimethamine in DMEM as single compound and in various combinations (T = 0 min).....	84
Figure 4.38:	Cumulative concentration of pyrimethamine as single compound and in various combinations in the acceptor chamber after 120 min	85

List of Tables

Table 2.1:	Description of the classes of the Biopharmaceutical Classification System.....	7
Table 2.2:	Descriptive solubility terms	8
Table 2.3:	Models to measure/predict drug permeation	24
Table 2.4:	Advantages and disadvantages of different <i>in vitro</i> models.....	25
Table 3.1:	Summary of the combinations of sulfadoxine and pyrimethamine and the tests conducted	41
Table 3.2:	Different solvents used for solubility studies of sulfadoxine and pyrimethamine as single compounds and in different ratio combinations	42
Table 4.1:	Average solubility of sulfadoxine and pyrimethamine as single compounds and in different combination ratios in water	53

List of Equations

Equation 2.1.....	9
Equation 2.2.....	10
Equation 3.1.....	49

List of Abbreviations

BCS	Biopharmaceutical Classification System
Caco-2	Human Caucasian colon adenocarcinoma cell line
DSC	Differential scanning calorimetry
DMEM	Dulbecco's Modified Eagle's medium
ECACC	European Collection of Cell Cultures
FDC	Fixed dose combination
GIT	Gastrointestinal tract
GSE	General solubility equation
¹ H-NMR	Proton nuclear magnetic resonance spectrometry
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPLC	High performance liquid chromatography
KRB	Krebs Ringer bicarbonate
MDCK	Madin-Darby canine kidney
NEAA	Non-essential amino acids
P	Pyrimethamine
PBSS	Phosphate buffer saline solution
PABA	Para-aminobenzoic acid
PAMPA	Parallel artificial membrane permeability assay
S	Sulfadoxine
SP	Sulfadoxine/Pyrimethamine combination
TEER	Trans epithelial electrical resistance
VTI	Vapour sorption analysis
WHO	World Health Organization
XRPD	X-ray powder diffraction

CHAPTER 1

INTRODUCTION AND AIM

1.1 Introduction

1.1.1 Sulfadoxine and pyrimethamine

Malaria remains a global problem, with transmission occurring in 97 countries and an estimate of 3.3 billion people at risk of infection annually, of which 1.2 billion are at high risk (>1 in 1000). In 2013, 198 million (uncertainty range: 124 – 283 million) cases of malaria occurred world-wide, which led to 584 000 deaths (uncertainty range: 367 000 – 755 000). Of all malaria deaths, 90% occur in the African region and 78% of all deaths are children under 5 years of age (WHO, 2014:2).

Artemisinin-combination therapies such as artesunate combined with sulfadoxine-pyrimethamine have been introduced by the World Health Organization (WHO) as recommended treatment for uncomplicated malaria caused by *Plasmodium falciparum* (WHO, 2010:16). Furthermore, the use of sulfadoxine-pyrimethamine as intermittent preventive treatment during pregnancy together with the utilization of insecticide-treated nets are included in malaria programme strategies in almost all endemic sub-Saharan countries in Africa (Lynch & Cibulskis, 2013:997). Although the sulfadoxine-pyrimethamine combination has become ineffective in South-east Asia and South America, it is still used in Africa as there are susceptible strains of the malaria parasite on this continent (Lynch & Cibulskis, 2013:997; Watkins *et al.*, 1997:459).

In the past decade, a decrease in the quality of anti-malarial products has been reported (Nayyar *et al.*, 2012:488), which may result in a reduction in the effective treatment of malaria with a potential increase in mortality and morbidity (Kaur *et al.* 2008:1; Newton *et al.*, 2006:602).

The information available on the water solubility of sulfadoxine and pyrimethamine refers to 'very slightly soluble' and 'practically insoluble', respectively (BP, 2015; Loutfy & Aboul-Enein, 1983:465). The relatively poor solubility of sulfadoxine and pyrimethamine may impact on their therapeutic effectiveness (Sinnaeve *et al.*, 2005:97).

1.1.2 Drug solubility and bioavailability

Drug release from a dosage form, drug dissolution under physiological conditions and gastrointestinal epithelial permeability are factors that have an influence on drug absorption and bioavailability (Ochekpe *et al.*, 2012:59). To reach the required blood levels of a drug after oral administration, sufficient permeation of the drug should occur across the intestinal epithelium (Hunter & Hirst, 1997:129). The diffusion of a drug across a biological membrane is determined by its physico-chemical properties (e.g. shape, charge, molecular size and solubility), the structure of the membrane and the interaction of the solute with the solvent (Bjarnason *et al.*, 1995:1566).

The Biopharmaceutics Classification System (BCS) is a system that classifies drugs according to their intestinal epithelial membrane permeability and aqueous solubility properties. This system is especially applicable for determining bioequivalence requirements and to allow bioavailability waivers for generic drug products. The BCS permits human pharmacokinetic studies and/or *in vitro* intestinal permeability methods for determination of the permeability classification. The latter includes cultured epithelial cell monolayers and excised animal intestinal tissues (Thiel-Demby *et al.*, 2008:11). Some of the most popular *in vitro* techniques for assessment of drug permeability across biological membranes include the parallel artificial membrane permeability assay (PAMPA), Madin-Darby canine kidney (MDCK) cell model, Caco-2 cell model (Nožinić *et al.*, 2010:323), and also excised intestinal tissues from animals such as rats and pigs. The most commonly used excised tissue techniques to screen membrane permeability properties of drugs include isolated sheets of intestinal mucosa mounted in Ussing type diffusion chambers or the use of everted intestinal rings (Ashford, 2013:337-341).

Drugs with a low aqueous solubility will exhibit a slow dissolution rate, which most likely will be the rate-controlling step during absorption. This can result in a relatively high variability in pharmacokinetics and biological activities within and between subjects, which has for example been observed for drugs with relatively low aqueous solubility such as chlorpropamide, digoxin, indomethacin, steroids and griseofulvin (Florence & Attwood, 1988:157).

The use of the BCS has been hindered due to the lack of information regarding the solubility and permeability of certain drugs. Some drugs, like sulfadoxine, have not yet been classified in the BCS. Sulfametoxazole, a sulfonamide, is classified as class II (low solubility and high permeability) and pyrimethamine as class II or IV (low solubility and low permeability) (Lindenberg *et al.*, 2004:265, 268-270).

1.2 Research Problem

Caira (2007:310-316) reported on numerous studies where sulfonamides have been used in complex or co-crystal formation with other drugs in a 1:1 molar ratio. Complex or co-crystal formation between two drugs like sulfadoxine and pyrimethamine could influence a range of pharmaceutical and biopharmaceutical properties, such as solubility, permeability, bioavailability and biological activity (Reilly, 2006:198). Seeing as the molecular weight of both compounds are close to one another, both the 1:1 molar and 1:1 weight combinations were the focus during most of the studies.

To investigate the possibility of physico-chemical interactions that may exist between different sulfadoxine and pyrimethamine ratio combinations that may change their solubility and dissolution properties need to be investigated. Furthermore, the effect that these ratio combinations may have on their membrane permeability properties is not yet known. These investigations will therefore contribute new information on the contribution of solubility changes of combinations of sulfadoxine and pyrimethamine on membrane permeability, which can be related to bioavailability and ultimately also to pharmacological effectiveness.

1.3 Aim & Objectives

The aim of this study is to identify potential physico-chemical interactions between sulfadoxine and pyrimethamine in different ratio combinations and to determine how these interactions may change the solubility and membrane permeability in comparison to that of each single drug.

The objectives are:

- To determine the solubility of each selected drug (i.e. sulfadoxine and pyrimethamine) alone and in different ratio combinations in various solvents.
- To conduct powder dissolution studies of each drug alone and in the different ratio combinations.
- To apply physico-chemical characterization techniques to each drug alone and to different ratio combinations including differential scanning calorimetry (DSC), Proton nuclear magnetic resonance spectrometry ($^1\text{H-NMR}$), X-ray powder diffraction (XRPD), isothermal microcalorimetry and vapour sorption analysis (VTI)
- To investigate the *in vitro* permeability of each drug alone and the different ratio combinations of sulfadoxine and pyrimethamine across excised pig intestinal tissues and Caco-2 cell monolayers.

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CHAPTER 2

LITERATURE OVERVIEW

2.1 Introduction

Solubility can be defined as the maximum amount of a substance that is completely dissolved in a certain volume of solvent at a specified temperature. In pharmaceutical sciences, the solubility of a substance becomes particularly important as it often plays a key role in a drug's bioavailability. For this reason, pharmaceutical scientists must have a good understanding of solubility phenomena, which can be used to assist in the development of suitable dosage forms (Gong *et al.*, 2010:1).

It is important, for several reasons, to understand not only the process of dissolution, but also the factors that govern and influence the solubility of excipients and drugs. Certain drugs must be formulated as a solution or as a powder for reconstitution whereafter it must be maintained in solution during use. Irrespective of the means by which a drug is administered, it must be dissolved in order for the drug molecules to move across biological membranes during absorption and/or distribution. Drugs with low aqueous solubility usually experience bioavailability problems (Florence & Attwood, 1988:131; Savjani *et al.*, 2012:2).

Drug release from the dosage form, drug solubility/dissolution under physiological conditions and gastrointestinal epithelial membrane permeability are the main factors that influence drug bioavailability (Ochekpe *et al.*, 2012:59). To reach the required blood levels of a drug after oral administration, sufficient permeation of the drug should occur across the intestinal epithelium (Hunter & Hirst, 1997:129). The diffusion of a drug across a membrane is determined by the solute's physico-chemical properties (e.g. molecular size, charge and solubility), the structure of the membrane and the interaction of the solute with the solvent as well as the membrane (Bjarnason *et al.*, 1995:1566).

The Biopharmaceutics Classification System (BCS) (Table 2.1) is a system that classifies drugs according to two properties namely intestinal membrane permeability and aqueous solubility. The BCS permits *in vitro* intestinal permeability methods for membrane permeability classification. *In vitro* permeability studies include cultured epithelial cell monolayers and excised animal intestinal tissue (Thiel-Demby *et al.*, 2008:11). The most popular *in vitro* techniques for assessment of drug permeability include the parallel artificial membrane permeability assay (PAMPA), Madin-Darby canine kidney (MDCK) cell assay, Caco-2 cell

assay (Nožinić *et al.*, 2010:324-325), and excised intestinal tissues from animals (Ashford, 2013:337). The most commonly used excised tissue techniques to screen membrane permeability properties of drugs include isolated sheets of intestinal mucosa mounted in Ussing type diffusion chambers or the use of everted intestinal rings (Ashford, 2013:337-341).

Drugs with a low aqueous solubility will exhibit a slow dissolution rate, which most likely will be the rate-controlling step during absorption. This can result in a relatively high variability in bioavailability and biological activities within and between subjects, which has been observed for chlorpropamide, digoxin, indomethacin, steroids and danazol (Florence & Attwood, 1988:157; Sunesen *et al.*, 2005:302).

Pyrimethamine is classified in the BCS system as both class II (low solubility and high permeability) and class IV (low solubility and low permeability). Sulfadoxine is not classified within the BCS yet, but sulfamethoxazole (another sulfonamide) is classified as class II (Mandell & Petri, 1996:1060; Lindenberg *et al.*, 2004:268). The available data make it difficult to assign a definite BCS class to the two drugs because of incomplete solubility or permeability data (Lindenberg *et al.*, 2004:265-270).

Table 2.1: Description of the classes of the Biopharmaceutical Classification System (Reddy & Karunakar, 2011:32)

Class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

2.2 Expressions of solubility

There exist several ways to quantitatively express a solute's solubility in a solvent, which are inter-convertible. Expressions such as parts of solute per parts solvent or terms such as "very slightly soluble", "insoluble" and "soluble" are frequently used by the British Pharmacopoeia and other pharmaceutical and chemical compendia (Table 2.2). For quantitative work, it is important to use concentration terms that are specific. For example, even substances that appear 'insoluble' in water quantitatively do have some degree of aqueous solubility, which can be measured and cited precisely (Florence & Attwood, 1988:131-132).

Table 2.2: Descriptive solubility terms (Aulton, 2005:23; BP, 2015)

Term	Weight of solvent (g) necessary to dissolve 1 g of solute
Very soluble	< 1
Freely soluble	Between 1 and 10
Soluble	Between 10 and 30
Sparsingly soluble	Between 30 and 100
Slightly soluble	Between 100 and 1000
Very slightly soluble	Between 1000 and 10 000
Practically insoluble	> 10 000

Concentration can quantitatively be expressed as percentage weight per volume (g per 100 ml), quantity per volume (mg/ml), parts per million (ppm), molarity (mol/l), molality (mol/kg), mole fraction or normality (N) (Gong *et al.*, 2010:2-3; Aulton, 2005:16-17).

2.3 The solubility process

The process by which a solid dissolves in a solvent can be explained by a simple model (refer to Figure 2.1). To dissolve a solid, the forces of attraction holding the solvent molecules together and those holding the solid particles together, must be overcome by the attraction forces between the solvent and solute molecules. For this to be a spontaneous process, the free energy needed to form a cavity in between the solvent molecules as well as the solid's lattice free energy must be exceeded by the solvation free energy before dissolution can occur. The solid's equilibrium solubility will be determined by the balance between the disruptive and attractive forces (Brittain, 2010:40).

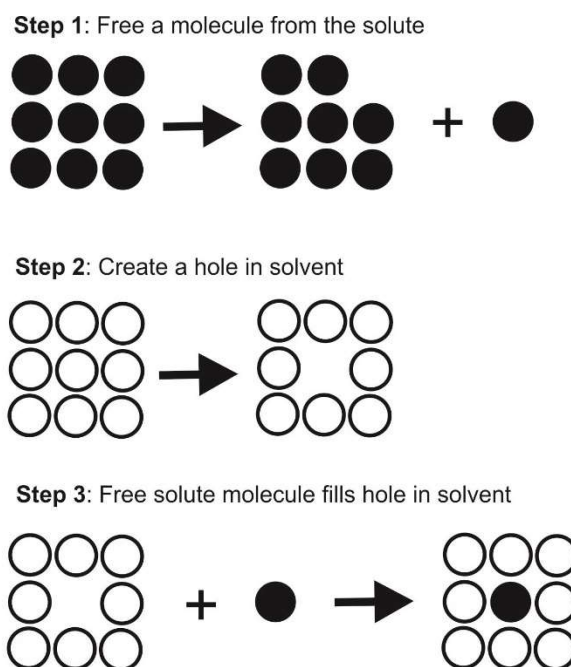


Figure 2.1: Model to illustrate the solution process (adapted from Gong *et al.*, 2010:10)

2.4 Measurement and prediction of solubility

For the measurement of solubility, a sufficient quantity of drug is added to a solvent in a suitable container to obtain a supersaturated solution. These containers are then sealed tightly and usually agitated in a temperature (usually 25 or 37°C) controlled water bath for a specified time depending on the design of the experiment. It is important that adequate time is allowed to reach equilibrium solubility prior to sample withdrawal for analysis. Samples can be analysed for drug content with any suitable analytical method (e.g HPLC or spectrophotometry) (Aucamp *et al.*, 2013:20).

Various methods have been developed to predict a drug's aqueous solubility from its chemical structure (Jorgensen & Duffy, 2002:355-356). These methods include a Monte Carlo simulation technique used by Jorgensen & Duffy (2000:1155-1158) and the general solubility equation by Ran & Yalkowsky (2001:357). The general solubility equation (GSE) is given as:

$$\log S_w = -0.01(MP - 25) - \log K_{ow} + 0.5 \quad \text{Equation 2.1}$$

It is used to estimate the molar solubility of a non-electrolyte organic compound in water (S_w) as a function of its octanol-water partition coefficient (K_{ow}) and melting point (MP) in degrees celcius. A data set of more than a thousand compounds validates the use of the GSE (Sanghvi *et al.*, 2003:258). This method of solubility prediction could prove invaluable with regards to new drugs of which little or no data is available.

2.5 Dissolution

The oral route of administration has been the main administration route for solid dosage forms for almost a hundred years. However, the importance of bioavailability of orally administered drugs and the importance of the dissolution processes in this was not recognised from the start. Since it was recognised that dissolution and bioavailability are pre-requisites for pharmacological action, physical chemists have been developing tests to investigate the dissolution process (Dokoumetzidis & Macheras, 2006:1).

The effective release of a drug from a solid dosage form such as a tablet for systemic absorption is dependent on the disintegration rate of the tablet and the deaggregation rate of the granules within the gastrointestinal tract. However, what is usually of greater importance is the dissolution rate of the solid drug particles. Regularly, dissolution is the rate-controlling or limiting step in absorption of poorly soluble drugs, since it is frequently the slowest of numerous steps involved in the movement of the drug molecules from the dosage form into the systemic circulation (Martin, 1993:330-331).

Noyes and Whitney were first in quantitatively describing the rate of dissolution of a solid in a solvent in 1897 (Equation 2.2), which was elaborated upon by other workers (Dokoumetzidis & Macheras, 2006:1-11). The Noyes Whitney equation can be written as:

$$\frac{dC}{dt} = \frac{DS}{Vh} (C_s - C) \quad \text{Equation 2.2}$$

in which S is surface area of the solid exposed to the dissolution medium, D the solute's diffusion coefficient, C_s the solid's solubility (i.e., the saturated solution concentration of the compound at the solid's surface at the experiment's temperature), h the diffusion layer's thickness, and C the solute concentration in the bulk solution at time t . The solution volume is V and the dissolution rate is dC/dt (Martin, 1993:330-331; Dokoumetzidis & Macheras, 2006:2).

The rate of dissolution can experimentally be measured by *in vitro* dissolution tests. These tests offer an opportunity to conduct reproducible and precise drug release measurements to differentiate between the same formulation of a drug after processing/formulation changes or the same drug in different formulations. *In vitro* testing can pinpoint factors important in formulation, which would influence drug release however; it doesn't replace the necessity for clinical work (Florence & Attwood, 1988:455).

In vitro dissolution methods can be divided into two types involving either forced convection or natural convection. A degree of agitation exists *in vivo* and therefore the most practical

dissolution methods fall in the forced convection category. A certain amount of agitation is introduced in forced convection methods, which can be divided into sink or non-sink conditions (Florence & Attwood, 1988:455).

According to the BP (2015), sink conditions can be obtained when the dissolution medium volume is 3 – 10 times the saturation volume. To maintain sink conditions during dissolution testing, dissolution medium can be replenished upon sample withdrawal. During non-sink conditions, the dissolution medium is not replenished when samples are withdrawn (Florence & Attwood, 1988:455).

2.6 Factors and approaches influencing solubility and dissolution

2.6.1 Temperature

When there is a positive change in enthalpy (ΔH), heat is usually absorbed during dissolution, rendering the process endothermic. If heating of the solvent occurs within this type of system, it will react by invalidating a rise in temperature. This is an example of Le Chatelier's principle. Therefore, an increase in the solubility of the solid will be obtained with a rise in temperature of the solvent, resulting in a positive heat of solution. In systems that display exothermic dissolution (systems that occur less commonly), a decrease will be observed in solubility upon increasing the temperature (Aulton, 2005:25). Murdande *et al.* (2011:188) emphasized the importance of temperature control as a practical precaution when solubility of a drug is measured.

The influence of temperature on drug solubility was illustrated in a study by Mota *et al.* (2009:506). During this study, the aqueous solubility of various drugs (i.e. budesonide, paracetamol, furosemide and allopurinol) was determined in temperatures ranging from 25°C to 42°C. For all four drugs, an increase in solubility was obtained with an increase in solvent temperature.

Figure 2.2 demonstrates the impact of temperature on drug solubility. It displays the solubility profile of the cholesterol-lowering drug, lovastatin, in a mixture of methanol and water as a function of temperature. It is clear that upon increasing the temperature, the solubility of the drug increases (Tung *et al.*, 2009:15).

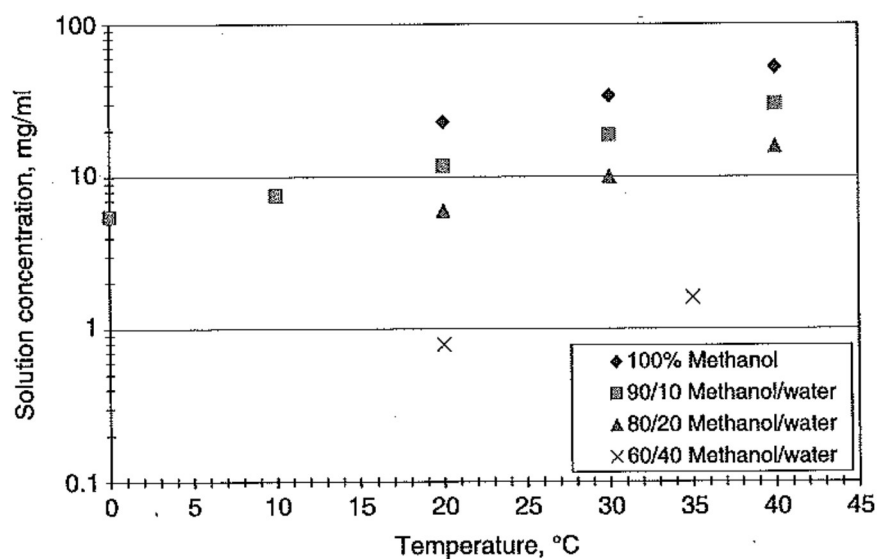


Figure 2.2: The solubility of lovastatin as a function of temperature in different solvents (Tung *et al.*, 2009:15).

2.6.2 Particle-size

Drug dissolution is often related to the particle size of the drug because there is an increase in the surface area to volume ratio upon reduction in particle size. An increase in surface area promotes solvent interaction resulting in an increased dissolution rate (Abdou, 1989:58-59; Savjani *et al.*, 2012:3).

Solubility is compound-specific, depending on temperature, the physico-chemical properties of the dissolution medium and of the drug or compound. However, this is only true for particles in the micrometer range. Solubility will increase when the particle size decreases below 1000 nm. Drug nanocrystals are a novel approach to improve the solubility of poorly water soluble drugs (Junyaprasert & Morakul, 2015:14).

In a study of the solubility of saquinavir mesylate nanoparticles, the drug was ball milled. In simulated saliva, the solubility increased 9-fold after milling for 3 h. This increase in solubility can be attributed to a change in the crystallinity of the drug. However, upon increasing milling time to 60 h, aqueous solubility exhibited a slight decrease rather than an improvement due to the formation of drug aggregates and or metastable amorphous solids (Branham *et al.*, 2012:201).

The increase in dissolution rate ceases with a decrease in particle size when the radius of the particles become very small and if the size is further decreased it will result in a decrease in dissolution rate. It has been suggested that this change is a result of an electrical charge present on the particles which becomes more important upon decreasing the particle size

(Aulton, 2005:27). The reason for a decrease in dissolution rate can also be attributed to agglomeration as proven by Branham *et al.* (2012:201).

2.6.3 Crystal properties

Drugs exist in the solid state as solvates, polymorphs or in amorphous forms, described collectively as different solid/crystal forms (Byrn *et al.*, 1999:3). Polymorphism is a term that describes the ability of a chemical compound to exist in different crystal forms (Borka, 1990:71).

When numerous crystal forms exist for a given substance, the polymorph considered the physically most stable at a certain temperature would be the least soluble polymorph at that temperature, deeming all other forms to be metastable. Each polymorph for a given substance has a specific solubility under fixed conditions of temperature, solvent composition and pressure. Even if two polymorphs have been produced, the tendency will always be that only the less soluble form will eventually be produced by the system (Byrn *et al.*, 1999:16-19).

In a study done by Aucamp *et al.* (2013:23-24), powder dissolution tests were performed for three forms of roxithromycin and one mixture of form I and III. Form I and the mixture (I / III) attained the lowest dissolved concentrations when compared to form II and form III, proving form I to be the stable solid-state form. These findings correlated well with the reported physico-chemical properties. During dissolution, the amorphous metastable form (form II) dissolved rapidly, producing a concentration of 373 µg/ml at 30 min, after which solubility decreased by 43% (to 159 µg/ml) at 720 min. Solution-mediated transformation of form II to form I is believed to have been the cause of a decrease in solubility. Form III produced similar results as form II with a concentration of 234 µg/ml attained after 120 min and a decrease in solubility (to 161 µg/ml) at 720 min, possibly due to solution-mediated transformation, implying that form III is also a metastable form.

2.6.4 Co-crystals

Another phenomenon that needs consideration is the formation of co-crystals. A pharmaceutical co-crystal forms via hydrogen bonding between a drug (such as carbamazepine) and another molecule (for instance saccharin). Co-crystallization aims to alter a drug's physical properties (Attwood & Florence, 2012:5). In recent years, interests regarding pharmaceutical co-crystal design have grown, resulting in the emergence of a method that could potentially enhance the bioavailability of drugs with poor aqueous solubility. Various techniques are employed to prepare co-crystals, these include: solution methods,

reaction crystallization, cooling crystallization, grinding methods and more recently supercritical fluid technology (Qiao *et al.*, 2011:1-7).

The role of co-crystals in changing drug physico-chemical properties has been demonstrated with distinct emphasis on dissolution and solubility. Co-crystals' crystalline nature offers the advantage to improve thermodynamic stability and solubility through solution phase chemistry. Co-crystallization is particularly advantageous in circumstances where salt formation is hindered by the fact that a drug does not have strong basic or acidic groups or sites, or where harsh basic or acidic conditions may cause drug degradation (Thakuria *et al.*, 2013:122).

In a study by Caira (2007:312), co-crystal formation between sulfadimidine (a sulfonamide) and trimethoprim occurred in a methanolic solution. As sulfadoxine is also a sulfonamide and pyrimethamine is related to trimethoprim, the possibility of co-crystallization between these compounds is a possible avenue for investigation.

In 1977, Schmidt *et al.* (1977:837) proved the existence of mutual activity enhancement between sulfadiazine (a sulfonamide) and pyrimethamine upon co-administration. It is therefore important, now more than ever, to exploit this avenue and others such as co-crystallization and solid dispersions to enhance the solubility of sulfadoxine and pyrimethamine. Increasing the solubility by means of solid dispersion manufacturing and/or co-crystallization will be significantly beneficial with regards to improvement of bioavailability.

2.6.5 Solid dispersions

A solid dispersion can be defined as one or more active ingredient(s) dispersed in an inert carrier/matrix in the solid state (Habib *et al.*, 2001:16). According to Katariya & Patil (2013:5) solid dispersions can be classified into several categories, which include eutectic mixtures, amorphous precipitations in a crystalline matrix, solid solutions, glass suspensions and glass solutions. Solid dispersions of several drugs were successfully manufactured and include candesartan cilexetil (Abbas *et al.*, 2014:265), domperidone (Abd Alaziz *et al.*, 2014:77) and itraconazole (Kapsi & Ayres, 2001:201).

The origin of solid dispersions can be traced back to 1961 with the formation of eutectic mixtures by Sekiguchi and Obi (Habib, 2001:4). Although this technique promised to be of value for the pharmaceutical industry, the commercial application proved to be very limited. Recently, the interest in the use of solid dispersions gained new attention due to new types of carriers and manufacturing technologies (Katariya & Patil, 2013:1-15; Vo *et al.*, 2013:799-813). The latest technologies in use for the formulation of solid dispersion systems include the direct capsule filling method, dropping method, supercritical fluid technology and

electrostatic spinning method (Katariya & Patil, 2013:3). Methods that are more regularly used in the preparation of solid dispersions include fusion, spray drying (Paudel *et al.*, 2013:253-284), solvent evaporation, melt agglomeration on process and surface-active carriers (Katariya & Patil, 2013:7-8).

The choice of carrier depends on the interactions between the drug and the carrier and also on the behaviour of the resulting solid dispersion. Paudel *et al.* (2013:253-284) presented an excellent overview on choices available for use when spray drying is used as technique to manufacture a solid dispersion. Polyethylene glycol and polyvinylpyrrolidone are two water-soluble polymers frequently used as carriers in the manufacturing process of solid dispersions (Owusu-Ababio, 2001:65).

2.6.6 Co-solvents

Co-solvents consist of mixtures of water miscible solvents and water, which are used to improve the solubility of poorly soluble compounds. Due to its simplicity in production and evaluation, co-solvency is one of the techniques most widely used to improve the solubility of drugs. Ethanol, polyethylene glycol and propylene glycol are solvents commonly used in co-solvency (Vemula *et al.*, 2010:42).

Miyako *et al.* (2010:48) used co-solvents of varying polarity in combination with water to improve drug solubilization of a range of hydrophobic drugs. In a study by Muela *et al.* (2010:93) the increase in solubility of thiabendazole was tested, using co-solvents in combination with solid dispersion. With the application of the solvent method, solid dispersions were prepared using polyvinyl pyrrolidone. Solubility was measured in non-aqueous (ethanol-ethyl acetate) and aqueous (ethanol-water) mixtures at 15-35°C. The aqueous solubility of thiabendazole was increased by the combination of the co-solvent and solid dispersion techniques to a larger extent when compared to each individual method.

2.6.7 pH

The solubility of weak acidic or basic drugs depends on the pH of the surrounding medium and the ionization constant (K_a) of the drug. These drugs possess an intrinsic solubility. The intrinsic solubility can be described as the solubility of the free acid or free base. This will also be the lowest solubility for the drug. The intrinsic solubility is obtained when the weak acid or weak base is predominantly in its unionized form (>99%). For weak acids this is approximated by the solubility at pH values more than two units below the pK_a . In the case of weak bases, it will be at pH values, two units above the pK_a value. An increase in the degree or extent of ionization of weak acidic and basic drugs is usually associated with an increase in solubility.

For weak acids an increase in ionization is obtained with an increase in pH while for weak bases the inverse relationship exists (Hörter & Dressman, 2001:78; Gaisford, 2013:376).

In the stomach (weak acids) and intestine (weak bases), the un-ionized species are absorbed, causing a disturbance in the equilibrium between ionized and un-ionized species. To restore equilibrium some of the ionized species is converted to the un-ionized form, absorption continues and the process is repeated until the drug is eventually absorbed (Jambhekar, 2013:67-78)

The aqueous solubilities of weak electrolyte drugs are pH dependent; hence with oral solid dosage forms containing these drugs, the drug's dissolution rate will be influenced by the pH in the diffusion layer that surrounds every dissolving solid drug particle. For a weak electrolyte, the microclimate pH (diffusion layer pH) will be affected by the dissolving drug's solubility and the pK_a . Thus one can expect different dissolution rate in different areas of the gastrointestinal tract (Ashford, 2005:237).

2.7 Factors influencing dissolution rate

2.7.1 Effective surface area (S)

Micronization, as a technique to reduce particle-size increases the effective surface area of the solid drug particles (Abdou, 1989:58-59). In a study performed by Vogt *et al.* (2008:283-288), several techniques were applied to improve the dissolution of the poorly soluble drug fenofibrate. Using jet milling (co-grinding with sodium lauryl sulphate, lactose or polyvinylpyrrolidone) and nano-sizing (bead milling with spray-drying) particle size reduction was obtained. It was found that the dissolution performance of the co-ground mixtures was significantly higher compared to the commercial products. This shows that nano-sizing and micronization deliver rapidly dissolving formulations of a drug that may otherwise be poorly soluble. The enhancement in the drug's dissolution using these techniques may lead to better bioavailability.

In another study, done by Varshosaz *et al.* (2008:222-228), the solvent change method was used to produce microcrystals of gliclazide. The results indicated that the dissolution rate was significantly higher for the treated samples in comparison to physical mixtures of the drug with stabilizers and the untreated sample. The results clearly indicated that drug release from the microcrystal formulations exhibited a pronounced increase in drug release compared to the untreated gliclazide powder.

However, micronization of drugs that are sparingly soluble is not guaranteed to give improved dissolution and bioavailability. This is proved in a study by Kornblum & Hirschorn on the

dissolution testing of a poorly water soluble quinazolinone compound employing two specific micronization methods: air-attrition and spray-drying (Kornblum & Hirschorn, 1970:606). The dissolution studies proved that the air-attritioned drug powder tended to form large aggregates, decreasing the dissolution rate (Kornblum & Hirschorn, 1970:609). It is therefore important to note that only increasing the surface area exposed to the dissolution medium (effective surface area) will result in an increase in dissolution rate (Abdou, 1989:60).

2.7.2 Solubility of the drug (C_s)

According to the equation by Noyes and Whitney (Equation 2.2), a drug's dissolution rate under sink conditions is directly proportional to the intrinsic solubility (C_s) of the drug in the diffusion layer that surrounds every dissolving drug particle (Ashford, 2005:237; Dokoumetzidis & Macheras, 2006:5).

The aqueous solubility of a drug is dependent on intermolecular interactions within the crystal lattice, changes in entropy associated with dissolution and fusion, and intermolecular interactions between the drug and solvent in which it dissolves (Ashford, 2005:237).

The solubility of a drug (weak electrolytes) is not only dependent on pH (see *pH* under 2.6.7), but also on factors such as polymorphism (see *Crystal properties* under 2.6.3), solvation, salt formation, and chemical structure to name a few. The assumption is made that similar solubilities for two compounds exist due to the similarity in their chemical structures. Figure 2.3 depicts the solubilities of simvastatin and lovastatin in a methanol/water system. The solubilities of the two compounds differ significantly, despite the only difference in structure being an extra methyl group in simvastatin's structure (Tung *et al.*, 2009:18).

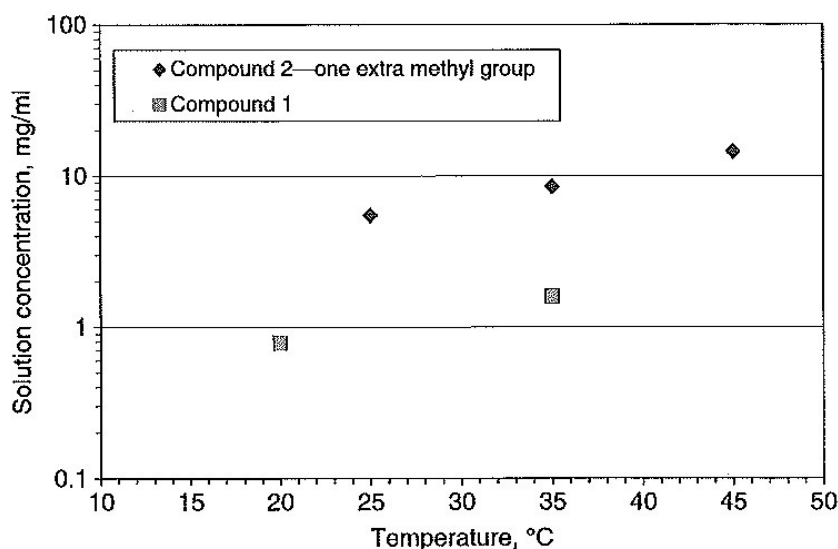


Figure 2.3: Impact of chemical structure on the solubility demonstrated for lovastatin (compound 1) and simvastatin (compound 2) with only one extra methyl group (Tung *et al.*, 2009:18)

If a compound has any basic or acidic functional groups, its solubility can be altered significantly by salt formation. Therefore, different salt types can have notably different solubilities (Tung *et al.*, 2009:18).

Since solubility can be significantly affected by variation in the salt form, the technique can be quite useful in conducting crystallization. For example, a compound's solubility in a specific solvent may be low; however, upon salt formation it may become completely soluble, hence increasing its solubility significantly. By adding an anti-solvent or cooling the solution, the desired pure salt can be crystallized. Alternatively, after dissolving the desired compound in a particular solvent, conversion to the salt form that has a lower solubility in the solvent mentioned, may occur. This approach is called reactive crystallization since the salt crystallizes from the solution. Both methods mentioned have been used in practice (Tung *et al.*, 2009:18).

Like solvent variation, salt formation also affects crystal properties including polymorphs, morphology, crystallization kinetics and drug stability (Tung *et al.*, 2009:18-19).

Different polymorphs (as discussed in 2.6.3 under *Crystal properties*) usually exhibit different behaviours and physical properties, such as different melting points and solubilities. With monotropic polymorphs, one polymorph is stable over the entire temperature range up to the melting point; consequently, there is no actual transition point below the melting point region. With enantiotropic crystal forms, there is a reversible transition point at a temperature below

the melting point of both polymorphs; therefore, both crystal forms have a definite temperature range over which they are stable below the melting point of either polymorph (Tung *et al.*, 2009:50) Figure 2.4 displays solubility curves of enantiotropic and monotropic polymorphs. For the monotropic polymorphs, polymorph II has higher solubility than polymorph I throughout the complete solubility range. For the enantiotropic polymorphs, polymorph III has lower solubility than polymorph I at the lower temperature but higher solubility than polymorph I at the higher temperature (Tung *et al.*, 2009:29-30).

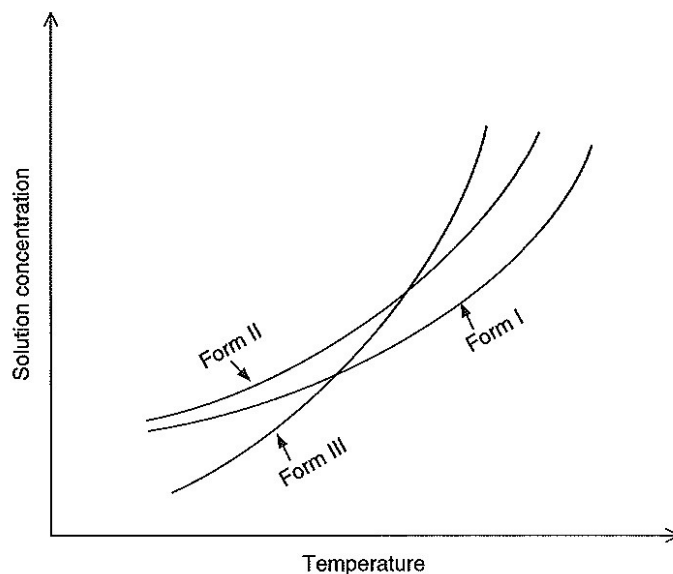


Figure 2.4: Solubility curves of monotropic and enantiotropic polymorphs (Form I, II and III) as a function of temperature (Tung *et al.*, 2009:30)

Solvation is when solvent molecules are incorporated into a compound's crystal lattice to form a different crystal. The original compound and the solvate are not chemically identical due to the presence of solvent molecules in the crystal lattice. Whilst "anhydrous" solid material can exist in either crystalline or amorphous forms, almost all solvates are in practice crystalline (Tung *et al.*, 2009:32).

In a study by Henwood *et al.* (2001:1029), the dissolution rates and solubility of several polymorphs, hydrates and solvates of rifampicin were found to be considerably different from each other. In phosphate buffer (pH 7.4), the 2-pyrrolidone solvate presented the highest solubility whilst amorph II presented the lowest solubility. The 2-pyrrolidone solvate also had the fastest dissolution and highest solubility in water, being the crystal form that presented the best dissolution behaviour overall.

2.7.3 Drug concentration in surrounding dissolution medium (C)

The drug's concentration, C , in solution in the gastrointestinal fluids will be effected by factors such as the rate at which the dissolved drug is removed by absorption and by the available volume of fluid for dissolution. The volume of fluid in the gastrointestinal tract depends on the region of the gastrointestinal tract as well as time of meal intake. The volume of fluid in the stomach will, for example, be influenced by fluid intake at the time of swallowing the drug. According to the equation of Noyes and Whitney, more rapid drug dissolution will occur if C has a low value by virtue of an increase in the value of the term " $C_s - C$ ". When the absorption of a drug is dissolution-rate limited, the value of C is generally kept very low by the absorption of drug molecules from the gastrointestinal fluids into the blood circulation. Therefore, dissolution takes place under sink conditions, i.e. $(C_s - C)$ approximates (C_s) (Ashford, 2005:236).

2.7.4 Diffusion coefficient and thickness of the diffusion layer (D/h)

According to Higuchi's diffusion layer model (Figure 2.5), equilibrium exists between the free and bound solute at the solid-solution interface (Higuchi, 1964:532-533). The pure solid's dissolution rate in a stirred solvent that contains a colloidal solubilizing agent is controlled by the diffusion of solubilized and free solute across a liquid stagnant diffusion layer (thickness h) (Abdou, 1989:22).

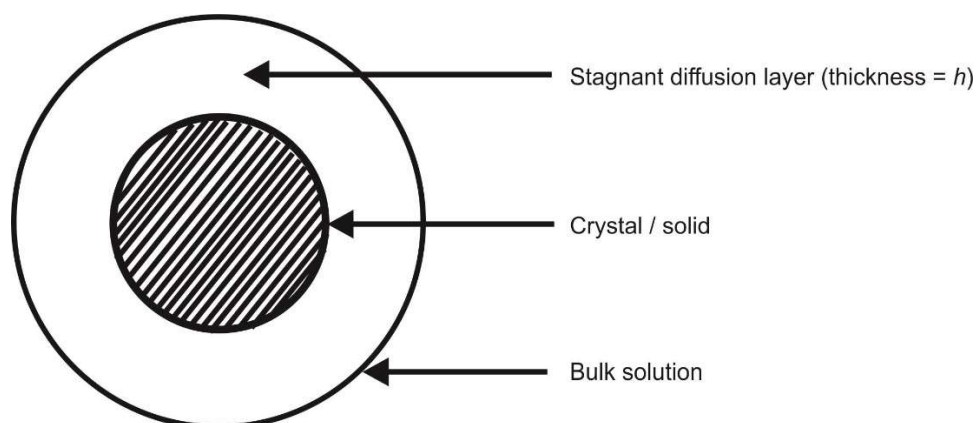


Figure 2.5: Higuchi's diffusion layer model (adapted from Abdou, 1989:12)

2.8 Pharmaceutical importance of solubility

A correlation exists between biological activity and the aqueous solubility of a drug. On the one hand, the aqueous solubility of a drug is inversely related to the drug's solubility in the biological lipid phases (e.g. membrane phospholipid bilayer). On the other hand, drugs with

a low aqueous solubility have slow dissolution rates, which will most likely be the rate-controlling step during absorption. A balance between hydrophilic and lipophilic properties is needed before a drug can reach the site of action in sufficient quantities to provide optimal pharmacological activity. A drug's physico-chemical properties therefore influence its biological properties (Florence & Attwood, 1988:157).

2.9 Membrane permeability

The ability of a drug to cross biological membranes is of particular importance, since the drug's pharmacokinetic properties (i.e. absorption, distribution and elimination) depends on its membrane permeability (Ingels *et al.*, 2010:180). Poor aqueous solubility in conjunction with poor intestinal membrane permeation cause low oral bioavailability, leading to poor plasma concentration control and high variability (Aungst, 2000:429).

The optimal use of the BCS has been hindered due to a lack of information regarding the solubility and permeability of some drugs (Lindenberg *et al.*, 2004:265).

2.9.1 Factors influencing absorption

2.9.1.1 Gastrointestinal pH and the influence of food

The bioavailability of an orally administered drug can be affected by the presence of food in the gastrointestinal tract due to its influence on the drug's solubility and the intestinal pH (Shargel *et al.*, 2005:391). The pH of the fluids in the gastrointestinal tract (GIT) varies considerably in the different regions along its length. Gastric fluid usually exhibits a pH in the range of 1 – 3.5 in the stomach in the fasted state of healthy individuals. Meal ingestion results in a gastric pH of 3 – 7 (depending on meal composition) as the gastric fluid is buffered. The pH will return to fasted-state values within 2 – 3 h after meal ingestion, depending on the size of the meal. Therefore a drug ingested soon after or with a meal will encounter higher pH values in the stomach, which is an important factor to consider regarding drug dissolution and absorption. Thus, the effect of a meal on pH and gastrointestinal residence time may cause variable drug absorption (Ashford, 2005:224; Fleisher *et al.*, 1999:236).

The dissolution rate of a drug is the determining factor for the drug concentration in solution and hence the concentration available for absorption within the intestinal lumen. The pH in the intestinal lumen can affect the dissolution rate of the drug. Weak acidic drugs have faster dissolution rates at a higher pH, whilst the opposite is true for weak basic drugs (Fleisher *et al.*, 1999:235-236).

Compared to the gastric pH value, the pH values in the rest of the intestine are higher. A gradual increase in pH occurs from the duodenum to the ileum due to the gastric acid being neutralized by bicarbonate ions, which is secreted into the small intestine by the pancreas (Ashford, 2005:224-225). The complexity of the effect of pH is increased *in vivo* due to changes in the pH with time and intestinal position (Löbenberg & Amidon, 2000:10).

In a study performed by Carver *et al.* (1999:718-724), the oral bioavailability and plasma concentration of indinavir was reduced severely by a high-calorie meal intake compared to the fasted state. This is consistent with drug precipitation due to elevation of the gastric pH. In another study, the presence of food increased the bioavailability of flurbiprofen (Pargal *et al.*, 1996:511-519). Studies have shown that chelation interactions occur between fluoroquinolones and metallic cations. These interactions together with the concurrent administration of antacids and ferrous sulphate proved to impair the bioavailability of quinolones (Depperman & Lode, 1993:65-72; Lomaestro & Balie, 1995:314-333).

In the upper intestine, viscosity proves to have the greatest impact on the diffusion of a drug upon the ingestion of a drug simultaneously with a highly viscous meal. By mid-jejunum, dilution effects and meal digestion will have significantly reduced luminal viscosity. Thus, for drugs absorbed in the upper intestine, viscosity effects can have a major impact by reducing drug absorption (Fleisher *et al.*, 1999:236).

2.9.1.2 Luminal enzymes

Pepsin is the primary enzyme present in gastric juice. In response to food ingestion, the pancreas secretes proteases, amylases and lipases into the small intestine. Proteases and pepsins are responsible for the degradation of peptide drugs and proteins in the lumen. Drugs may also be subjected to enzymatic degradation, which will decrease their bioavailability. In fat/oil-containing dosage forms, drug release may be affected by lipases (Ashford, 2005:225).

Enzymatic hydrolysis remains a major limitation to the oral administration of protein and peptide drugs (i.e. insulin). In an effort to bypass this limitation, various formulations, including liposomal and microsphere encapsulation have been investigated (Fleisher *et al.*, 1999:236). Two important approaches in the formulation of orally administered peptides and proteins include the use of enzymatic inhibitors and absorption enhancers (Muheem *et al.*, 2016:417).

2.9.1.3 Disease state and physiological disorder

The absorption and bioavailability of oral drugs are likely to be influenced by physiological disorders and disease states of the GIT. Certain diseases can lead to an alteration in gastric

pH, hence affecting dissolution, absorption and stability of a drug. Furthermore, surgery to the GIT can result in variations in drug bioavailability compared to that in normal individuals (Ashford, 2005:226).

The abnormal absorption of drugs also features in conditions of the lower small intestine such as Crohn's disease. Drug absorption rate and concentration may vary from one patient to another with the same disease. Data regarding the abnormal plasma concentration over time curve of clindamycin, trimethoprim and sulphamethoxazole (all orally administered) in Crohn's disease, small bowel diverticulosis and coeliac disease suggests a similar mechanism is responsible for abnormalities in absorption of these physico-chemically unrelated drugs (Parsons, 1977:45-60).

2.9.2 Permeability models

There is an abundance of methods available to measure or estimate the perfusion rate of drugs across membranes (summarised in Table 2.3) in order to gain information regarding membrane permeability and oral absorption (Ashford, 2013:337).

Table 2.3: Models to measure/predict drug permeation (adapted from Ashford, 2013:337; Nožinić *et al.*, 2010:323; Luo *et al.*, 2013:209)

Method / Model	Model type	Name
Computational	<i>In silico</i>	clog <i>P</i>
		mlog <i>P</i>
Physico-chemical	<i>In vitro</i>	Partition coefficient
		Immobilized artificial membrane
Biological	<i>In vivo</i>	Intestinal loop
	<i>In situ</i>	Closed loop method
		Thiry-Vella fistula
		Intestinal single-pass perfusion
		Intestinal recirculation perfusion
		Intestinal perfusion with venous sampling
		Vascularly perfused intestine-liver
		Loc-I-Gut
	<i>In vitro</i>	Parallel artificial membrane permeability assay
		Madin-Darby canine kidney cells (MDCK)
		Caco-2 monolayer
		HT-29
		Everted sacs
		Everted intestinal rings
		Ussing chamber
		Isolated sheets

For the purpose of this study, the focus of discussion will be *in vitro* methods. The different *in vitro* methods can be categorized according to three different screening systems namely membrane-based, cell-based and tissue-based methods (Ashford, 2013:337; Ingels *et al.*, 2010:181-185). These models with their advantages and disadvantages are listed in Table 2.4. The two *in vitro* models used during this study were Caco-2 monolayer and excised pig intestinal tissue. These models are discussed in sections 2.9.2.1 and 2.9.2.2 respectively.

Table 2.4: Advantages and disadvantages of different *in vitro* models (Ingels *et al.*, 2010:183).

	Advantages	Disadvantages
Membrane-based systems (PAMPA)	Easy; low cost; high- or medium throughput	Only passive diffusion
Cell-based systems (Caco-2 monolayer, HT-29, MDCK)	Good screening tool; good correlation with human fraction absorbed; high- or medium throughput; cells may have receptors; widely recognized; evaluation of compound toxicity; absorption enhancing strategies and transport mechanism.	No cellular heterogeneity; mucus or natural tissue environment (blood vessels, nerves, hormone regulation); culture conditions can influence presence of receptors.
Tissue-based systems (Ussing chambers, everted sacs & intestinal rings, isolated sheets)	Segmental absorption evaluation; evaluation of transport mechanism and absorption enhancing strategies; comparison between species is possible; enzymes and transporters as <i>in vivo</i> ; other tissues (nasal, buccal).	Viability of tissue; limited database; limited access to tissue (human); low throughput.

2.9.2.1 Caco-2 monolayer model

The Caco-2 cell line (human colon adenocarcinoma) is the most well-established cell model for drug permeation and its use has shown remarkable growth in the last two decades. In culture, these cells undergo spontaneous enterocytic differentiation to produce a polarized monolayer of cells with well-established tight junctions. The polarized monolayer of enterocytes resemble those present in the small intestine, containing similar transport systems (i.e. P-glycoprotein efflux transporter) and microvilli. The tight junctions, however, is more comparable to those found in the colon (Ashford, 2013:338; Balimane & Chong, 2005:337).

Hidalgo *et al.* (1989:736-749) characterized the Caco-2 cell line as a valuable transport model for intestinal epithelial permeability. The Caco-2 cell line was validated to determine intestinal permeability of a drug in accordance with the BCS by Bock *et al.* (2003:7). Comparing Caco-2 drug transport with *in vivo* intestinal drug transport, the results indicated that these monolayers are useful in predicting drug transport across the intestinal epithelium by different pathways. Of these pathways, the passive transcellular route produced the best correlation between Caco-2 and *in vivo* transport (Artursson *et al.*, 2001:39).

There are several advantages and disadvantages pertaining to the Caco-2 cell line specifically. Disadvantages include that the physiological factors such as bile salts, mucous and cholesterol that influence transport are not present; the model only contain one cell type and the cells have a tumoral origin. The advantages are that Caco-2 cells are from human origin; it can be used for drug screening; the test drug can be exposed to either apical or basolateral side and it is a flexible model (Le Ferrec *et al.*, 2001:662).

2.9.2.2 Excised pig intestinal tissue model

Transport studies across excised animal tissue have been used to explore nutrient absorption from the intestine since the 1950s. This widely used *in vitro* method, was first demonstrated by Ussing and Zerahn (1951:110-127). The method involves the isolation of the intestinal tissue, which is cut into strips of the appropriate size and clamped onto a suitable device and the drug transport rate is then measured across the tissue. During the course of the experiment, the electric resistance can be measured (Balimane *et al.*, 2000:305). In 1987, a new diffusion cell was developed for measuring tissue permeability. Derived from the Ussing chamber, the apparatus is used to evaluate the transport of compounds across epithelial/mucosal barriers, i.e. gastrointestinal tissue (Grass & Sweetana, 1988:372).

As with the Caco-2 cell monolayer model, there are several disadvantages with regard to excised animal tissue. These include the rapid loss of tissue viability during the experiment; the lack of blood and nerve supply; changes in morphology and constant oxygenation (Balimane *et al.*, 2000:305-306). Advantages of this model are the possibility of metabolism studies; the test drug can be exposed to either apical or basolateral side and drug absorption at specific intestinal sites are possible (Le Ferrec *et al.*, 2001:661).

2.10 Antimalarial drugs

2.10.1 Pyrimethamine

2.10.1.1 Introduction and history

Pyrimethamine (or pyrimethaminum) was produced by Burroughs Wellcome & Co. and marketed under the trade name “Daraprim®”, which was regarded as the new synthetic antimalarial of the early 1950's. It was tested by Wilson & Edeson (1953) at the Malaria Research Division of the Institute for Medical Research, Kuala Lumpur, Federation of Malaya. Falco *et al.* (1951) found activity of this drug against *Plasmodium berghei*, *P. gallinaceum* and *P. cynomolgi* in small doses and reported slight toxicity (Wilson & Edeson, 1953: 253-255; Falco *et al.*, 1951:199).

2.10.1.2 Pharmacological classification

Pyrimethamine is a 2,4-diaminopyrimidine, which is submissive to slow but adequate absorption from the gastrointestinal tract. Peak plasma levels are reached 2-6 h after oral intake of pyrimethamine, which binds to plasma proteins and boasts a 3.5 day elimination half-life. Because of its long half-life, pyrimethamine only needs to be administered once a week and before excretion, it is extensively metabolized. Both sulfadoxine and pyrimethamine are excreted primarily by the kidneys and peak plasma levels for both components are detectable in urine within 2-8 hours post dose (Rosenthal, 2009:908, 909).

2.10.1.3 Mechanism of action

Due to pyrimethamine's inhibition of dihydrofolate reductase in the parasite, plasmodia can neither synthesize nor use nucleic acid precursors necessary for growth of the developing parasite. Pyrimethamine provides slow action in the prevention of the erythrocytic phase development, which is not of much value in suppressing acute attacks. It is mostly used in combination with sulfadoxine, as suppressive prophylactic in chloroquine resistant regions to prevent *P. falciparum* clinical attacks. It breaks the parasite's life cycle by rendering it unable to sporulate within the mosquito and boasts a success rate of even 95 % in certain regions. For toxoplasmosis, pyrimethamine is combined with trisulfapyrimidines as treatment of choice (Nichols, 2000:1548).

2.10.1.4 Physico-chemical properties

Pyrimethamine is a tasteless, odourless, white, crystalline powder with the chemical name 2,4-diamino-5-(p-chlorophenyl)-6-ethylpyrimidine (Figure 2.6) (Loutfy & Aboul-Enein, 1983:464-465).

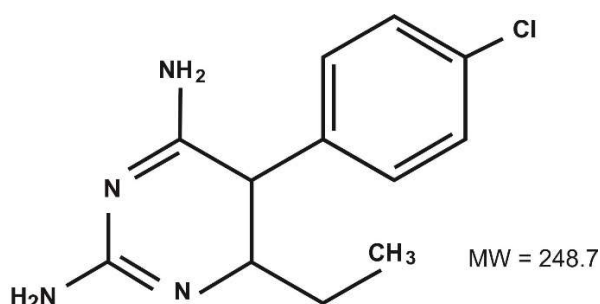


Figure 2.6: The chemical structure of pyrimethamine (BP, 2015).

Pyrimethamine is slightly soluble in dilute hydrochloric acid (HCl), chloroform, ethanol and acetone and basically insoluble in water. It has a melting range between 233°C and 242°C (Loutfy & Aboul-Enein, 1983:465).

2.10.2 Sulfadoxine

2.10.2.1 Introduction and history

Sulfanilamide, otherwise known as *p*-aminobenzenesulfonamide, was synthesized in 1908 for the first time; however the discovery of its therapeutic value occurred many years later. In 1932 a red dye, *p*'-sulfamylchrysoidine or 4-(4'-sulfamylphenylazo)-*m*-phenylenediamine was prepared by a German firm, whilst in 1935 the incredible curative effects of sulfanilamide (sulfadoxine) was reported by Domagk and he named the product "Prontosil®". In that same year, a team of French investigators discovered the drug's antibacterial property, related to the molecule's *p*-aminobenzenesulfonamide portion (Nichols, 2000:1511).

It was only in the 1960's that the sulfadoxine-pyrimethamine combination made its debut in South America and Southeast Asia for the treatment of *falciparum* malaria resistant to chloroquine. However, by the 1980's this combination had become ineffective in these areas and it is only in Africa, that the combination's extensive use is recent, as there are sulfadoxine-pyrimethamine susceptible strains of the malaria parasite (Watkins *et al.*, 1997:459).

2.10.2.2 Pharmacological classification

Sulfadoxine is a sulfonamide with a 7 – 9 day half-life, thus a long-acting antimalarial drug. Absorbed sufficiently after oral intake, sulfadoxine is excreted very slowly via the urinary tract (due to protein binding higher than 85% and extensive tubular reabsorption) hence serum drug levels are prolonged. Sulfadoxine, in combination with pyrimethamine, is used as second-line treatment for malaria and it is available as Fansidar® (Chambers, 2001:794, 795).

2.10.2.3 Mechanism of action

Sulfonamides are structural analogues of para-aminobenzoic acid (PABA), acting to competitively antagonise PABA's bacterial utilization in the synthesis of folic acid also known as pteroylglutamic acid. The bacterial enzyme dihydropteroate synthase, responsible for incorporating PABA into folic acid's immediate precursor, dihydropteroic acid, is competitively inhibited by sulfonamides. Bacteria that are capable of using preformed folate are unaffected by this. This is not the case for micro-organisms synthesizing their own folic acid. PABA competitively counteracts sulfonamide induced bacteriostasis. Since mammalian cells cannot synthesize their own folate and are dependent on preformed folic acid, this mechanism of the sulfonamides does not affect them. Because they use preformed folic acid, these mammalian cells are similar to sulfonamide-insensitive bacteria (Mandell & Petri, 1996:1058).

2.10.2.4 Physico-chemical properties

Sulfadoxine is a white or creamy-white, odourless, crystalline powder and chemically it is known as N' – (5,6-dimethoxy-4-pyrimidinyl)sulfanilamide (Figure 2.7) (Kapoor, 1988:573). Synonyms for sulfadoxine are sulfadoxin, sulphorthodimethoxine, sulphormethoxine, sulforthomidine and sulformethoxine (Sweetman, 2007:301).

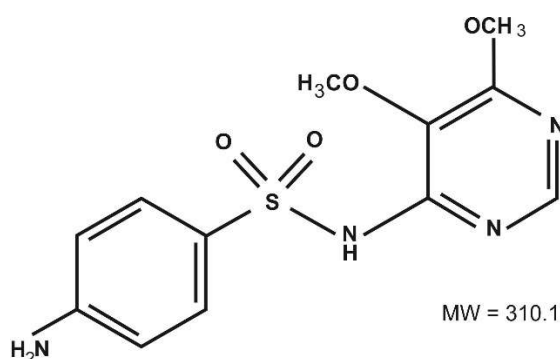


Figure 2.7: The chemical structure of sulfadoxine (BP, 2015).

Sulfadoxine is slightly soluble in methanol and ethanol and very slightly soluble in water. It dissolves in dilute mineral acids and in solutions alkali hydroxides. It melts with decomposition at about 198°C (BP, 2015).

2.11 Summary

Solubility and dissolution of drugs are important to ensure optimal absorption and bioavailability of drugs in the biological system. A drug presenting with a low aqueous solubility will have a slow dissolution rate. Dissolution rate will then be the rate-limiting step during absorption of a drug (Florence & Attwood, 1988:157). If the same drug has poor intestinal membrane permeation, the result will be low bioavailability, poor plasma concentration control and high variability (Aungst, 2000:429). Several factors or formulation approaches such as temperature, pH, particle size and solid dispersions influence solubility and dissolution. These factors can be manipulated to improve the solubility, dissolution or dissolution rate of a drug, which in turn will be advantageous with regards to intestinal membrane permeation and bioavailability.

Drugs are categorized according to their aqueous solubility and intestinal membrane permeability in a system known as the Biopharmaceutics Classification System (Thiel-Demby *et al.*, 2008:11). Its optimal use has been hindered due to lack of this information regarding some drugs (Lindenberg *et al.*, 2004:265). Pyrimethamine is classified as both class II and IV while sulfadoxine hasn't been classified yet due to lack of solubility and permeability data (Lindenberg *et al.*, 2004:265-270).

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CHAPTER 3

METHODOLOGY

3.1 Introduction

Relatively little is known about the solubility and dissolution characteristics of sulfadoxine and pyrimethamine, especially in combination. Even though these two drugs have been available on the market for a relatively long period of time for malaria treatment, they are still on the World Health Organization (WHO) list of essential medicines (WHO, 2015:17), contrary to the fact that resistance to these drugs is a reality. This argues the fact that both these drugs are still of great therapeutic value in certain parts of the world, more specifically in Africa.

Detailed and comprehensive solubility and dissolution studies were performed for both sulfadoxine and pyrimethamine as single components and in different combination ratios to investigate the effects that they may have on each other's solubility and dissolution characteristics under specific conditions.

Since preliminary results showed a mutual solubility enhancement of sulfadoxine and pyrimethamine in combination, several physico-chemical characterization techniques were employed to further investigate the potential interaction that occurs between these two drugs (e.g. formation of co-crystals, complexes or eutectic mixtures). Finally, *in vitro* permeability studies were performed to investigate if a relationship between the solubility and permeability could be established. The methods used for the experimental work in this study are described in this chapter.

3.2 Materials

Sulfadoxine and pyrimethamine were acquired from DB Fine Chemicals (Pty) Ltd. (Johannesburg, South Africa). All other chemicals were of analytical grade and obtained from reputable suppliers in South Africa. Filters with a pore size of 0.45 μm were used for filtration.

3.3 Study design

During this study, sulfadoxine and pyrimethamine as single compounds and in different combination ratios were prepared for various tests (described in sections 3.4 – 3.7). A summary of the different combination ratios and tests is displayed in Table 3.1.

Table 3.1: Summary of the combinations of sulfadoxine and pyrimethamine and the tests conducted

Combination	Tests
S (single compound)	solubility, dissolution, all physico-chemical, permeability
P (single compound)	solubility, dissolution, all physico-chemical, permeability
SP (500:25 wt)	solubility, dissolution, DSC, permeability
SP (1:1 mol)	solubility, dissolution, all physico-chemical, permeability
SP (70:30 wt)	solubility, DSC, permeability
SP (60:40 wt)	solubility, DSC, permeability
SP (1:1 wt)	solubility, dissolution, all physico-chemical, permeability
SP (40:60 wt)	solubility, DSC, permeability
SP (30:70 wt)	solubility, DSC, permeability

S = sulfadoxine, P = pyrimethamine, wt = weight, mol = molecular weight, DSC = differential scanning calorimetry

As shown in Table 3.1, the various combinations range in increments of 10 with the exception of SP (500:25 wt). It was decided to include SP (500:25 wt) as this is the commercially available fixed dose combination. Furthermore, in Table 3.1 reference is made to “all physico-chemical” tests which refers to the solid state investigation performed under section 3.6.

3.4 Solubility studies

The first set of solubility studies that were performed ranged in different concentration ratios of sulfadoxine and pyrimethamine and the solvent used was water. From these results it was decided to focus on the single compounds and the 1:1 weight combination ratio. For the next set of solubility studies, the single compounds were compared to the 1:1 weight combination ratio using a different solvent for each solubility study. All the solubility studies were performed at 37°C (± 2°C) in a water bath containing a rotating aluminium axis on which test tubes can be affixed. Individual samples (four per experiment) of a pre-determined weight (sufficient to ensure oversaturation) were weighed off for sulfadoxine (S1-S4), pyrimethamine (P1-P4) and for each of the different combinations as shown in Table 3.1 (SP1-SP4). The samples were transferred to glass test tubes and 10 ml of the relevant solvent was pipetted into each glass

tube. The glass tubes were sealed with Parafilm® (a thermoplastic flexible plastic film) (Neenah, USA) and a screw cap was fitted to each tube to prevent leakage. Each test tube was affixed to the rotating axis in the water bath and switched on to rotate at 54 rpm for 24 h. After the 24 h period, the motor of the rotating axis was switched off and from each test tube, a sufficient volume of sample was collected, filtered and analysed on the high performance liquid chromatograph (HPLC, method described in section 3.8). If necessary, dilutions of the samples were made to ensure useful chromatograms were obtained. Any dilutions were compensated for during the processing of the data.

3.4.1 Solubility determination of various combinations

Solubility studies were performed where sulfadoxine and pyrimethamine were combined in different concentration ratios and compared to both single compounds. This was done to obtain information regarding the solubility of these two drugs. Samples of both single compounds and various combinations of sulfadoxine and pyrimethamine were prepared. The combination samples varied in weight from 30% sulfadoxine and 70% pyrimethamine to 70% sulfadoxine and 30% pyrimethamine (by increments of 10%). The 1:1 weight and 1:1 molecular weight combinations were also tested. The solvent used for this study was water.

3.4.2 Solubility determination in different solvents

Solubility studies were performed in different solvents on sulfadoxine and pyrimethamine as single compounds and in various combinations. A summary of the solubility studies in different solvents and combinations are displayed in Table 3.2.

Table 3.2: Different solvents used for solubility studies of sulfadoxine and pyrimethamine as single compounds and in different ratio combinations

Solvent	Drug combination
Water	S and P (single components), SP (1:1 wt), SP (1:1 mol), SP (500:25 wt)
0.1 N HCl	S and P (single components), SP (1:1 wt)
0.1 N NaOH	S and P (single components), SP (1:1 wt)
Ethanol	S and P (single components), SP (1:1 wt)
Methanol	S and P (single components), SP (1:1 wt)
Octanol	S and P (single components), SP (1:1 wt)

S = sulfadoxine, P = pyrimethamine, wt = weight, mol = molecular weight

3.5 Dissolution

Dissolution studies were performed to establish whether there exists a relationship between the solubility and dissolution profiles obtained for the single compounds and the combination ratios (as outlined in Table 3.1) of sulfadoxine and pyrimethamine.

For dissolution testing during this study the method described for sulfadoxine and pyrimethamine tablets according to the International Pharmacopoeia (Ph.Int., 2015) was used. A VanKel700 dissolution bath (Carry, USA) equipped with paddles (USP apparatus 2) was used, the rotational speed was set at 75 rpm and the temperature at 37°C. The dissolution medium used during this study was distilled water and after withdrawals at predetermined time intervals, all samples were filtered into vials and analysed by HPLC.

3.5.1 Powder dissolution

Dissolution studies were performed on single compound powders as well as three different sulfadoxine and pyrimethamine combinations as indicated in Table 3.1. Powder dissolutions were conducted in triplicate and glass beads, $\leq 106\ \mu\text{m}$ (Sigma–Aldrich, South Africa) were added to each test tube containing the relevant single compound or combination ratio. Glass beads are used during powder dissolutions to prevent aggregation (Lötter *et al.*, 1983:56-66). Each vessel was filled with 1000 ml of dissolution medium (distilled water). From each vessel, 5 ml samples of solution were withdrawn, at time intervals: 5, 10, 15, 20, 30, 45 and 60 min. No dissolution medium was replaced after sample withdrawal.

3.5.1.1 Powder dissolution (commercial product combination ratio)

For this dissolution, the same combination of sulfadoxine (500 mg) and pyrimethamine (25 mg) as in the commercially available product (Fansidar®) was tested. Approximately 500 mg of sulfadoxine powder was weighed into each of three 10 ml test tubes. To this, approximately 250 mg glass beads, $\leq 106\ \mu\text{m}$ (Sigma–Aldrich, South Africa) was added (S_A1 - S_A3). The same was done for pyrimethamine, where a weight of approximately 25 mg was added to each test tube to which approximately 12.5 mg glass beads were added per test tube (P_A1 - P_A3). For the combination of sulfadoxine and pyrimethamine, approximately 500 mg and 25 mg were weighed respectively of each compound and added together into each of three 10 ml test tubes (SP_A1 - SP_A3). Again, glass beads (approximately 262.5 mg) were added. A volume of 5 ml of dissolution medium (37°C) was added to each test tube after which a vortex mixer (Celsius Scientific, Glenvista, South Africa) was used to agitate each mixture for 2 min at the highest speed. After agitation, each suspension was introduced to separate dissolution

vessels. Sample withdrawals were made at time intervals as specified in section 3.5.1 and the samples were filtered, placed in vials and analysed using HPLC (according to the method described in section 3.8).

3.5.1.2 Powder dissolution (1:1 weight combination)

Approximately 250 mg of sulfadoxine powder was weighed into each of three 10 ml test tubes. To this, approximately 125 mg glass beads, $\leq 106\ \mu\text{m}$ (Sigma–Aldrich, South Africa) were added (S_B1 - S_B3). The same was done for pyrimethamine with a weight of approximately 250 mg per test tube, adding to that approximately 125 mg glass beads per test tube (P_B1 - P_B3). For the 1:1 weight combination of sulfadoxine and pyrimethamine, approximately 250 mg were weighed for each drug and added together into each of three 10 ml test tubes (SP_B1 - SP_B3). Again, glass beads (approximately 250 mg) were added. To each test tube, 5 ml of dissolution medium (37°C) was added after which a vortex mixer was used to agitate each mixture for 2 min. After agitation, each mixture was introduced to separate dissolution vessels. Sample withdrawals were made at time intervals as specified in section 3.5.1 and these samples were filtered, placed in vials and analysed using HPLC (according to the method described in section 3.8).

3.5.1.3 Powder dissolution (1:1 molecular weight combination)

Approximately 310 mg of sulfadoxine powder was weighed into each of three 10 ml test tubes. To this, approximately 155 mg glass beads, $\leq 106\ \mu\text{m}$ (Sigma–Aldrich, South Africa) were added (S_C1 - S_C3). The same was done for pyrimethamine with a weight of approximately 248 mg per test tube, adding to that approximately 124 mg glass beads per test tube (P_C1 - P_C3). For the 1:1 molecular weight combination of sulfadoxine and pyrimethamine, approximately 310 mg and 248 mg were weighed respectively for each compound and added together into each of three 10 ml test tubes (SP_C1 - SP_C3). Again, glass beads (approximately 278 mg) were added. To each test tube 5 ml of dissolution medium (37°C) was added after which a vortex mixer was used to agitate each mixture for 2 min. After agitation, each mixture was introduced to separate dissolution vessels. Sample withdrawals were made at time intervals as specified in section 3.5.1 and the samples were filtered, placed in vials and analysed using HPLC (according to the method described in section 3.8).

3.6 Solid state investigation

The following techniques were employed to characterise the physico-chemical properties of sulfadoxine and pyrimethamine alone (single compounds) and in the different combinations

(as outlined in Table 3.1): Differential scanning calorimetry (DSC), x-ray powder diffraction (XRPD), nuclear magnetic resonance (^1H NMR) spectrometry, isothermal microcalorimetry and vapour sorption analysis as described below.

3.6.1 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) is used to gain information about the melting point, glass transition temperature (T_g) and energy changes, which may be associated with phase transformations. A DSC-60 Shimadzu instrument (Shimadzu, Kyoto, Japan) was used to record the DSC thermograms. Samples weighing approximately 3 – 6 mg were placed in aluminium crimp cells with pierced lids and heated to the desired temperature (max 300°C) and heating rate (10°C/min), with a nitrogen gas purge of 35 ml/min. DSC was employed to investigate the possible existence of eutectic mixtures in the binary combinations of sulfadoxine and pyrimethamine.

Samples of both single compounds and various combinations of sulfadoxine and pyrimethamine were prepared. The combination samples varied in weight from 30% sulfadoxine and 70% pyrimethamine to 70% sulfadoxine and 30% pyrimethamine (by increments of 10%). The 1:1 weight and 1:1 molecular weight combinations were also characterised.

3.6.2 X-Ray Powder Diffraction (XRPD)

X-ray powder diffraction (XRPD) is used to identify and characterise the crystalline or amorphous state of the drug. Samples were evenly distributed on a zero background sample holder and then placed into the PANalytical Empyrean diffractometer (PANalytical, Almelo, Netherlands) for analysis. The measurement conditions were as follows: target, Cu; voltage, 40 kV; current, 30 mA; divergence slit, 2 mm; antiscatter slit, 0.6 mm; detector slit, 0.2 mm; monochromator; scanning speed, 2° /min (step size, 0.025°; step time, 1.0 sec). The data was analysed using HighScore Plus software version 3.0a.

The samples used for XRPD identification included both sulfadoxine and pyrimethamine single compounds and the 1:1 molecular weight combination.

3.6.3 ^1H Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is employed to elucidate the chemical structures of substances and to indicate if interactions (i.e. complex formation) occur in solution. Deuterium oxide (D_2O) and deuterium chloride (DCI in D_2O) (Sigma-Aldrich,

Steinheim, Germany) were used as solvents. Proton (^1H) NMR spectra was recorded on a Bruker advance 600 NMR spectrometer at 600.17 MHz. Chemical shifts are reported in parts per million (δ). Three samples were prepared: sulfadoxine and pyrimethamine 1:1 molecular weight combination (SP 1:1 mol) and sulfadoxine and pyrimethamine single compounds. Saturated solutions were prepared for all samples in D_2O . A small quantity of DCI was added to each sample in order to enhance the solubility of the drugs in the D_2O . The solutions were then filtered into 5 mm NMR tubes for analysis.

3.6.4 Isothermal microcalorimetry

The influence of water on the single compounds (sulfadoxine and pyrimethamine) or combinations of the sulfadoxine and pyrimethamine was determined using a 2277 Thermal Activity Monitor (TAM III) (TA Instruments, USA) which was equipped with an oil bath with a stability of $\pm 100\ \mu\text{K}$ over 24 hours. In each 4 ml ampoule, approximately 50 mg of the single compounds, the 1:1 weight combination or the 1:1 molecular weight combination was weighed. To each ampoule 1 ml of water was accurately pipetted into the ampoule and weighed. Each ampoule was sealed with a rubber stopper and aluminium crimp cap.

3.6.5 Vapour Sorption Analysis

A VTI-SA vapour sorption analyser (TA Instruments, USA) was used for the moisture sorption analysis. A 100 mg standard weight was used to calibrate the microbalance prior to each vapour sorption run and the microbalance was set to zero before the sample was weighed into the quartz sample container. Each sample was carefully placed into the sample container and distributed evenly. A drying phase was included (40°C) and drying was performed until the sample weight variation was less than 0.0001%. TA Instruments Isotherm software was used to set the percentage relative humidity (%RH) / temperature program. The %RH was set to ramp from 5% to 95% RH and the ramp criteria was set to 2 minute stability of weight gained or lost or 0.0001% weight change. The temperature was set to be constant at 25°C . To calculate all adsorption and desorption isotherms, an ExcelTM macro supplied by TA Instruments, was used.

3.7 Permeability studies

Permeability studies were performed to investigate if the solubility enhancement as a result of combining sulfadoxine and pyrimethamine had an effect on the membrane permeability of these drugs. During this study, two *in vitro* permeability models were employed to determine the permeability of sulfadoxine and pyrimethamine as single compounds and in different

combinations (as outlined in Table 3.1), namely excised pig intestinal tissues and Caco-2 cell monolayers. The most suitable model was employed for each of the two drugs as determined by preliminary permeation studies.

3.7.1 Excised pig intestinal tissue model

The *in vitro* permeability of sulfadoxine and pyrimethamine as single compounds and in different ratio combinations (as outlined in Table 3.1) was determined across excised pig jejunum tissue in the apical to basolateral direction. A piece (approximately 30 cm) of pig jejunum was collected from the abattoir directly after slaughtering of the pig and washed with cold (below 8°C) Krebs Ringer bicarbonate (KRB) buffer. The clean section of the jejunum was placed in a container filled with cold KRB buffer for transportation to the laboratory. Preparation of the tissue was completed in the laboratory within half an hour from collection at the abattoir.

The piece of tissue was carefully pulled over a glass tube and washed again. The serosal layer was removed by blunt dissection and the tissue inspected for weaknesses or thickened areas (e.g. Payer's patches). The mesenteric border was used as guideline to dissect the jejunum, producing a flat sheet of tissue. The tissue was then washed from the glass tube with KRB buffer onto a sheet of filtration paper with the apical side facing upward.

The tissue sheet was cut into small strips of approximately 1.5 cm in width and mounted between two half cells of a Sweetana-Grass diffusion apparatus. The surface area of the mucosal tissue that was available for transport equalled 1.78 cm². The assembled chambers were mounted onto the heating plate of the diffusion apparatus.

Pre-heated (37°C) KRB buffer (7 ml) was placed in both the apical and basolateral chambers of the apparatus, with medical oxygen bubbling constantly through the liquid in the chambers. The prepared drug solutions were heated to 37°C whilst sufficient time was allowed for the equilibration (30 min) of the tissue in the KRB buffer. After equilibration, the buffer was removed from the chambers. The basolateral side was then refilled with 7 ml of fresh KRB buffer (37°C). The apical side was filled with 7 ml of test solution and the integrity of the tissue measured at the start and again at the end of the study, using a Millipore™ Millicell® ERS-2 epithelial volt/ohm meter to measure trans-epithelial electrical resistance (TEER).

A 200 µl sample was withdrawn from the acceptor chamber every 20 min over a period of 120 min. The relevant chamber from which the sample was withdrawn was immediately replenished by the same volume of fresh pre-heated KRB buffer solution. The samples were

placed into HPLC vials containing inserts and immediately refrigerated. All samples were then analysed by means of HPLC (according to the method described in section 3.8).

3.7.2 Caco-2 cell monolayer model

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC), by Sigma Aldrich, Johannesburg, South Africa. The cells were cultured in high-glucose DMEM (Separations, Randburg, South Africa) supplemented with 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 10% foetal bovine serum (Merck, Modderfontein, South Africa), 1% penicillin/streptomycin (Separations, Randburg, South Africa), 1% amphotericin B (250 µg/ml) (The Scientific Group, Johannesburg, South Africa) and 1% 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa).

The Caco-2 cells were cultured at 37°C with 95% humidified air and 5% carbon dioxide in a Forma Steri-cycle CO₂ incubator (Life Technologies, Fairland, South Africa). The cells were inspected by means of a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) to ensure the absence of contamination and to determine the percentage confluence. The growth medium was replaced every second day upon which it was decanted and removed from the cell culture flask and then replaced with 10 ml of growth medium (pre-warmed at 37°C) by means of a pipettor and a serological pipette. This was done in a laminar flow hood equipped with HEPA filters, under sterile conditions.

The Caco-2 cells were sub-cultured, until reaching 50% confluency (Natoli *et al.*, 2012:1244) by means of trypsinisation. Phosphate buffered saline solution (PBSS) (Sigma Aldrich, Johannesburg, South Africa) and growth medium were pre-warmed in a circulating water bath to 37°C. The growth medium was decanted and removed from the cell culture flask and the cells rinsed twice with 10 ml PBSS using serological pipettes and a pipettor. This was done in a laminar flow hood under sterile conditions. A volume of 3 ml of Trypsin-Versene (EDTA) mixture (Whitehead Scientific, Cape Town, South Africa) was added to the cell culture flask and the mixture distributed evenly on the cell layer. The cell culture flask was then placed in the CO₂ incubator at 37°C for 5 min. Cell detachment was determined by means of a light microscope. Upon complete cell detachment, 6 ml of growth medium (pre-warmed at 37°C) was added to the content of the flask in order to inactivate the trypsin mixture's action. The cell suspension was gently agitated by means of a pipette to ensure that all cells were rinsed from the bottom of the flask and divided accordingly. Cells were maintained under normal cell culturing conditions prior to seeding. The Caco-2 cells were seeded onto Transwell® 6-well membrane filters (Corning Costar® Corporation, Tewksbury, USA) with a surface area of 4.67 cm² and a pore diameter of 0.4 µm. A cell suspension was obtained by trypsinisation

with Trypsin-Versene as previously described. After cell detachment, 6 ml of growth medium (pre-warmed at 37°C) was added to the flask and the suspension agitated extensively by means of a pipette to ensure de-agglomeration and complete detachment, resulting in a suspension consisting of single cells. The single cell suspension was transferred to a 50 ml tube. To ensure a homogenous distribution of the cells, a Pasteur pipette was used to agitate the suspension. Cells were counted employing a haemocytometer using Trypan blue (Sigma Aldrich, Johannesburg, South Africa) as staining agent to monitor the percentage cell death. In a 2.5 ml Eppendorf tube, a volume of 15 µl PBSS, 10 µl cell suspension and 25 µl Trypan blue were mixed and incubated for 3 min, after which a volume of 10 µl of the mixture was placed on either side of the cover slip of the haemocytometer. Cells were counted on five of the nine squares on the haemocytometer's grid on each side by means of a light microscope. The average number of cells was calculated (per square), which was then multiplied with the dilution factor (5×10^4). This established the total number of cells per ml in the cell suspension. The suspension was diluted to a concentration of approximately 20 000 cells per ml using equation 3.1.

$$C_1 \times V_1 = C_2 \times V_2 \quad \text{Equation 3.1}$$

Where V_1 and V_2 represent the initial and final volumes, respectively and C_1 and C_2 represent the initial and final concentrations, respectively. Cell seeding onto Transwell filter membranes occurred in a laminar flow hood under sterile conditions by pipetting 2.5 ml of the final cell suspension into each apical chamber of the membrane filter plate wells. The Caco-2 cells were grown for 21 to 24 days on the membrane filters to produce intact epithelial cell monolayers. The growth medium was replaced every second day under sterile conditions.

Prior to the transport studies, the TEER of each cell monolayer was measured using a Millcell ERS II meter (Millipore, Billerica, Massachusetts, USA) to ensure that confluent monolayers were formed. A resistance of at least 150Ω per well was required to commence the experiment.

For the transport studies, the growth medium was removed from the basolateral chamber and replaced with 2.5 ml of pre-warmed (37°C) DMEM buffered with HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (Scientific group, Biochrom, Randburg, South Africa) and incubated at 37°C for 30 min, after which TEER measurements were performed. The growth medium was removed from the apical chamber and replaced with 2.5 ml of test solution (each drug solution was tested in triplicate), after which TEER measurements were performed.

For all test solutions, supersaturated solutions were prepared where approximately 100 mg of pyrimethamine single compound or 200 mg of the different combinations were weighed off in test tubes and 12.5 ml of DMEM added as solvent and pre-heated to 37°C.

A 200 µl sample was withdrawn from the basolateral chamber every 20 min over a period of 120 min. The chamber from which the sample was withdrawn was immediately replenished by the same volume of fresh pre-heated DMEM. The samples were placed into HPLC vials containing inserts. To ensure that the monolayer integrity was maintained, final TEER measurements were performed at the end of the study. All samples were then analysed by means of HPLC (according to the method described in section 3.8).

3.8 High-performance liquid chromatography (HPLC)

The samples obtained from the solubility, dissolution and permeability studies were analysed using a Shimadzu (Kyoto, Japan) UFLC (LC-20AD) high performance chromatographic system. The apparatus consisted of a SPD-M20A UV/VIS Photodiode Array detector, an auto-sampler (SIL-20AC) with a sample temperature controller and a solvent delivery module (LC-20AD). The mobile phase consisted of 20 ml of glacial acetic acid (100 %) and 1 ml triethylamine dissolved in approximately 1600 ml of water, diluted to 2000 ml and the pH adjusted with sodium hydroxide (10 N) to a value of 4.2. Of this solution, 1600 ml were mixed with 400 ml of acetonitrile. The final solution was degassed and filtered. A Phenomenex Luna C₁₈ (250 mm x 4.6 mm) column with a particle size of 5 µm was used at a detection wavelength of 254 nm. The flow rate was set at 2 ml per min and the injection volume was 20 µl.

For the solubility and dissolution studies, stock solutions were prepared for sulfadoxine and pyrimethamine from which dilutions were made to construct standard curves. Approximately 50 mg of sulfadoxine was weighed off accurately and transferred to a 50 ml volumetric flask to which 20 ml acetonitrile was added. This sulfadoxine stock solution (S_{stock}) was then sonicated for 10-20 min. In the same way, approximately 25 mg of pyrimethamine was weighed off and transferred to a 100 ml volumetric flask to which 35 ml acetonitrile were added. This pyrimethamine stock solution (P_{stock}) was then sonicated for 10-20 min. After sonication both stock solutions were allowed to cool to room temperature after which mobile phase was used to fill the solutions to volume. For the standard curves, dilutions were prepared using mobile phase as diluent.

For the permeability studies, stock solutions approximately 200 mg of sulfadoxine was weighed off and transferred to a 100 ml volumetric flask to which 40 ml acetonitrile was added. This sulfadoxine stock solution (S_{stock}) was then sonicated for 10-20 minutes. In the same

way, approximately 80 mg pyrimethamine was weighed off and transferred to a 100 ml volumetric flask to which 35 ml acetonitrile was added. This pyrimethamine stock solution (P_{stock}) was then sonicated for 10-20 minutes. After sonication both stock solutions were allowed to cool to room temperature after which mobile phase was used to fill the solutions to volume. The necessary dilutions were prepared using mobile phase as diluent to produce of two standard solutions (one for each drug). For the standard curve each standard solution was filtered, placed in a vial and analysed on the HPLC, increasing injection volumes (10 – 120 μl).

3.9 References

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The results obtained from the solubility studies of the sulfadoxine and pyrimethamine as single compounds and in different combination ratios are depicted and discussed in this chapter. The physico-chemical characterization of the individual compounds and the combinations were interpreted to identify potential interactions between the compounds in the different combinations that may be used to explain the solubility results in more detail. The results obtained from the dissolution studies were discussed with reference to the solubility results and were also used to indicate potential effects on membrane permeation. The *in vitro* permeability are also presented and discussed as solubility enhancement effects of combinations between sulfadoxine and pyrimethamine may impact on their pharmacokinetic properties.

4.2 Solubility studies

4.2.1 Solubility determination of various combinations

Solubility studies were performed on sulfadoxine and pyrimethamine as single compounds and in different concentration ratios in water as solvent. Table 4.1 displays a summary of the average concentrations obtained for each single compound and each combination tested.

Table 4.1: Average solubility of sulfadoxine and pyrimethamine as single compounds and in different combination ratios in water

Compound / Combination	Average drug concentration ($\mu\text{g/ml} \pm \text{SD}$)	
	Sulfadoxine	Pyrimethamine
Single compound	269.12 $\pm$ 10.39	29.02 $\pm$ 2.69
1:1 weight	502.54 $\pm$ 10.58	269.68 $\pm$ 4.49
1:1 mol	500.67 $\pm$ 0.62	275.13 $\pm$ 0.99
500:25 weight Combination	490.93 $\pm$ 1.20	258.62 $\pm$ 3.22
60:40 weight	502.05 $\pm$ 10.87	252.16 $\pm$ 3.87
40:60 weight	505.38 $\pm$ 13.15	249.71 $\pm$ 4.63
70:30 weight	473.68 $\pm$ 18.66	250.37 $\pm$ 6.17
30:70 weight	481.43 $\pm$ 23.43	255.85 $\pm$ 2.77

From Table 4.1 it is clear that for both drugs, higher average solubility values were obtained in the different combinations when compared to that of the single compounds. For pyrimethamine the highest solubility was obtained for the 1:1 mol combination, while the 40:60 weight combination produced the highest solubility value for sulfadoxine, albeit similar to its solubility in the 1:1 weight and 1:1 mol combinations. For sulfadoxine, a 1.9-fold increase in solubility was obtained for the 1:1 weight, 1:1 mol, 60:40 and 40:60 combination ratios. A pronounced 9.5-fold increase in the solubility of pyrimethamine was obtained for the 1:1 mol combination ratio and an 9.3-fold increase for the 1:1 weight combination ratio. It is therefore clear that pyrimethamine and sulfadoxine improves each other's solubility in combination. This might indicate a possible interaction between these two drugs. Differential scanning calorimetry (DSC) results (see section 4.4.1) displayed a single endotherm for the 1:1 mol and 1:1 weight combinations, which is indicative of a eutectic mixture that formed between sulfadoxine and pyrimethamine in these particular combinations. For this reason, the 1:1 weight combination ratio were further tested in various solvents to establish whether this enhanced solubility phenomenon can occur in other media than water as well.

4.2.2 Solubility determination in different solvents

4.2.2.1 Solubility in water (single compounds and 1:1 weight ratio)

The solubilities (i.e. concentrations in $\mu\text{g/ml}$) obtained for sulfadoxine as single compound and in a 1:1 weight ratio for four replicates are presented in Figure 4.1 and in Figure 4.2 for pyrimethamine.

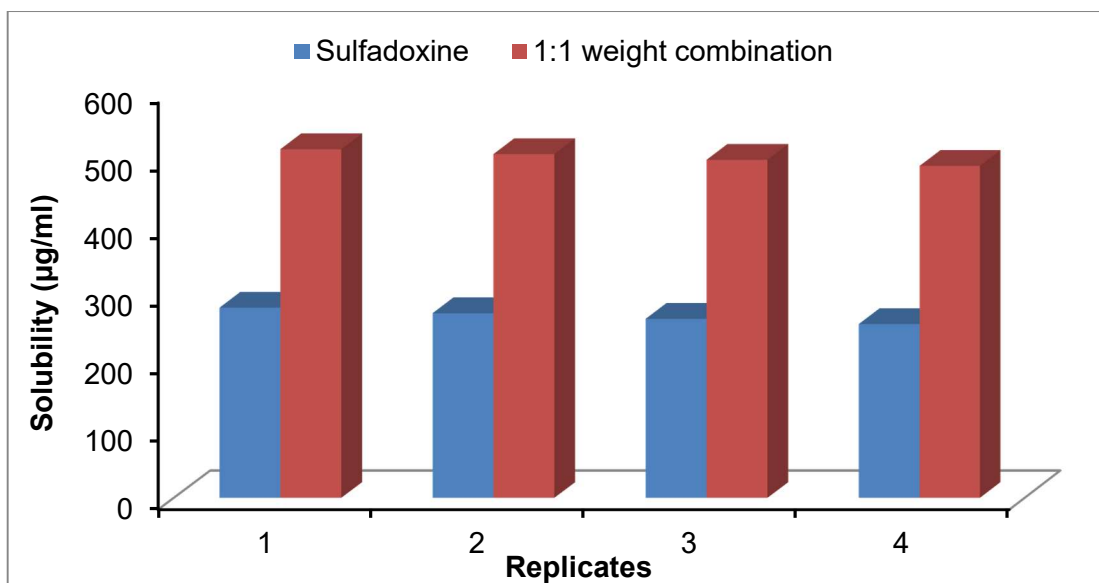


Figure 4.1: Graphical presentation of the concentrations obtained for sulfadoxine in water as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine

It is clear from Figure 4.1 that the solubility of sulfadoxine is almost doubled when combined with pyrimethamine in a 1:1 weight ratio. The repeatability of this solubility enhancement effect is indicated by the relatively low standard deviation ($SD = 10.58 \mu\text{g/ml}$) of the concentrations obtained for the four replicates.

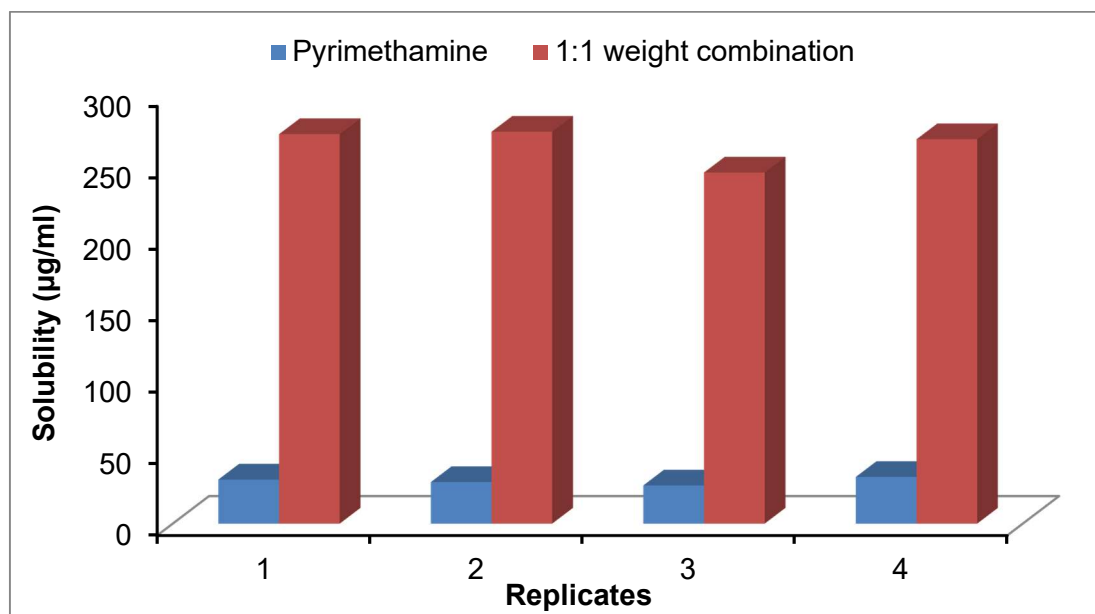


Figure 4.2: Graphical presentation of concentrations obtained for pyrimethamine in water as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine

It is clear from Figure 4.2 that the solubility of pyrimethamine in the 1:1 weight mixture with sulfadoxine mixture was increased almost ten-fold ($SD = 2.52 \mu\text{g/ml}$).

4.2.2.2 Solubility in water (single compounds and 1:1 mol ratio)

The solubility concentrations obtained for the single compounds and for the 1:1 mol ratio (that is ± 103.4 mg sulfadoxine and ± 82.9 mg pyrimethamine) are presented in Figures 4.3 and 4.4.

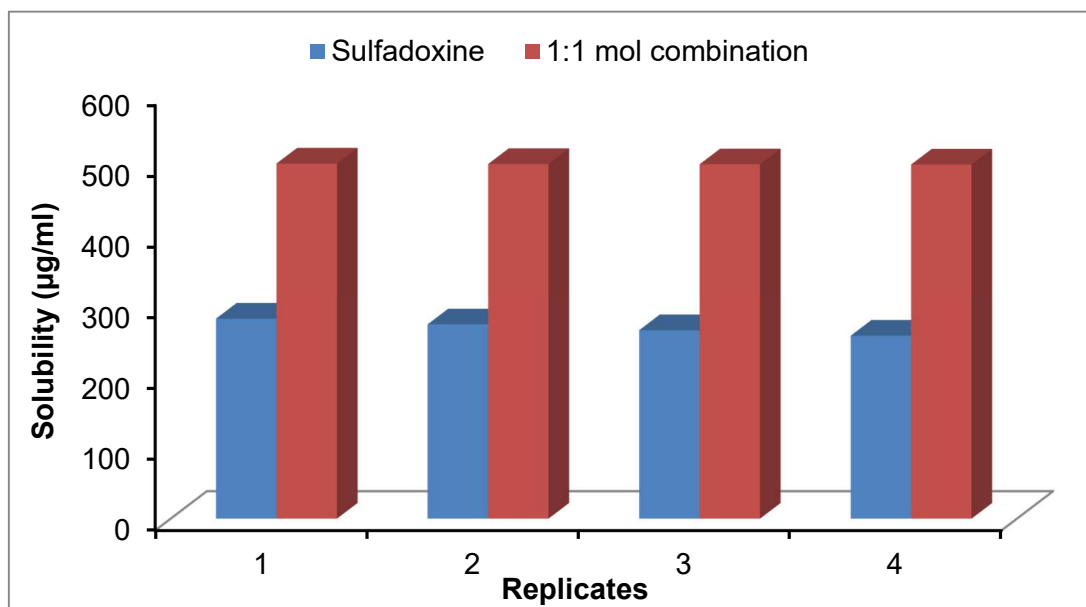


Figure 4.3: Graphical presentation of the concentrations obtained for sulfadoxine in water as single compound and sulfadoxine in a 1:1 mol combination with pyrimethamine

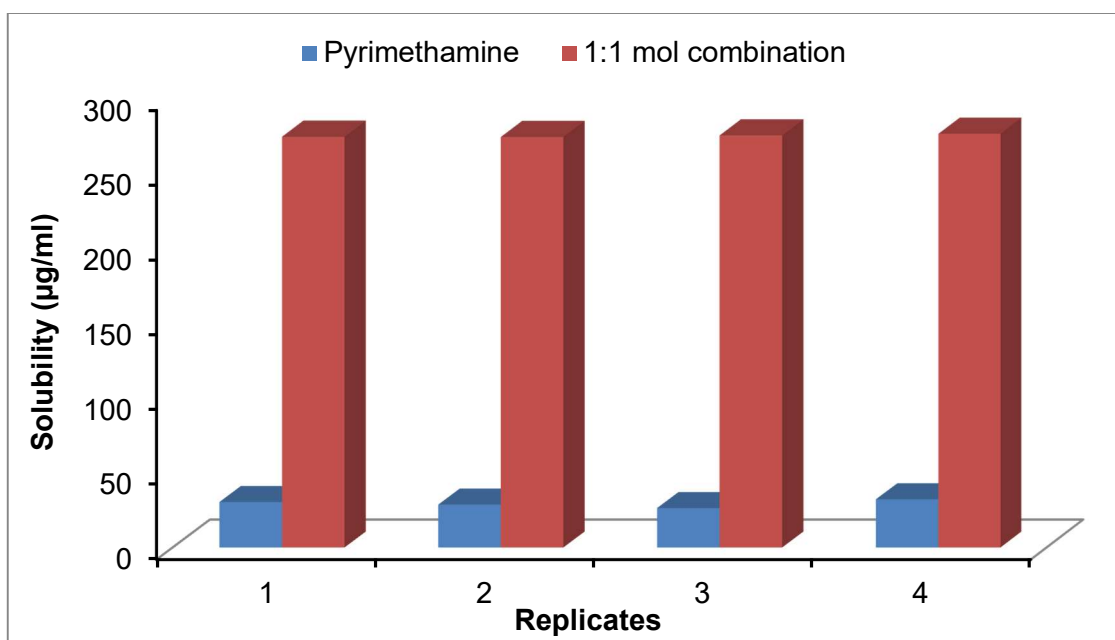


Figure 4.4: Graphical presentation of concentrations obtained for pyrimethamine in water as single compound and pyrimethamine in a 1:1 mol combination with sulfadoxine

The solubility concentration obtained for the 1:1 mol combination ratio in water produced a similar value to that obtained for the 1:1 weight combination. This could be due to the fact that these two combination ratios are relatively close to one another in terms of the weight of each compound used as the molecular weights are in the same range.

4.2.2.3 Solubility in water (single compounds and 500:25 weight ratio)

The reason why the solubility of a 500:25 weight combination between sulfadoxine and pyrimethamine was determined, is that this specific combination is commercially available (Fansidar®) where 500 mg sulfadoxine and 25 mg pyrimethamine is formulated in a single tablet. The concentrations obtained are presented in Figures 4.5 and 4.6.

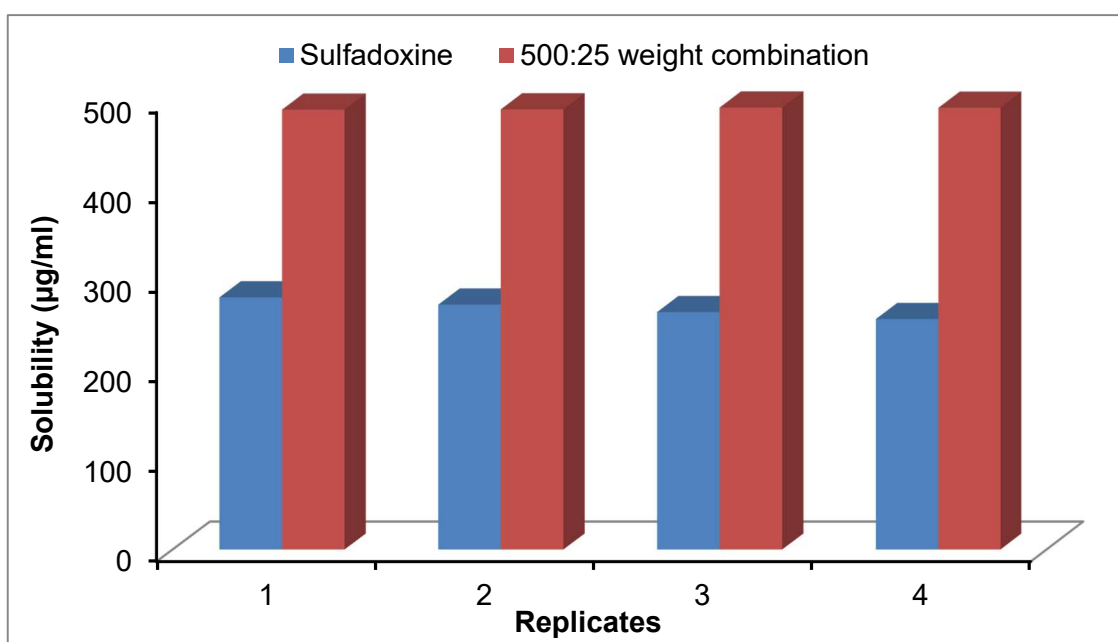


Figure 4.5: Graphical presentation of the concentrations obtained for sulfadoxine in water as single compound and sulfadoxine in a 500:25 weight combination with pyrimethamine

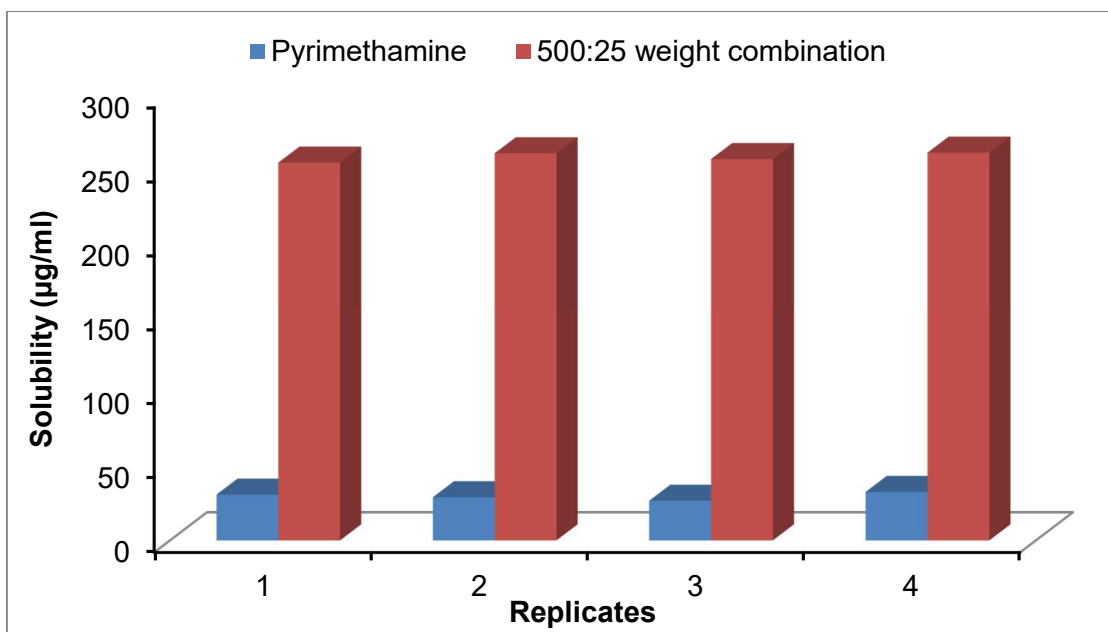


Figure 4.6: Graphical presentation of concentrations obtained for pyrimethamine in water as single compound and pyrimethamine in a 500:25 weight combination with sulfadoxine

The concentrations obtained for the 500:25 weight combination also exhibited a pronounced increase in solubility compared to the single compounds. This correlates well with the results obtained for the 1:1 weight and 1:1 mol combinations.

4.2.2.4 Solubility in 0.1 N HCl (single compounds and 1:1 weight ratio)

The solubility results obtained for the single compounds and 1:1 weight combination in 0.1 N HCl are displayed in Figures 4.7 and 4.8.

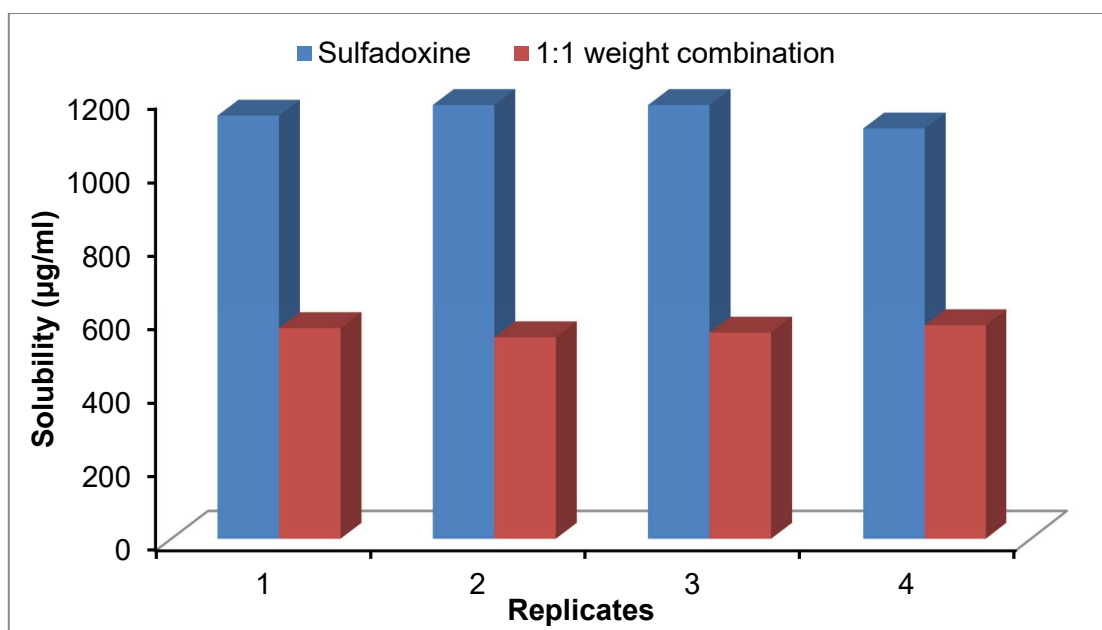


Figure 4.7: Graphical presentation of the concentrations obtained for sulfadoxine in 0.1 N HCl as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine

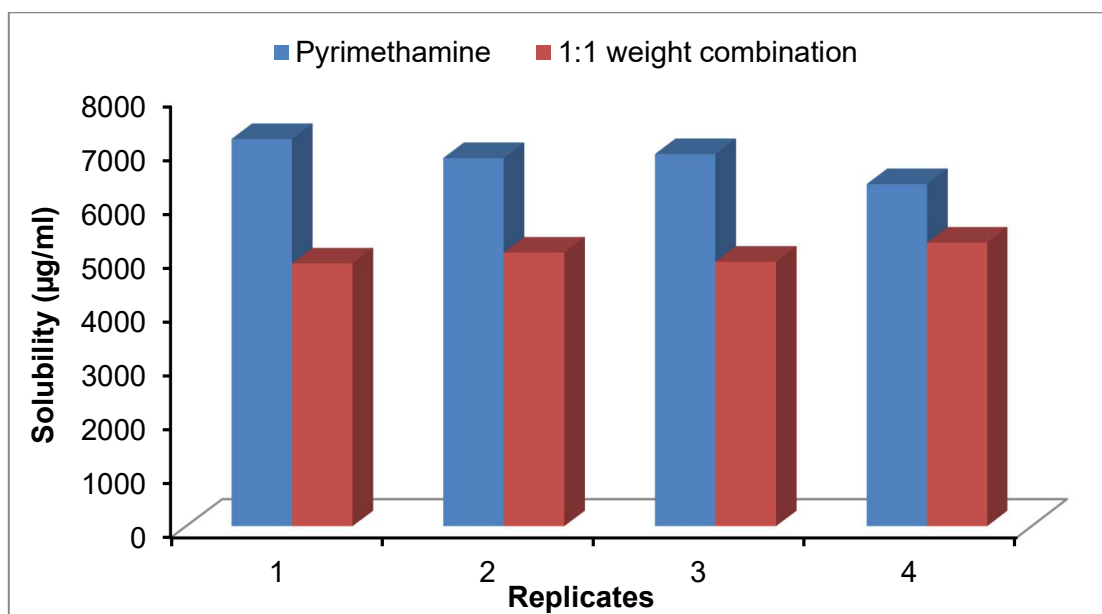


Figure 4.8: Graphical presentation of concentrations obtained for pyrimethamine in 0.1 N HCl as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine

It is clear from Figures 4.1 – 4.8 that the solubility of the single compounds (sulfadoxine and pyrimethamine) was substantially higher in 0.1 N HCl when compared to that in water. The average concentration obtained for sulfadoxine as single compound was 1154.93 ± 30.13 µg/ml and for sulfadoxine in 1:1 weight combination with pyrimethamine is 562.71 ± 14.22 µg/ml. For pyrimethamine single compound and pyrimethamine in 1:1 weight combination with sulfadoxine, the average concentrations obtained in 0.1 N HCl were

6 825.65 ± 347.01 µg/ml and 5 041.54 ± 177.60 µg/ml, respectively. It is interesting to note that a 1:1 weight combination of sulfadoxine and pyrimethamine exhibited a lower solubility in 0.1 N HCl in comparison to the single compounds. The increased solubility of the single compounds in a medium with an acidic pH might be attributed to the ability of the compounds to act as proton acceptors in an acidic environment. Pyrimethamine is a weak base and will therefore exhibit an increase in solubility with a decrease in pH. The possibility exists that charge transfer occurs between the amino and/or sulfa groups of these two compounds. Because sulfadoxine dissolves in both acidic and alkaline environments, it acts as an ampholyte (Martin, 1993:149). In some instances sulfadoxine acts as an acid (donating protons) and in other as a base (accepting protons), influencing the intermolecular interaction between sulfadoxine and pyrimethamine. This may increase the solubility of the combination in certain solvents, while in others (in this case HCl) the solubility of both drugs in combination decreases. Furthermore, ionization has a large effect on the aqueous solubility of ionisable compounds (Aulton 2005:27). It seems possible that, in water, sulfadoxine lowers the pH thus leading to the ionisation of pyrimethamine. This ionisation may, at least in part, explain the increase in the solubility observed for pyrimethamine.

4.2.2.5 Solubility in 0.1 N NaOH (single compounds and 1:1 weight ratio)

The solubility results obtained for sulfadoxine and pyrimethamine as single compounds and in 1:1 weight combination in 0.1 N NaOH are displayed in Figures 4.9 and 4.10.

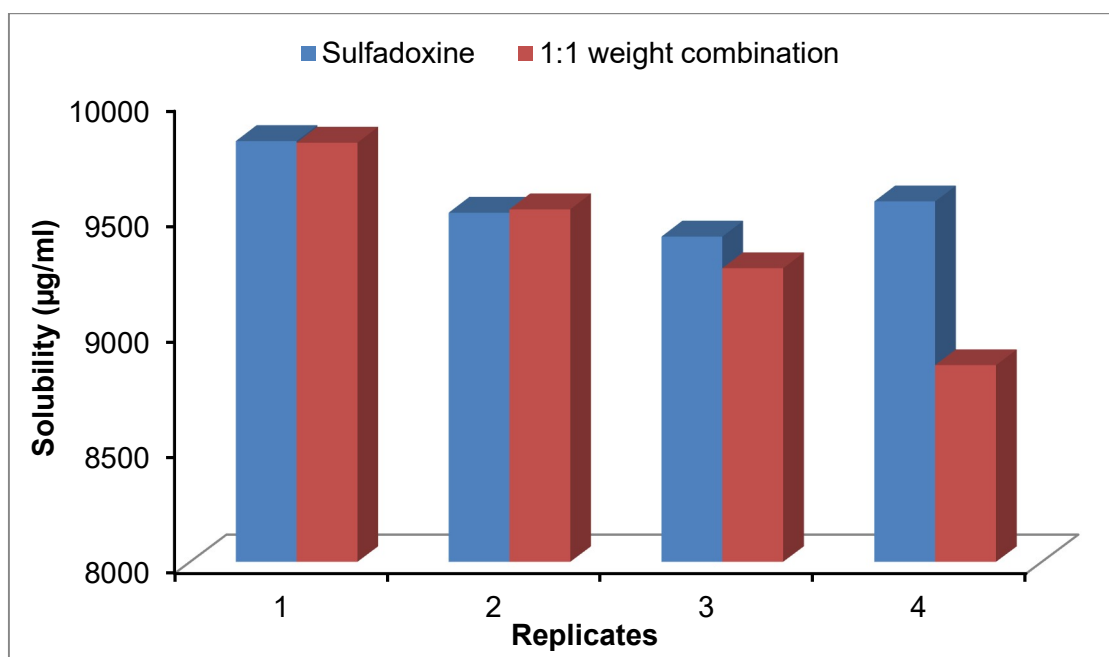


Figure 4.9: Graphical presentation of the concentrations obtained for sulfadoxine in 0.1 N NaOH as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine

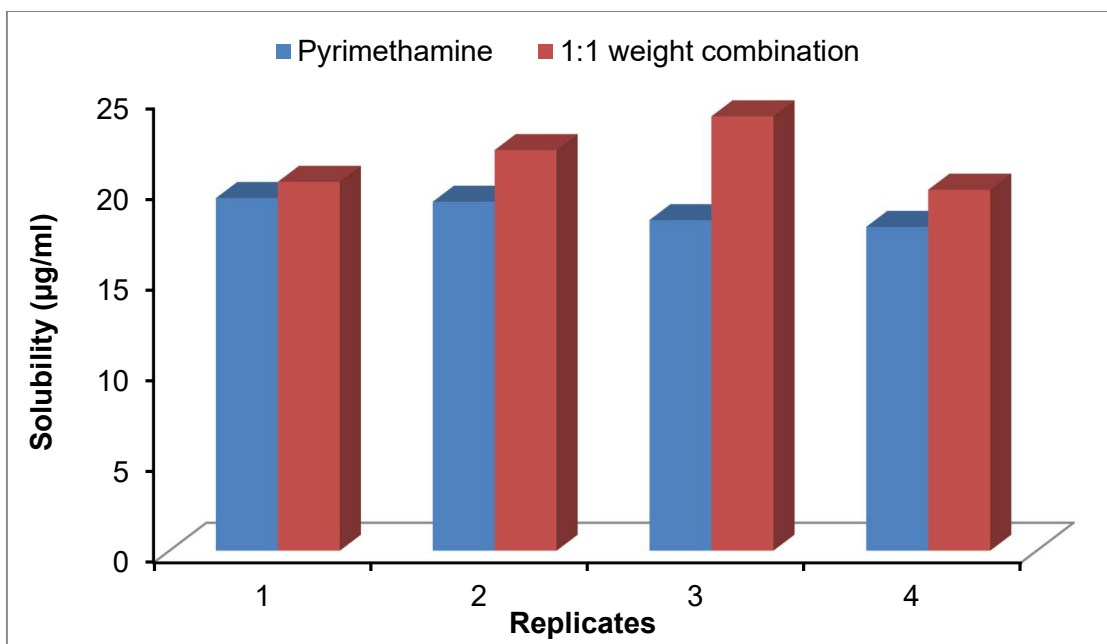


Figure 4.10: Graphical presentation of concentrations obtained for pyrimethamine in 0.1 N NaOH as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine

Sulfadoxine as single compound reached a concentration of $9\,576.10 \pm 174.52$ µg/ml in 0.1 N NaOH, which is substantially higher than the solubility obtained in other solvents such as water and 0.1 N HCl.

The average concentration obtained for pyrimethamine single compound in 0.1 N NaOH was 18.69 ± 0.77 µg/ml, which is substantially lower than the concentration obtained in water and HCl. Pyrimethamine is a weak base and therefore exhibits higher solubility in an environment with an acidic pH compared to an environment with an alkaline pH, explaining why pyrimethamine exhibited higher solubility in 0.1 N HCl.

The average concentrations obtained for sulfadoxine and pyrimethamine in the 1:1 weight combination were $9\,367.19 \pm 407.18$ µg/ml and 21.56 ± 1.85 µg/ml, respectively. The solubility of sulfadoxine in 1:1 weight combination with pyrimethamine was similar than that obtained for sulfadoxine as single compound, as was the solubility obtained for pyrimethamine in 1:1 weight combination with sulfadoxine compared to that obtained for pyrimethamine as single compound.

4.2.2.6 Solubility in ethanol (single compounds and 1:1 weight ratio)

The solubility results obtained for the single compounds and 1:1 weight combination in ethanol are displayed in Figures 4.11 and 4.12.

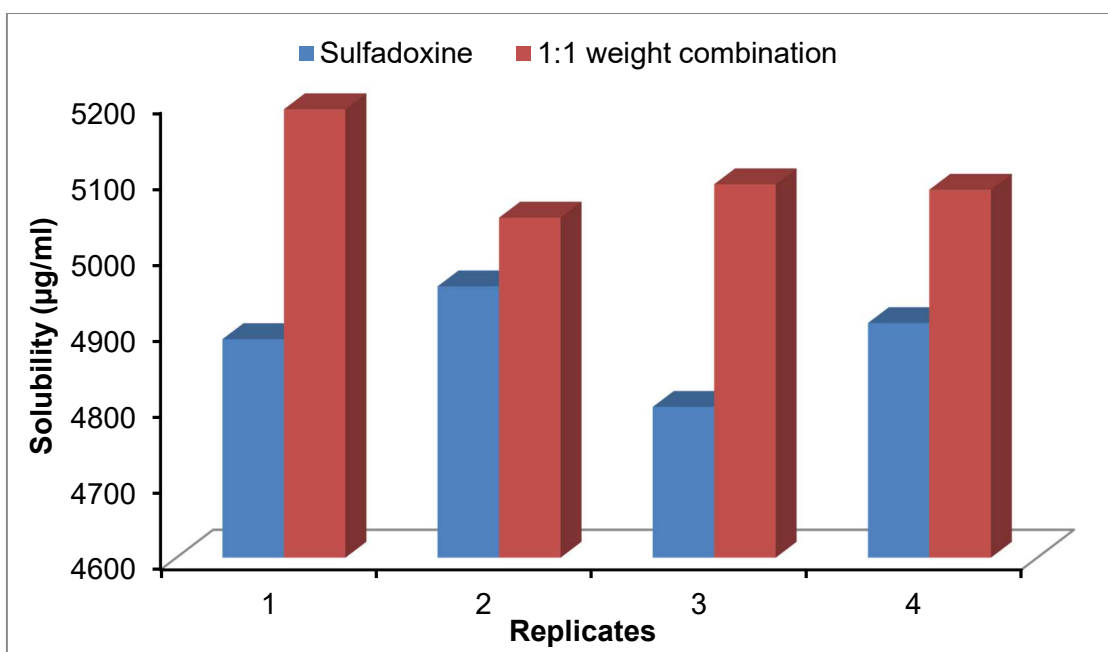


Figure 4.11: Graphical presentation of the concentrations obtained for sulfadoxine in ethanol as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine

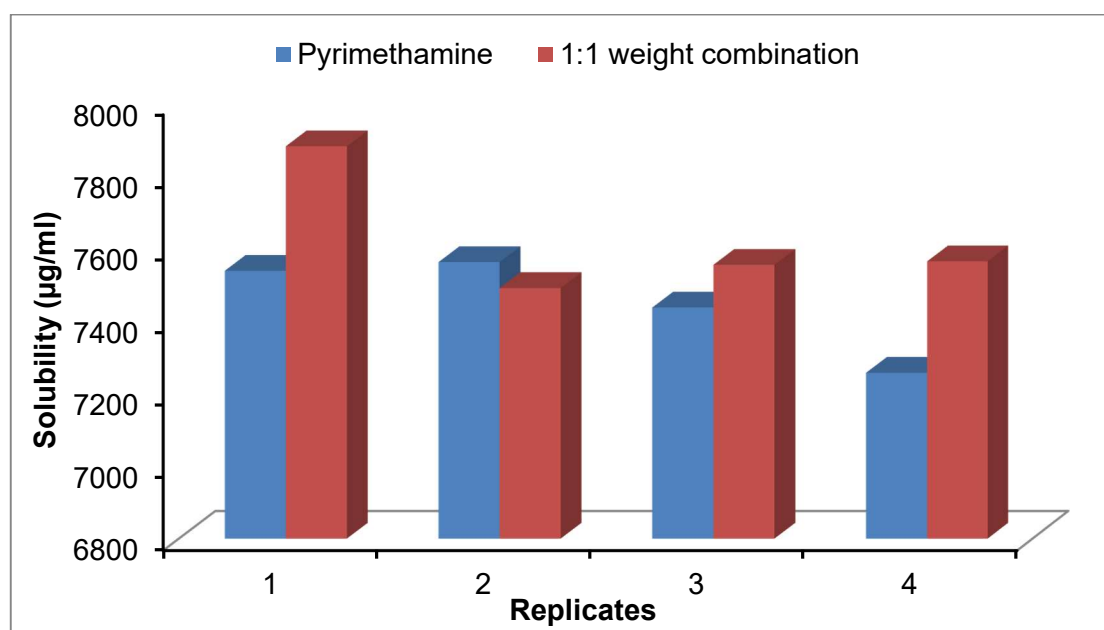


Figure 4.12: Graphical presentation of concentrations obtained for pyrimethamine in ethanol as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine

The solubility concentrations obtained for sulfadoxine single compound and sulfadoxine in 1:1 weight combination with pyrimethamine were $4887.49 \pm 66.37 \mu\text{g/ml}$ and $5102.72 \pm 60.94 \mu\text{g/ml}$ respectively, producing a slight increase in the solubility for sulfadoxine in combination. This proves that the solubility is enhanced in combination but to a lesser extent than achieved in water.

For pyrimethamine single compound and pyrimethamine in 1:1 weight combination with sulfadoxine, the concentrations were $7\,448.19 \pm 138.61 \mu\text{g/ml}$ and $7\,622.28 \pm 175.44 \mu\text{g/ml}$ respectively, also producing a slight increase in solubility for pyrimethamine in combination.

The improved solubility of both drugs in ethanol in comparison to water might be related to the polarity of the solvents. To investigate the possible role of the polarity on the enhanced solubility of both drugs, methanol was used as solvent and the solubility of the single compounds and the 1:1 weight combination were determined. Ethanol is less polar than methanol, which in turn is less polar than water (Martin, 1993:225).

4.2.2.7 Solubility in methanol (single compounds and 1:1 weight ratio)

The solubility results obtained for sulfadoxine and pyrimethamine as single compounds and the 1:1 weight combination in methanol are displayed in Figures 4.13 and 4.14.

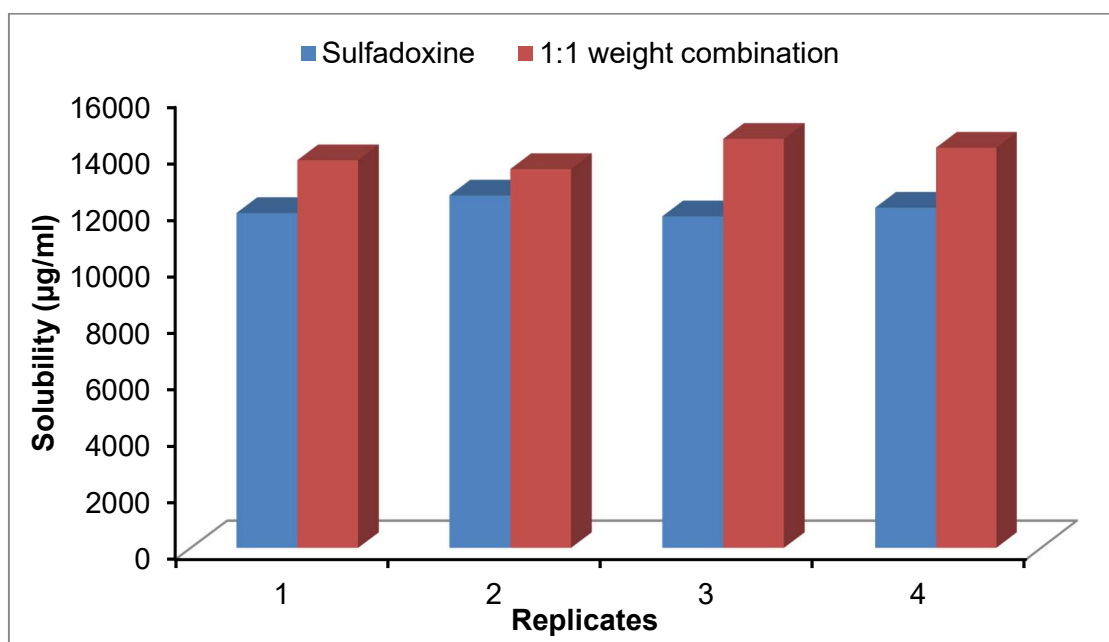


Figure 4.13: Graphical presentation of the concentrations obtained for sulfadoxine in methanol as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine

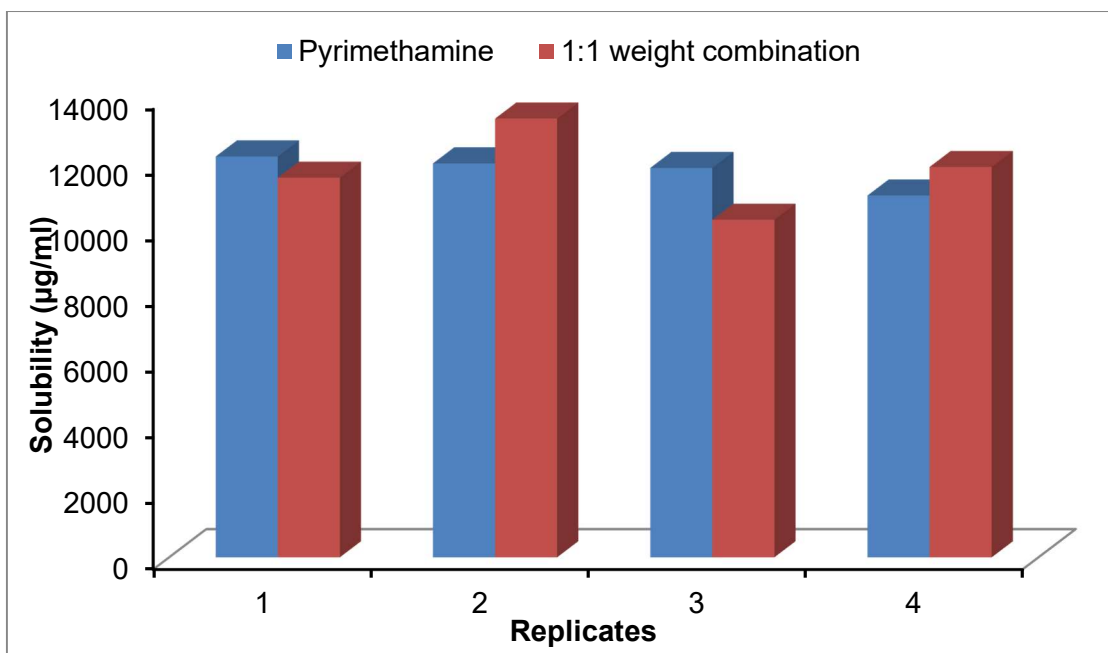


Figure 4.14: Graphical presentation of concentrations obtained for pyrimethamine in methanol as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine

The concentrations obtained for sulfadoxine single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine were $12\,029.25 \pm 322.33 \mu\text{g/ml}$ and $13\,940.36 \pm 471.67 \mu\text{g/ml}$ respectively. Sulfadoxine in combination produced solubility results of almost $2\,000 \mu\text{g/ml}$ higher than the single compound. This was not the case with pyrimethamine however, as the average concentration obtained for the single compound was $11\,758.53 \pm 520.89 \mu\text{g/ml}$ and for pyrimethamine in 1:1 weight combination with sulfadoxine $11\,760.73 \pm 1\,265.52 \mu\text{g/ml}$.

The results for water, ethanol and methanol showed an increase in solubility of the combination in comparison to the single compounds, with the exception of pyrimethamine in methanol as solvent. It might be due to the polarity of the solvents and for this reason solubility testing was performed in octanol, a non-polar solvent.

4.2.2.8 Solubility in octanol (single compounds and 1:1 weight ratio)

The solubility results obtained for sulfadoxine and pyrimethamine as single compounds and in 1:1 weight combination in octanol are displayed in Figures 4.15 and 4.16.

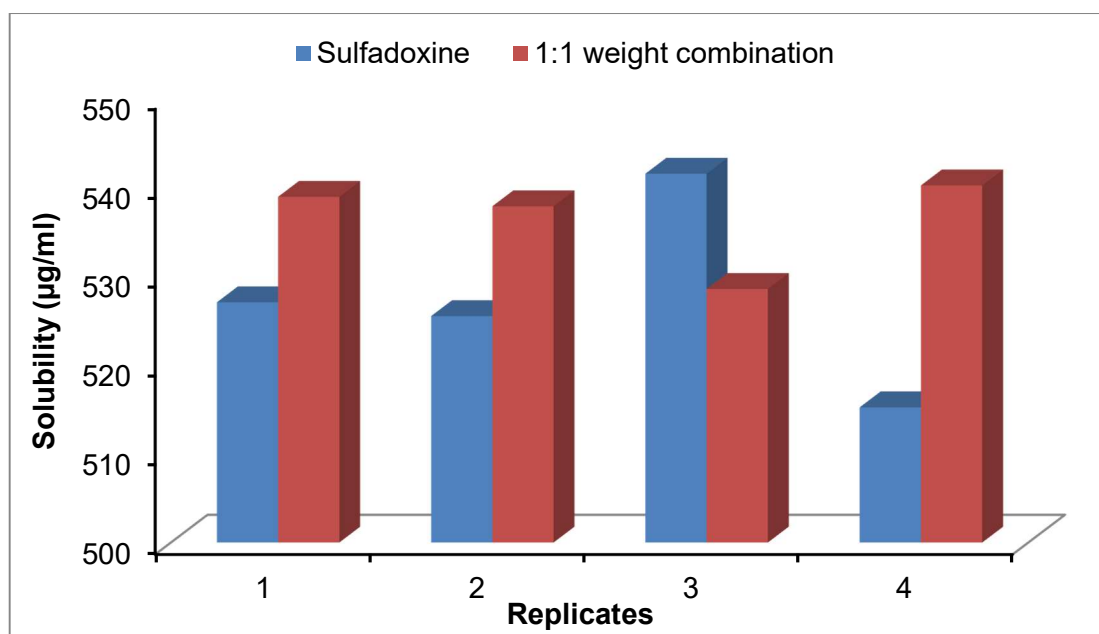


Figure 4.15: Graphical presentation of the concentrations obtained for sulfadoxine in octanol as single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine

The concentrations obtained for sulfadoxine single compound and sulfadoxine in a 1:1 weight combination with pyrimethamine were 527.24 ± 10.80 µg/ml and 536.28 ± 5.29 µg/ml respectively, producing a slight increase in the solubility of sulfadoxine in the combination. For pyrimethamine single compound and pyrimethamine in 1:1 weight combination with sulfadoxine the average concentrations obtained were $3\ 319.95 \pm 376.98$ µg/ml and $2\ 386.24 \pm 693.76$ µg/ml respectively, producing a pronounced decrease in the solubility of pyrimethamine in the 1:1 weight combination.

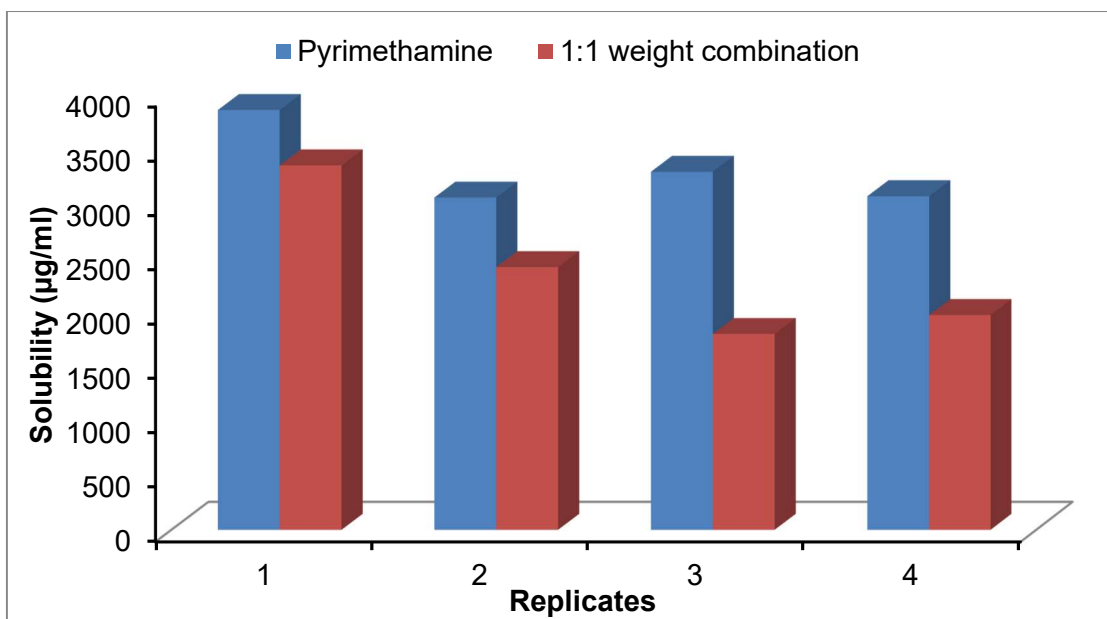


Figure 4.16: Graphical presentation of concentrations obtained for pyrimethamine in octanol as single compound and pyrimethamine in a 1:1 weight combination with sulfadoxine

It is clear that the mutual solubility enhancement trend is observed in polar solvents, while a decrease is observed in non-polar solvents with regards to pyrimethamine in the 1:1 weight combination. From the results obtained during this study, it seems that the enhancement in solubility is most significant in water as both drugs produced improved solubility results in combination in comparison with the single compounds. For the purpose of this study dissolution testing was performed in water as solvent.

4.3 Dissolution

Dissolution studies were performed on sulfadoxine and pyrimethamine as single compounds and in different combinations to establish what the solubility enhancing effect was on the dissolution profiles of each compound. In each case, the same amount of single compound was used as for every combination (see section 3.5.1 in Chapter 3). The dissolution medium was water and a volume of 1000 ml was used in each vessel.

4.3.1 Powder dissolution

4.3.1.1 Powder dissolution (500:25 weight ratio)

The dissolution profiles of sulfadoxine and pyrimethamine as single compounds and in combination (500:25 weight) are displayed in Figures 4.17 and 4.18, respectively.

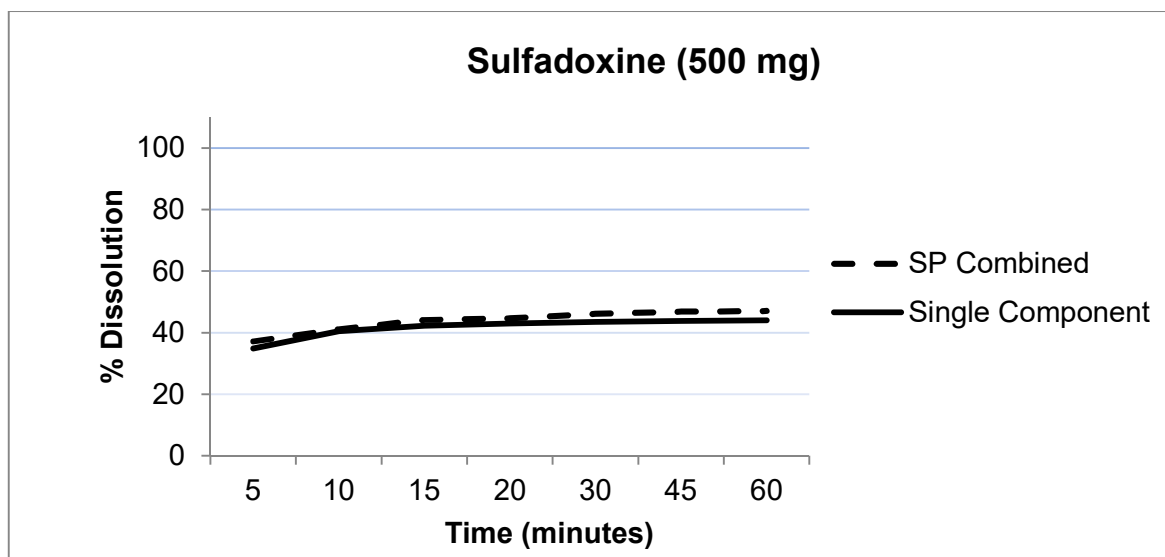


Figure 4.17: Dissolution profile of sulfadoxine as single compound and in a 500:25 weight combination with pyrimethamine

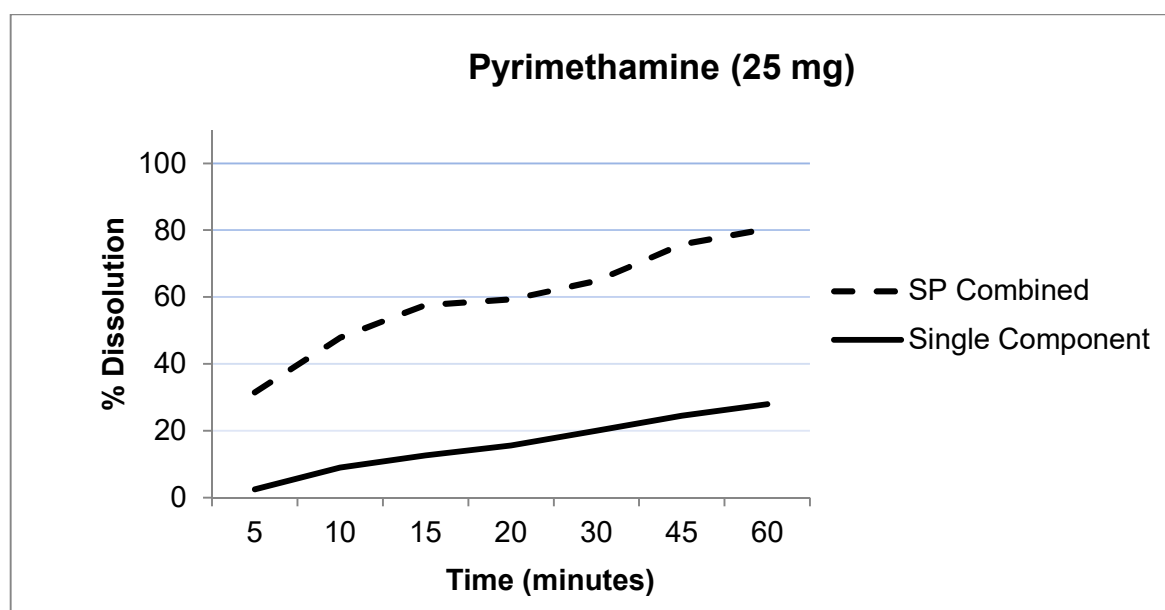


Figure 4.18: Dissolution profile of pyrimethamine as single compound and in a 500:25 weight combination with sulfadoxine

For sulfadoxine single compound and sulfadoxine in a 500:25 combination with pyrimethamine 43.98% and 47.06% dissolution was achieved after 60 min, respectively. There is a slight increase in dissolution with regards to sulfadoxine in the 500:25 combination with pyrimethamine. For pyrimethamine single compound 27.89% dissolution was achieved whilst for pyrimethamine in 500:25 combination with sulfadoxine 80.49% dissolution was achieved, almost three fold that of the pyrimethamine single compound.

4.3.1.2 Powder dissolution (1:1 weight ratio)

The dissolution profiles of sulfadoxine and pyrimethamine as single compounds and in combination (1:1 weight) are displayed in Figures 4.19 and 4.20, respectively.

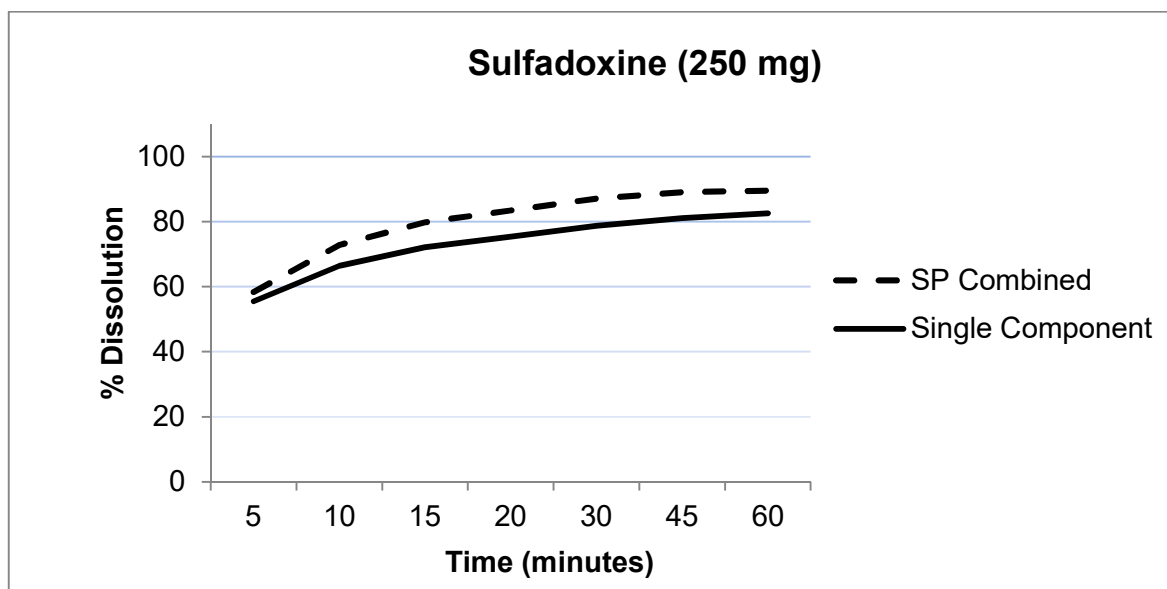


Figure 4.19: Dissolution profile of sulfadoxine as single compound and in a 1:1 weight combination with pyrimethamine

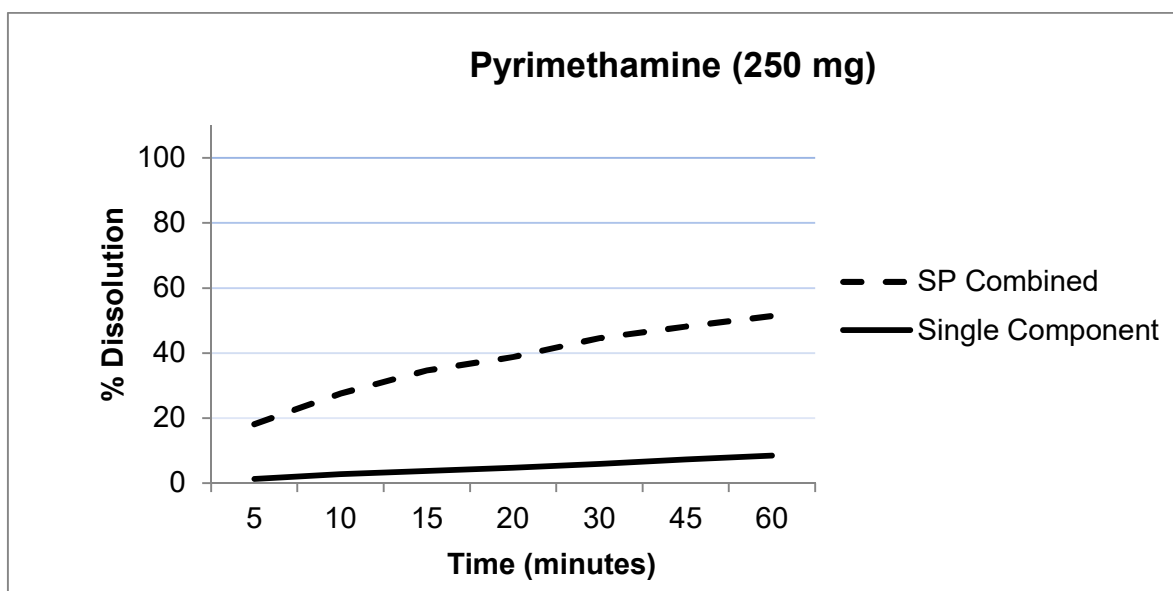


Figure 4.20: Dissolution profile of pyrimethamine as single compound and in a 1:1 weight combination with sulfadoxine

For sulfadoxine single compound 82.52% dissolution was achieved and for sulfadoxine in the 1:1 weight combination with pyrimethamine 89.62% was dissolved after 60 min. Sulfadoxine

in the 1:1 weight combination with pyrimethamine produced an increase in dissolution compared to sulfadoxine single compound. However, the dissolution profile for pyrimethamine (Figure 4.20) displays a larger difference between pyrimethamine single compound (8.47% dissolution) and pyrimethamine in the 1:1 weight combination with sulfadoxine (51.44% dissolution), the latter producing a six-fold increase in dissolution compared to pyrimethamine single compound.

4.3.1.3 Powder dissolution (1:1 mol ratio)

The dissolution profiles of sulfadoxine and pyrimethamine as single compounds and in combination (1:1 mol) are displayed in Figures 4.21 and 4.22, respectively.

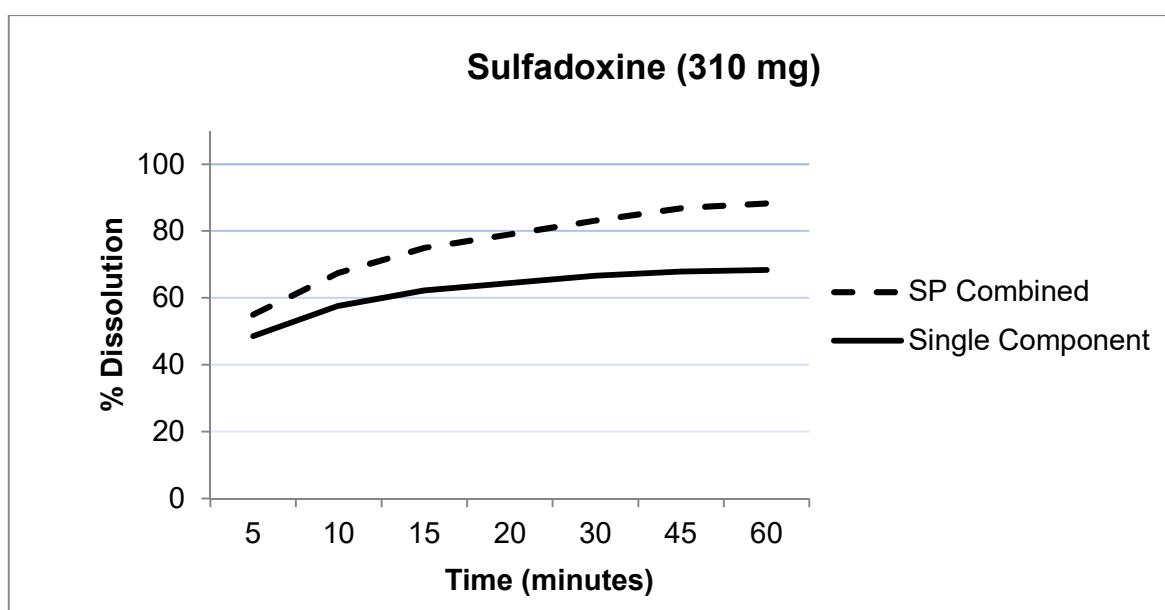


Figure 4.21: Dissolution profile of sulfadoxine as single compound and in a 1:1 mol combination with pyrimethamine

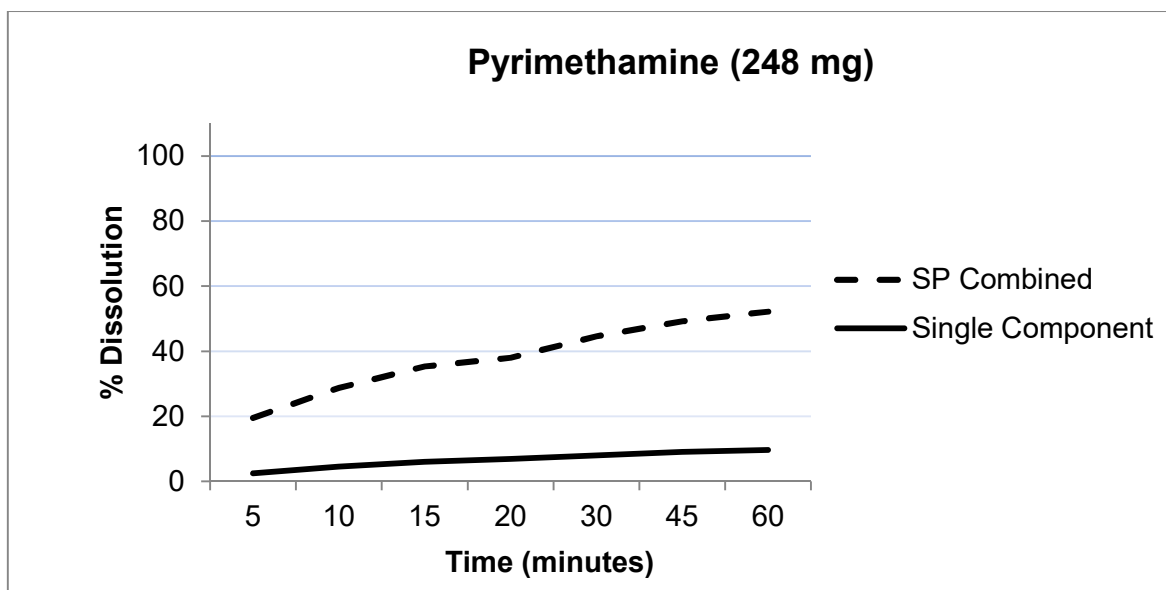


Figure 4.22: Dissolution profile of pyrimethamine as single compound and in a 1:1 mol combination with sulfadoxine

Compared to the other dissolutions, the dissolution profile for sulfadoxine single compound (68.35% dissolution) and sulfadoxine in the 1:1 mol combination with pyrimethamine (88.34% dissolution) in Figure 4.21 displays the most pronounced difference. Sulfadoxine in a 1:1 mol combination with pyrimethamine achieved 20% higher dissolution compared to sulfadoxine single compound. The dissolution profile for pyrimethamine single compound and pyrimethamine in a 1:1 mol combination with sulfadoxine (Figure 4.22) is similar to the profile obtained for the single compound and pyrimethamine in the 1:1 weight combination with sulfadoxine (Figure 4.20). For pyrimethamine single compound 9.61% dissolution was achieved and for pyrimethamine in the 1:1 mol combination with sulfadoxine, 52.25% dissolution was achieved.

It is clear from the dissolution results that when sulfadoxine is combined with pyrimethamine in different ratios, their dissolution rate is increased to different extents. This is in accordance with the solubility results where combinations between sulfadoxine and pyrimethamine resulted in an increase in their solubilities.

4.4 Solid state investigation

As is evident from the solubility as well as the dissolution results, the combination of sulfadoxine and pyrimethamine resulted in an increase in solubility and dissolution. As this might be related to an interaction between these two drugs, the following techniques were employed to characterise the physico-chemical properties of sulfadoxine and pyrimethamine alone and in different combinations:

- Differential Scanning Calorimetry (DSC)
- X-Ray Powder Diffraction (XRPD)
- Nuclear Magnetic Resonance (NMR)
- Isothermal microcalorimetry
- Vapour sorption analysis

4.4.1 Differential Scanning Calorimetry (DSC)

From the solubility results obtained during this study, it was evident that a possible interaction occurred between sulfadoxine and pyrimethamine. For this reason DSC was used to investigate the interaction between the two drugs. Residues were obtained from the solubility study performed on the 1:1 weight and 1:1 mol ratio combinations in water and the results are displayed in Figure 4.23.

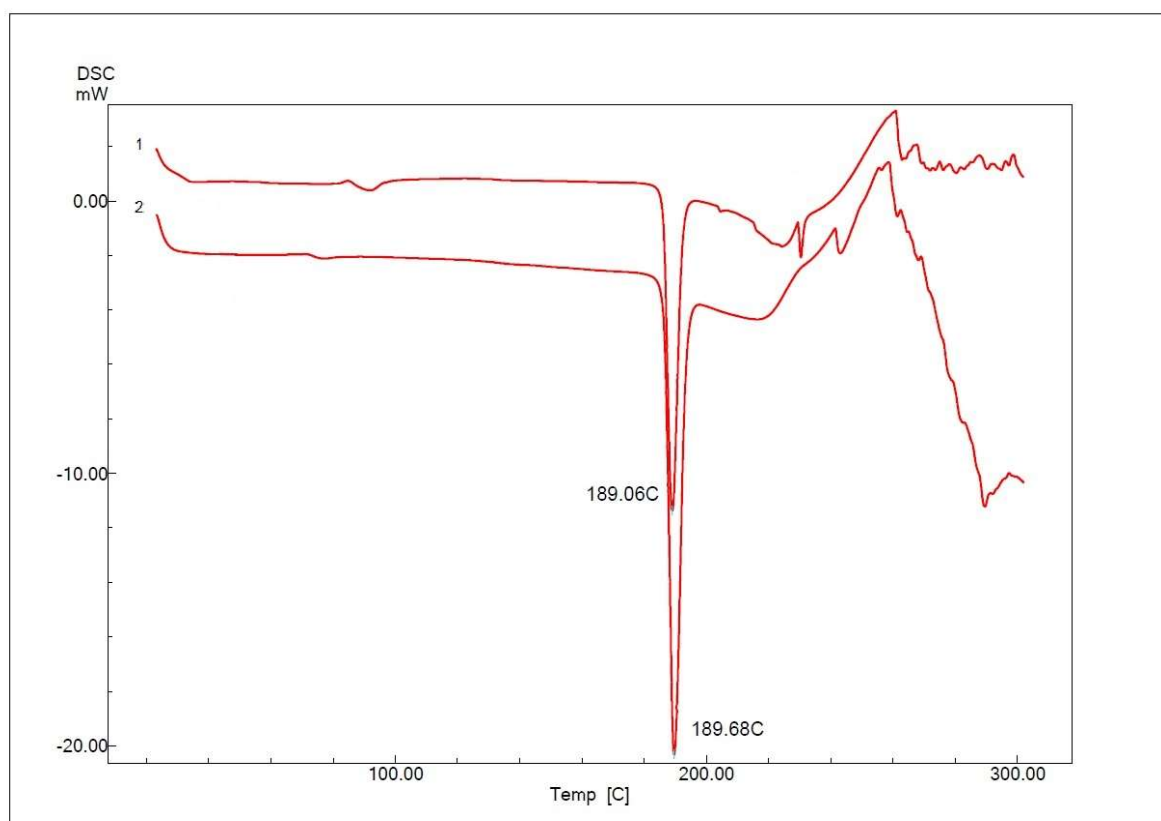


Figure 4.23: Differential scanning calorimetry thermogram for the 1:1 weight ratio combination (1) and the 1:1 mol ratio combination (2) from solubility residues

From the results it is clear that in both cases a single endotherm was obtained. For this reason DSC was used in the characterization of various powder combinations and the single

compound powders. The 1:1 weight and 1:1 mol ratio powder combinations together with that of the single compound powders are displayed in Figure 4.24. The results for all the other combinations (all powder mixtures) are illustrated in appendix 1 (Figure A.1 – A.8).

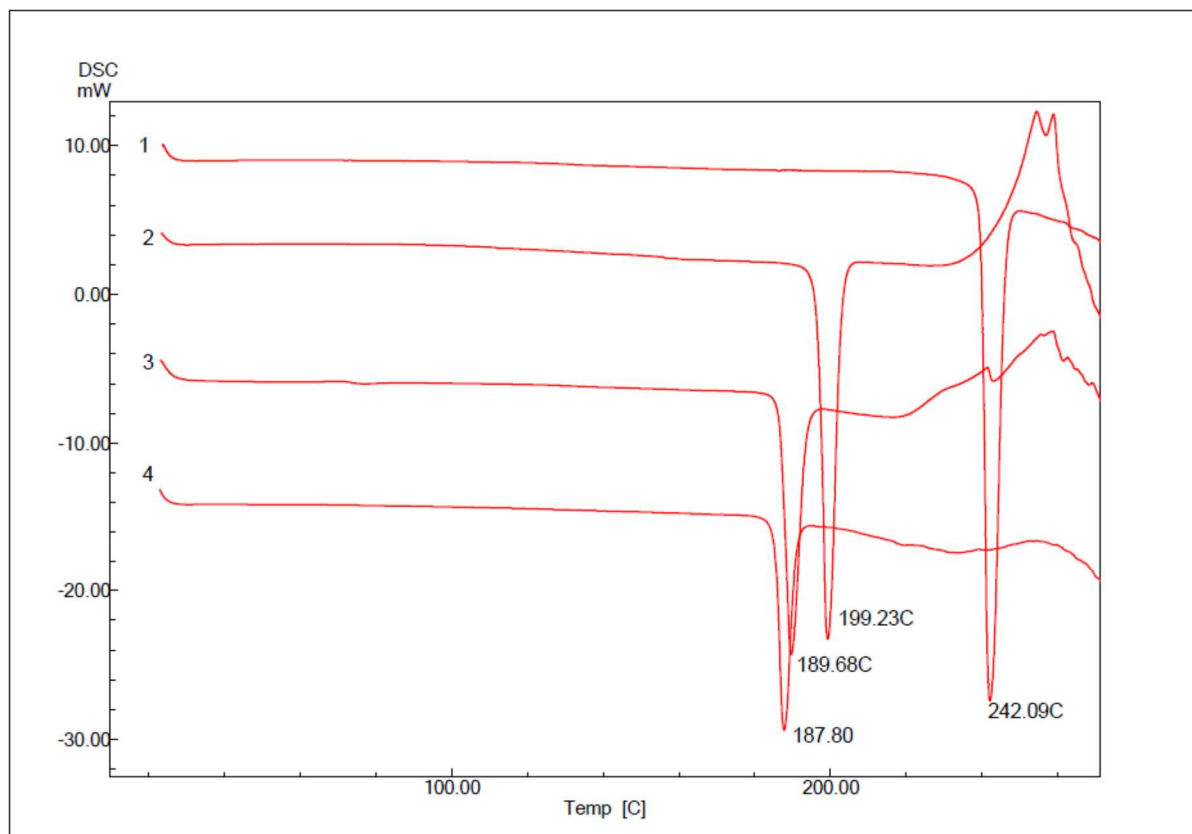


Figure 4.24: Differential scanning calorimetry thermogram for pyrimethamine (1) and sulfadoxine (2), the 1:1 mol ratio combination (3) and the 1:1 weight ratio combination (4)

Both sulfadoxine and pyrimethamine have melting points higher than that of the 1:1 mol and 1:1 weight combinations as shown in Figure 4.24 (sulfadoxine = 199.23°C, pyrimethamine = 242.09°C). The 1:1 mol and 1:1 weight combinations each produced a single endotherm at lower temperatures (1:1 mol combination = 189.68°C, 1:1 weight combination = 187.80°C) as shown in Figure 4.24. This single endotherm is indicative of a eutectic mixture that had formed (Habib *et al.*, 2001:17). From the DSC thermograms it may be indicative that eutectic mixtures were formed in the 1:1 mol and 1:1 weight ratio combinations, but other types of interactions may have occurred in the other ratio combinations. It appears that pyrimethamine dissolved in sulfadoxine during melting as part of the thermal analysis. This is characteristic of a solid solution, a form of solid dispersion where a solid solute (in this case pyrimethamine) dissolves in a solid solvent (i.e. sulfadoxine) (Habib *et al.*, 2001:18). This solid solution occurs spontaneously but the occurrence can be compared to the fusion-solvent method whereby the

carrier (sulfadoxine) is melted and the drug (pyrimethamine) is incorporated in solution form (Habib *et al.*, 2001:24). It is clear from the DSC results that no co-crystals were formed in these binary systems. Upon viewing the data obtained for the other combinations (Figure A.1 – A.8), it is clear that two endotherms were produced by each of these binary mixtures.

4.4.2 X-Ray Powder Diffraction (XRPD)

The samples used for XRPD identification included both sulfadoxine and pyrimethamine single compounds and the 1:1 mol ratio combination of which the XRPD pattern is shown in Figure 4.25. Since the 1:1 weight ratio combination produced similar results to that of the 1:1 mol ratio combination, only the 1:1 mol ratio combination results are presented for comparison with the single compounds.

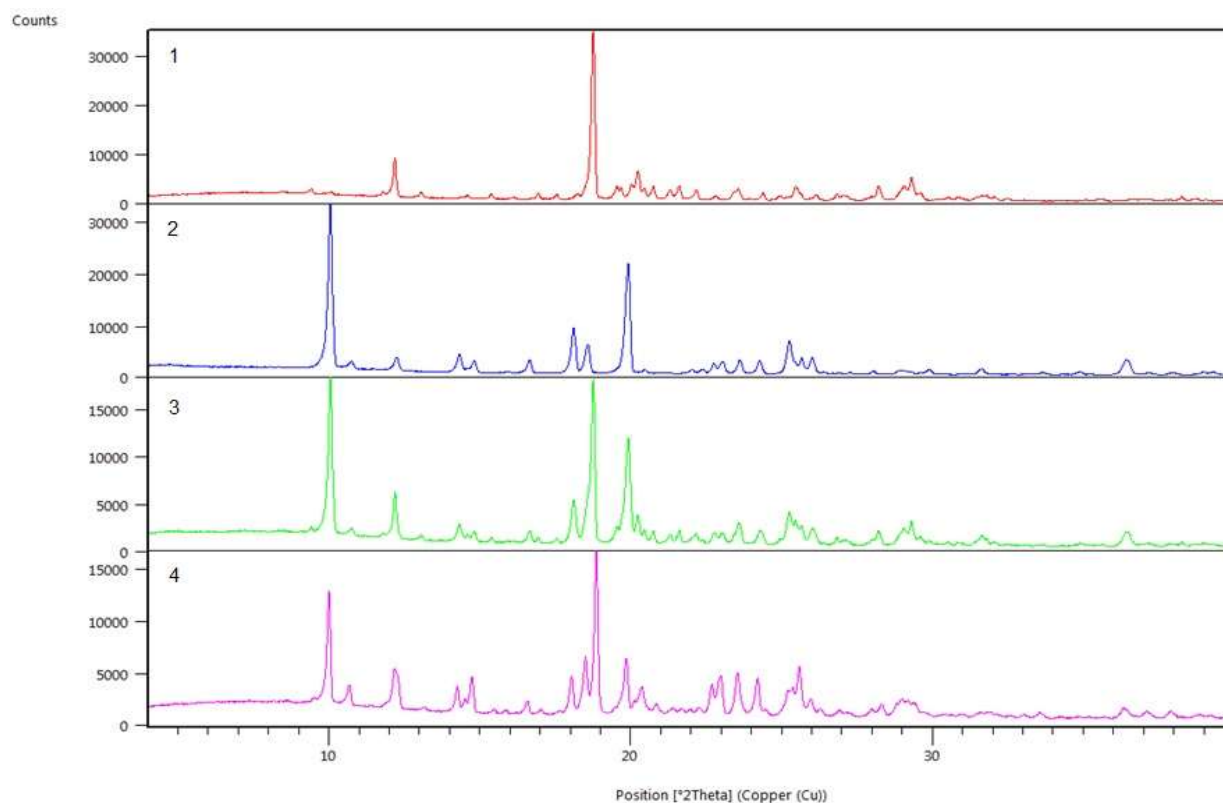


Figure 4.25: X-ray powder diffraction pattern for pyrimethamine (1) and sulfadoxine (2) as single compounds, the predicted combined pattern (3) and the sulfadoxine-pyrimethamine 1:1 mol combination residues after solubility testing (4)

The XRPD pattern confirmed that no co-crystals were formed during solubility testing (Habib *et al.*, 2001:28). Furthermore, the predicted combined pattern and the 1:1 mol combination pattern are very similar to each other. However, negligible displacement of diffraction peaks occurred with the 1:1 mol combination as evident from Figure 4.25.

4.4.3 Nuclear Magnetic Resonance (NMR)

The ^1H -NMR spectra of sulfadoxine and pyrimethamine single compounds as well as the 1:1 mol ratio combination are shown in Figure 4.26. As the 1:1 weight ratio combination produced similar results to that of the 1:1 mol ratio combination, only the 1:1 mol ratio combination results are presented for comparison with the single compounds.

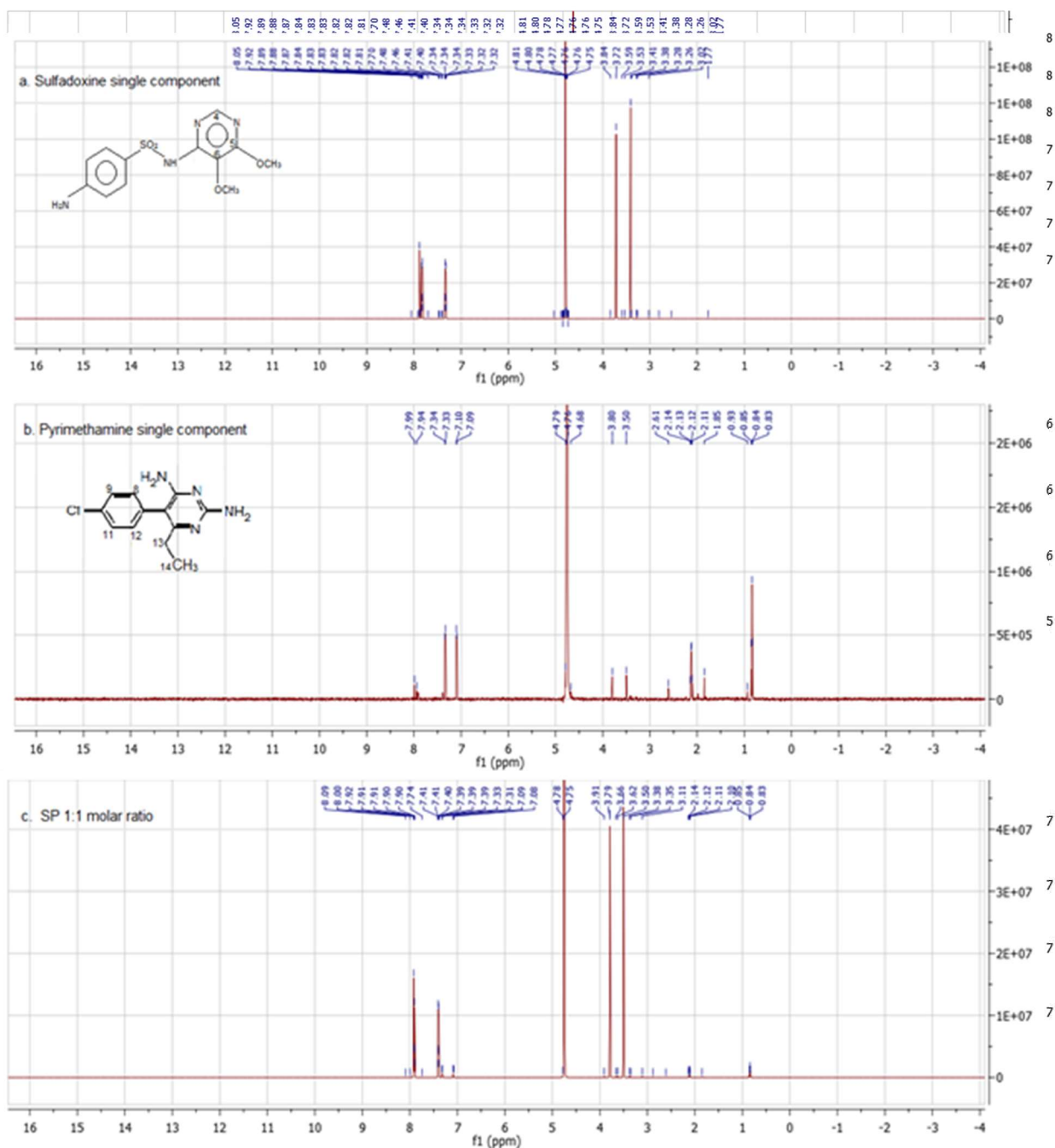


Figure 4.26: ^1H -NMR spectra for sulfadoxine (a), pyrimethamine (b) and 1:1 mol combination (c)

The ^1H -NMR spectra (Figure 4.26) showed that for sulfadoxine the two doublets at $\delta = 7.33 - 7.82$ ppm correspond to the protons in the benzenesulfonamide group ($J = 8.7 - 9.0$ Hz) and the singlets at $\delta = 3.41$ ppm and 3.72 ppm correspond to the protons on the methoxy groups connected at position 5 and 6 on the pyrimidinyl-ring. The singlet at $\delta = 7.88$ ppm corresponds to the proton at position 4 on the pyrimidinyl-ring. For pyrimethamine the two doublets at $\delta = 7.09 - 7.34$ ppm correspond to the protons of the chlorophenyl-ring ($J = 8.3 - 8.7$ Hz), the triplet at $\delta = 0.83 - 0.85$ ppm ($J = 7.5$ Hz) correspond to the CH_3 protons and the quartet at $\delta = 2.11 - 2.14$ ppm ($J = 7.5$ Hz) correspond to the CH_2 protons.

The ^1H -NMR spectrum of sulfadoxine in the sulfadoxine-pyrimethamine combination (1:1 molar) displayed a downfield shift in the spectra. For sulfadoxine the singlets of the protons on the methoxy groups shift to $\delta = 3.50$ ppm and 3.79 ppm respectively and the two doublets of sulfadoxine shift to $\delta = 7.39 - 7.92$ ppm. The shifts observed were, however, relatively small and were only evident in the sulfadoxine part of the NMR spectrum, indicating a possible weak intermolecular interaction between sulfadoxine and pyrimethamine.

4.4.4 Isothermal microcalorimetry

From all the above discussed data it became evident that some sort of interaction between sulfadoxine and pyrimethamine occurs when these two drugs are combined with one another and dissolved in distilled water. However, from DSC and XRPD data no complex formation was identified within the solid-state. For this reason microcalorimetry was used to investigate if an interaction occurs once these drugs are in the presence of water. Microcalorimetry is considered a powerful analytical technique to detect incompatibilities, interactions or instabilities between combinations of drugs or combinations of drugs and excipients. Microcalorimetry is based on the measurement of heat flow. All physical or chemical processes show associated heat flow properties, which can be measured by the microcalorimeter (Schmitt *et al.*, 2001:176). During this part of the study isothermal calorimetry was used. Samples were prepared as described in Chapter 3, section 3.6.4.

The heat flow data obtained for sulfadoxine in combination with distilled water did not show any significant indication of an interaction between the drug and water, although a deviation from the slope of the measured heat flow curve versus the theoretically calculated curve was observed. The interaction integral was determined to be -4.28 J/g and an interaction error of 62.72 $\mu\text{W/g}$. The heat flow data is depicted in Figure 4.27.

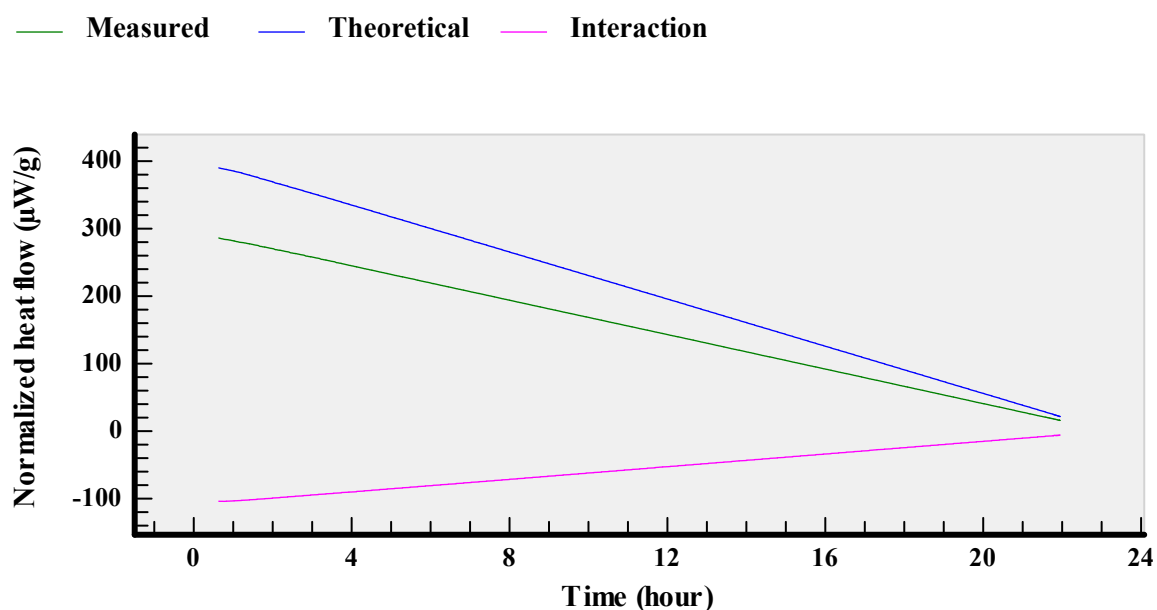


Figure 4.27: Heat flow data obtained with the sample in which sulfadoxine was combined with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.

On the other hand the combination of pyrimethamine with distilled water showed that an interaction between the drug and water does occur, with an interaction integral of -22.21 J/g. The interaction error calculated was also significantly higher at 331.94 $\mu\text{W/g}$. The subsequent heat flow graph is depicted in Figure 4.28.

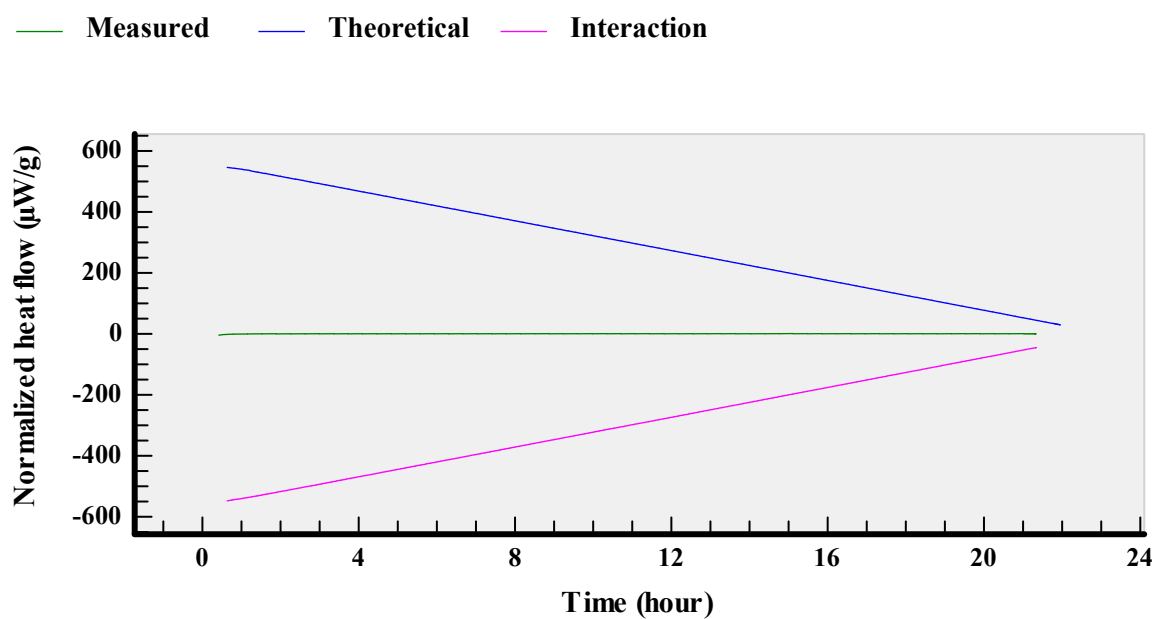


Figure 4.28: Heat flow data obtained with pyrimethamine combined with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.

The possibility of an interaction between water and the 1:1 weight and 1:1 mol combination ratios of sulfadoxine and pyrimethamine were also tested under the same isothermal conditions. The heat flow data of both combinations showed that an interaction between the three components did occur, as depicted in Figures 4.29 and 4.30. In the instance where a 1:1 weight ratio of sulfadoxine and pyrimethamine was tested in combination with distilled water an interaction integral of -22.62 J/g was calculated with a significantly high interaction error of 338.01 $\mu\text{W/g}$. For the interaction of the 1:1 mol ratio of sulfadoxine : pyrimethamine and water an interaction integral of -21.87 J/g and an interaction error of 326.82 $\mu\text{W/g}$ were obtained. The 1:1 mol and 1:1 weight ratio combinations produced similar isothermal microcalorimetric results, probably due to the fact the weights of the individual compounds in these two combinations, are similar.

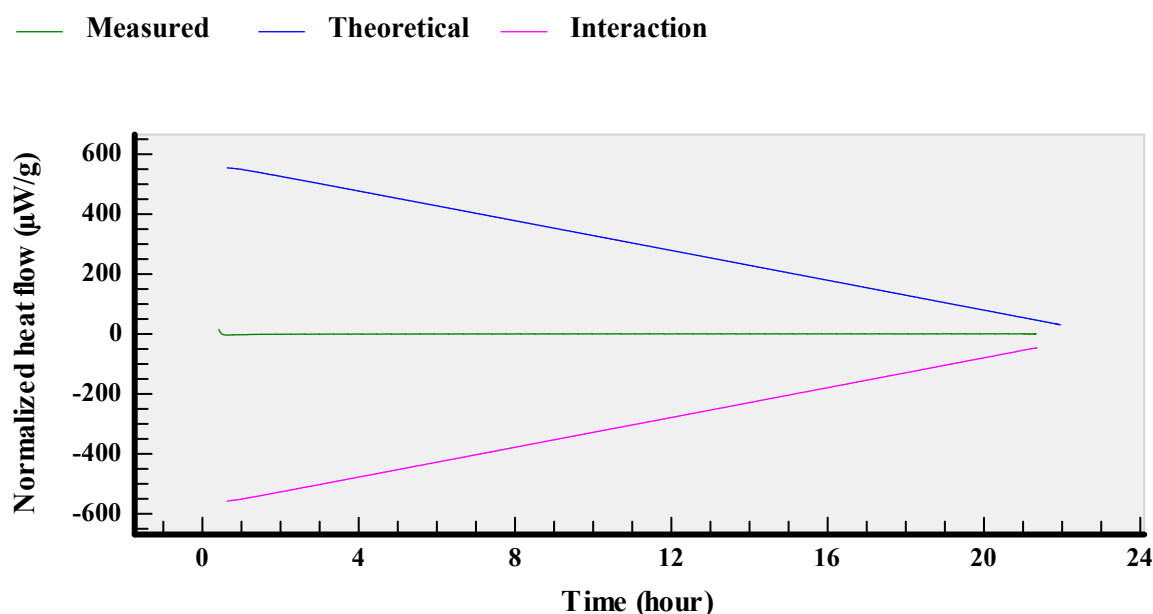


Figure 4.29: Heat flow data obtained with a 1:1 weight combination ratio of sulfadoxine and pyrimethamine with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.

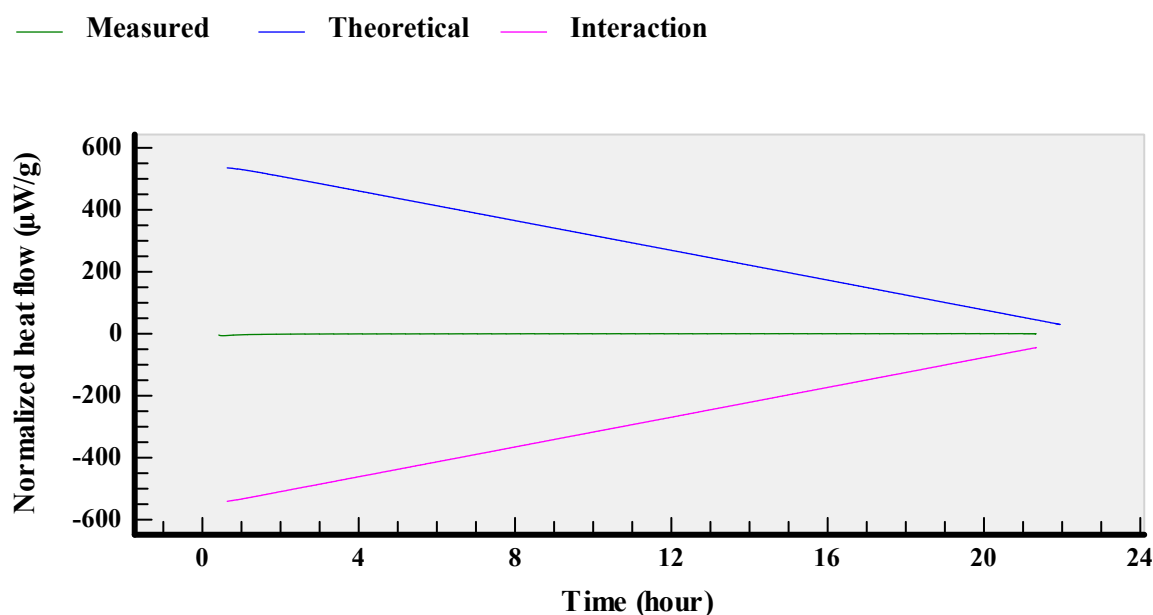


Figure 4.30: Heat flow data obtained with a 1:1 mol combination ratio of sulfadoxine and pyrimethamine with distilled water. The heat flow was measured over a period of 22 hours at an isothermal temperature of 37°C.

4.4.5 Vapour Sorption Analysis

From the isothermal microcalorimetry data it became evident that water has some kind of influence on the behaviour of both drugs and from the data interactions were identified, although the specific interaction or interaction mechanism wasn't identified. This led to the investigation of the behaviour of the single drugs or the combinations, when exposed to high relative humidity. These investigations were done in order to confirm how sensitive these two drugs are in terms of exposure to water or an environment high in water content.

The moisture sorption isotherms obtained with sulfadoxine single compound without drying phase are depicted in Figure 4.31. The initial adsorption isotherm (Adsorption 1 – Figure 4.31) shows a critical humidity (RH_c) point where a sudden weight loss occurred. A weight loss of 0.14% was observed from 50% RH to 70% RH. This was followed by a gradual decrease in weight with a total weight loss of 0.18%.

The desorption phase (95% - 5%) followed an inverse behaviour of that observed during the adsorption phase with an increase in weight up to 75% RH followed by a sudden weight loss up to 70% RH. After 55% RH there was also a sudden decrease in sample mass, followed by a gradual weight loss up to 5% RH. A total moisture loss of 0.07% occurred during the complete desorption phase. After the desorption phase a second adsorption phase followed with almost no weight gain or loss. The second adsorption step followed Type II or III isotherm

behaviour, indicating adsorption on macroporous adsorbent with weak adsorbate-adsorbent interactions (Guillard *et al.*, 2013:3-33). The moisture sorption isotherms of sulfadoxine single compound shows atypical behaviour and further studies must be endeavoured in order to conclude on whether the critical RH points and weight loss is due to some degree of molecular rearrangement or re-organising due to an interaction between sulfadoxine and water.

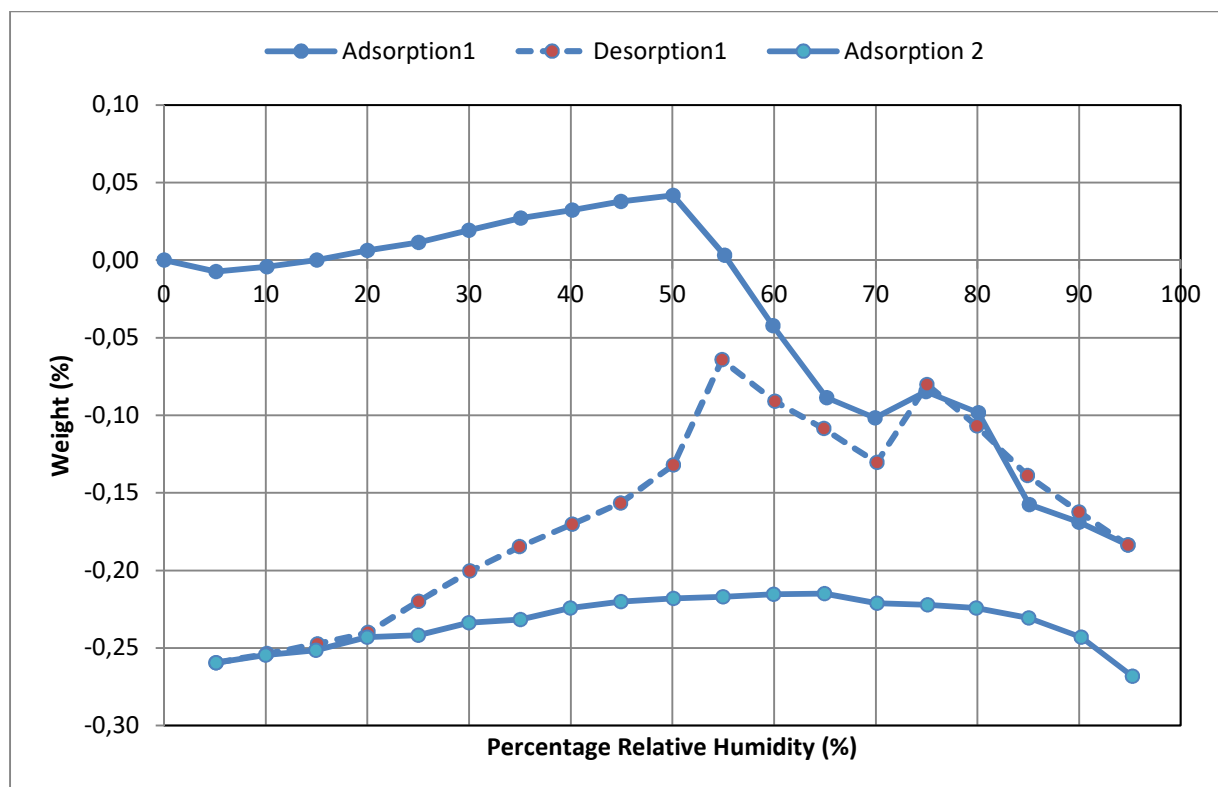


Figure 4.31: Moisture sorption isotherms obtained with sulfadoxine single compound at 25°C, without drying phase

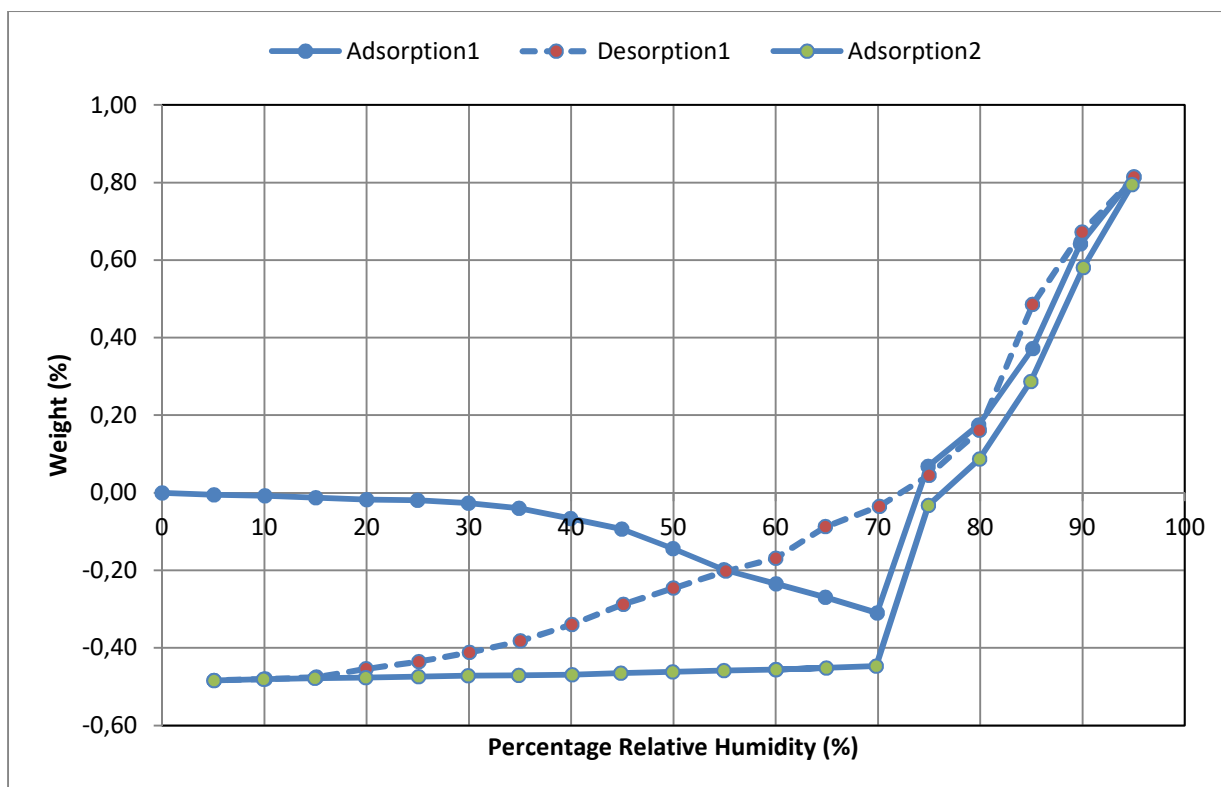


Figure 4.32: Moisture sorption isotherms obtained with pyrimethamine single compound at 25°C

The moisture sorption isotherms obtained for pyrimethamine single compound are depicted in Figure 4.32. The adsorption phase shows a critical RH of 45%, where a mentionable decrease in weight occurred up to 70% RH. A total weight loss of 0.88% occurred from 45 – 70% RH. This was followed by an increase in sample weight from 70 – 95% RH, indicating adsorption of moisture (1.48%). The subsequent desorption of pyrimethamine followed a sigmoidal curve and correlate well with a Type V van der Waals isotherm. A total moisture loss of 1.65% was obtained during the desorption phase. The subsequent adsorption phase once again showed a critical RH point of 70% RH and an increase in sample weight of 1.69% from 70 – 95% RH.

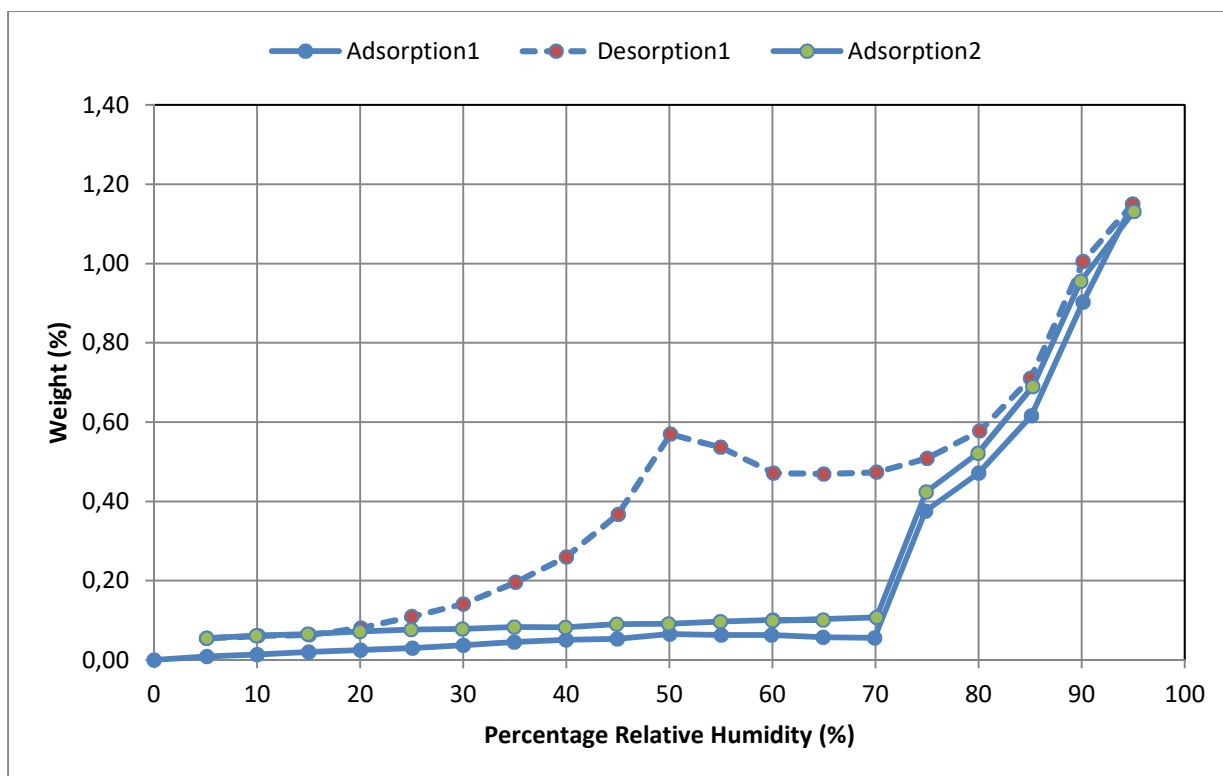


Figure 4.33: Moisture sorption isotherms obtained with the 1:1 weight combination ratio at 25°C

Figures 4.33 and 4.34 depict the moisture sorption isotherms obtained for the 1:1 weight and 1:1 mol combination ratios respectively. The first adsorption step of the two combination ratios showed similar sorption results. The critical RH point of 45% RH was not as pronounced with the combinations, indicating that the presence of sulfadoxine influenced the vapour sorption behaviour of pyrimethamine. However, a weight loss from 45% RH to 70% RH was still observed, with the next critical RH point of 70% RH correlating well with that observed with pyrimethamine single compound (Figure 4.34).

In the desorption phase, the critical RH point of 70% RH is still visible in the 1:1 weight combination ratio and even more pronounced, this is not the case in the 1:1 mol combination. Furthermore, it should be noted that the percentage weight variation is more pronounced with only pyrimethamine single compound while with the combinations it is evident that the presence of sulfadoxine lower the weight loss capacity of the combination.

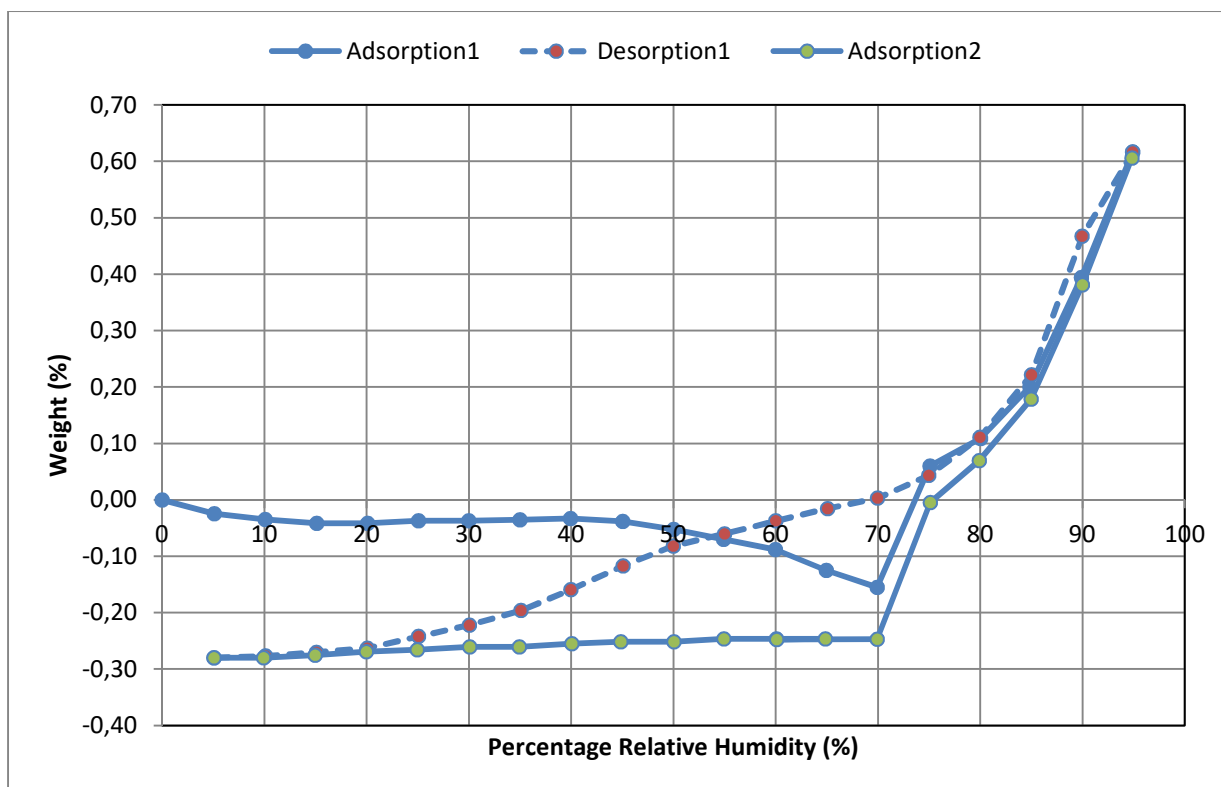


Figure 4.34: Moisture sorption isotherms obtained with the 1:1 mol combination ratio at 25°C

In conclusion, it is evident that sulfadoxine and pyrimethamine influence the vapour sorption behaviour of each other, with sulfadoxine having a limiting influence on pyrimethamine. One could hypothesize the possibility of intermolecular rearrangement due to incorporation or loss of water from the molecular structure.

From all the data obtained during the solid state investigation, it is clear that an intermolecular interaction occurs between sulfadoxine and pyrimethamine. However, it is challenging to establish the kind of interaction when these two drugs are combined in a solution. It is important to state that although DSC results proved a possible eutectic mixture, the focus of this study was to investigate the solubility and permeability of sulfadoxine and pyrimethamine physical mixtures and not to produce a eutectic mixture.

4.5 Permeability studies

During this study, two *in vitro* permeability models were employed for the analysis of sulfadoxine and pyrimethamine transport, namely excised porcine intestinal tissue in diffusion chambers (for sulfadoxine) and Caco-2 cell monolayers in Transwell plates (for pyrimethamine). The transport of both sulfadoxine and pyrimethamine as single compounds and various combinations were tested.

4.5.1 Excised pig intestinal tissue studies

The *in vitro* permeability of sulfadoxine and pyrimethamine as single compounds and in different ratio combinations was determined across excised pig jejunum tissue in the apical to basolateral direction in a Sweetana-Grass diffusion chamber apparatus. The solubility (in KRB) of the supersaturated solutions ($T = 0$ min) and permeation results ($T = 120$ min) obtained for sulfadoxine as single compound and in different combinations are displayed in Figures 4.35 and 4.36 respectively. Pyrimethamine transport was very low across the excised pig tissues and was below the limit of detection.

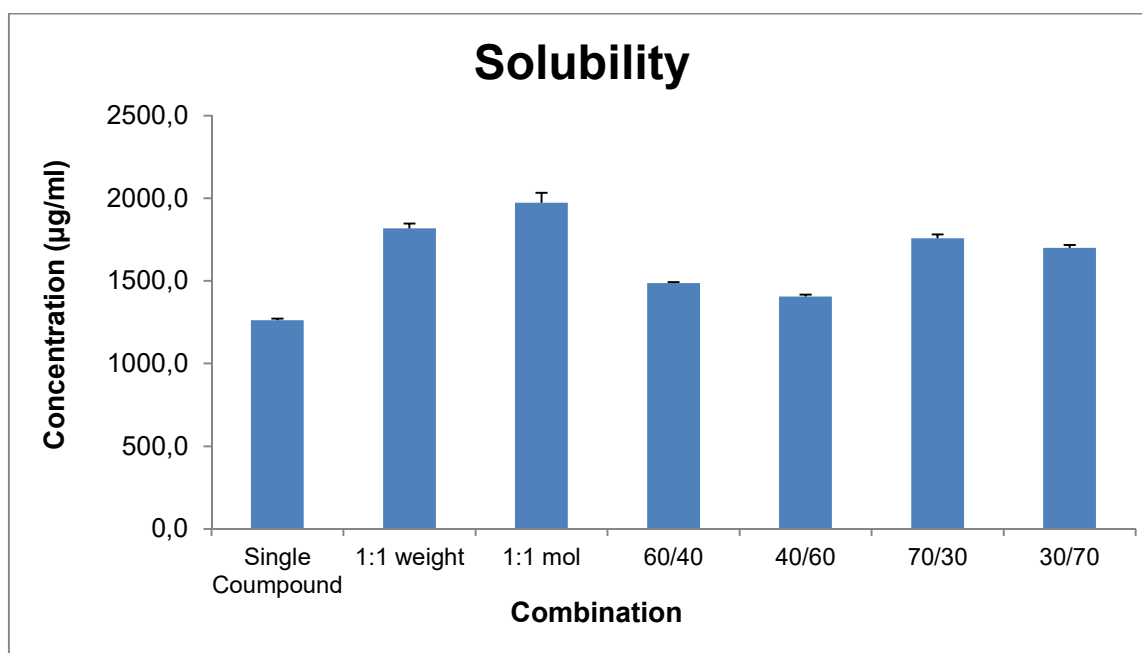


Figure 4.35: Graphical presentation of concentrations obtained for sulfadoxine in KRB as single compound and in various combinations ($T = 0$ min)

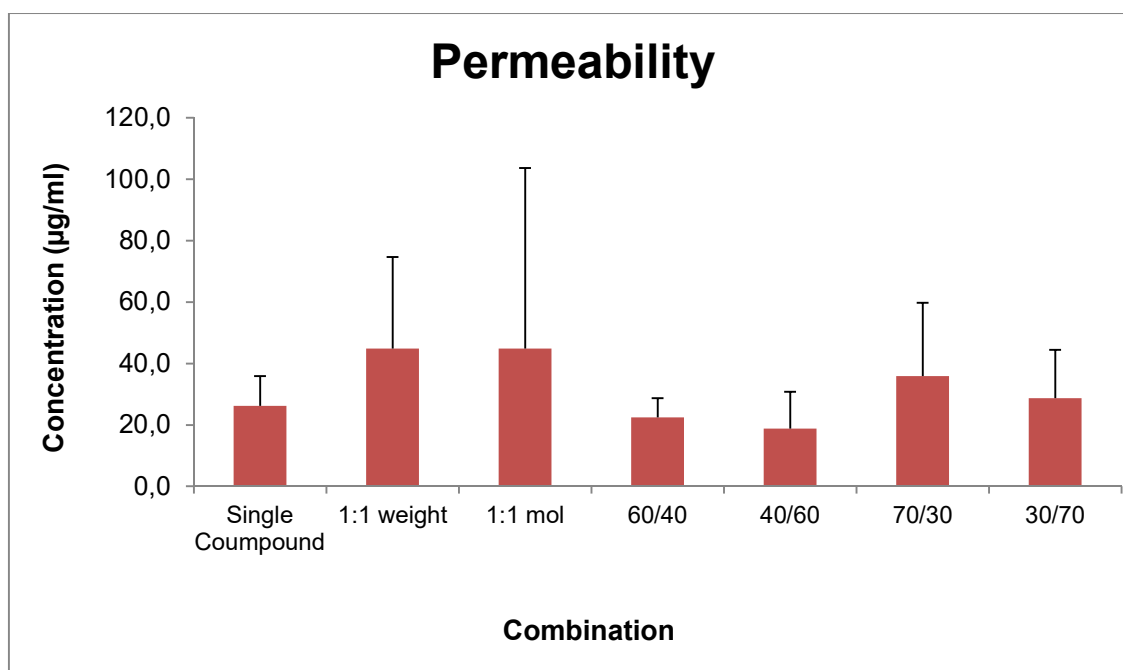


Figure 4.36: Cumulative concentration of sulfadoxine as single compound and in various combinations in the acceptor chamber after 120 min

From the permeation results, it is clear that the 1:1 weight, 1:1 mol and the 70/30 and 30/70 combinations produced higher permeation for sulfadoxine than the single compound. Of these combinations the 1:1 weight and 1:1 mol combination displayed the highest increase in permeation compared to the single compound. For the 1:1 weight, 1:1 mol combinations the increase in permeation was 1.7 times higher than that of sulfadoxine single compound. This indicates that a correlation exists between the solubility and permeability of sulfadoxine, which was expected due to the increased solubility of the combinations as compared to the single compound. The increase in permeation of sulfadoxine across the excised pig intestinal tissue when combined with pyrimethamine may be attributed to the increased solubility that caused a higher concentration gradient, which is the driving force behind passive diffusion. It is, however, noteworthy to mention that the standard deviations are higher with the permeability results of sulfadoxine across excised pig intestinal tissue compared to that of the permeability results of pyrimethamine across Caco-2 cell monolayers. The probable reasons for the higher standard deviations in the case is of the excised pig intestine include multiple cell layers, whereas Caco-2 cells are grown in single layers. Furthermore, the Caco-2 cell monolayer studies were performed in a sterile environment with less variables (i.e. mucus, thickness of the intestine, age of the animal, etc.) compared to the excised pig intestinal tissue studies.

4.5.2 Caco-2 cell monolayer studies

The Caco-2 monolayer cell line was used to determine the *in vitro* permeability of pyrimethamine as single compound and in different ratio combinations in the apical to basolateral direction. The solubility (in DMEM) of the supersaturated solutions ($T = 0$ min) and permeation results ($T = 120$ min) obtained for pyrimethamine as single compound and in different combinations are displayed in Figures 4.37 and 4.38 respectively.

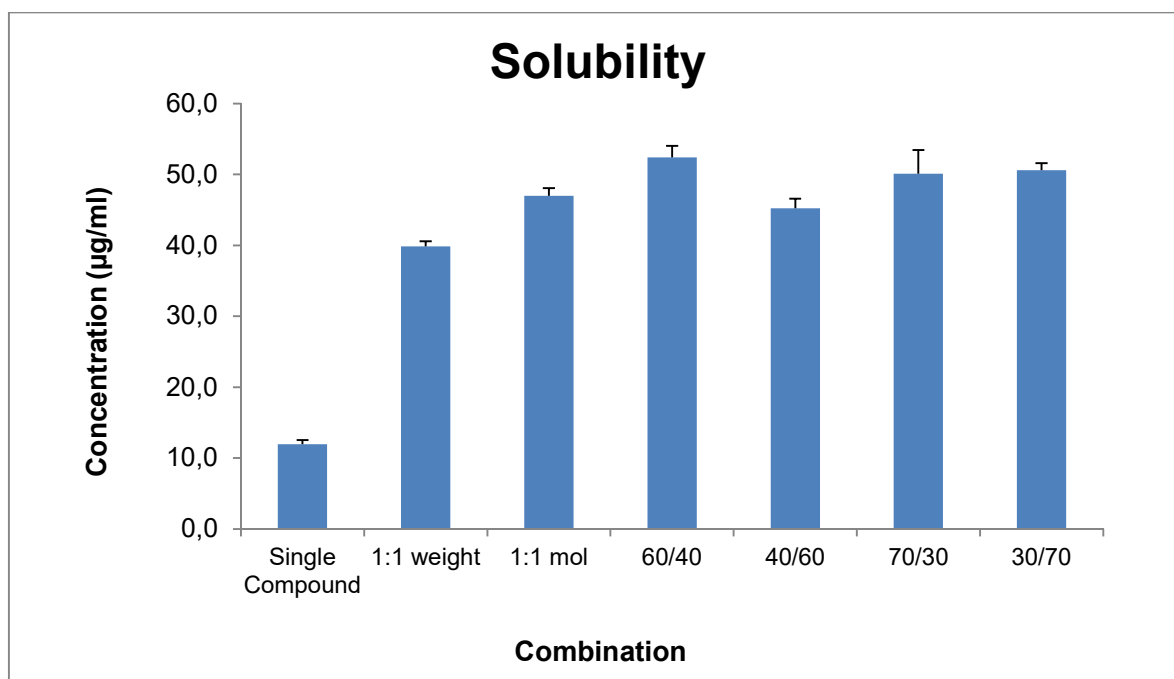


Figure 4.37: Graphical presentation of concentrations obtained for pyrimethamine in DMEM as single compound and in various combinations ($T = 0$ min)

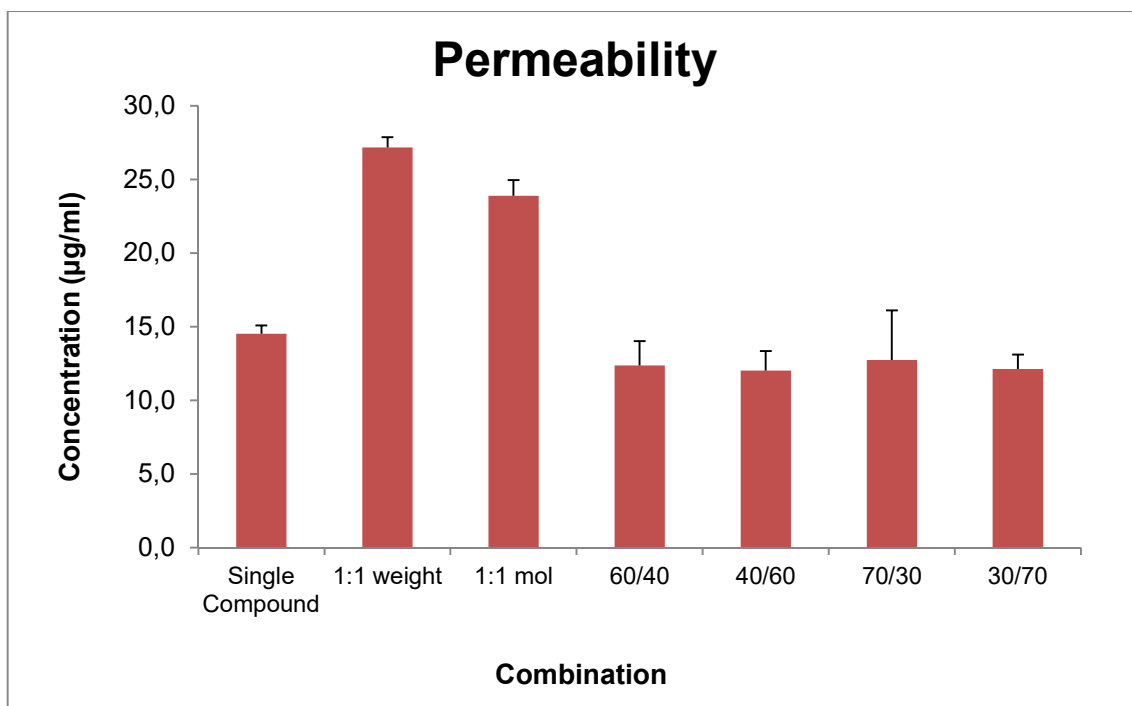


Figure 4.38: Cumulative concentration of pyrimethamine as single compound and in various combinations in the acceptor chamber after 120 min

The permeability results obtained for pyrimethamine show an increase in permeation concentration for the 1:1 weight (1.9 times) and 1:1 mol (1.6 times) compared to the single compound. These two combinations were the only combinations that achieved higher permeation concentrations compared to that of the single compound. It is therefore clear that a correlation exists between the solubility and the permeability for pyrimethamine as single compound and in the 1:1 weight and 1:1 mol combinations. As mentioned before, the increase in permeation of pyrimethamine across Caco-2 cell monolayers when combined with sulfadoxine may be attributed to the increased solubility that caused a higher concentration gradient, which is the driving force behind passive diffusion.

4.6 Conclusion

From the solubility studies (solubility determined for various combinations and in different solvents) it is clear that pyrimethamine and sulfadoxine increase each others' solubility in the majority of the solubility tests performed, of which pyrimethamine's solubility is most affected. This is in accordance with the dissolution results obtained where the different combination ratios showed an increase in dissolution compared to the single compounds. The NMR data indicated a possible weak intermolecular interaction between sulfadoxine and pyrimethamine. The DSC results portray single endotherms for the 1:1 mol and 1:1 weight combinations, a possible indication of eutectic mixtures (Habib *et al.*, 2001:17). Both XRPD and DSC ruled

out the possibility of co-crystal formation. Furthermore, it is apparent that this intermolecular interaction only occurs in solution, as evident from solubility testing, dissolution testing and DSC data, as no complex formation was detected with XRPD. The increase in solubility and dissolution with regards to the combination of these two drugs, could be due to the formation of a complex (Reilly 2006:198)

The results obtained for the *in vitro* permeability studies (excised pig intestinal tissue and caco-2 monolayer studies) proved a relationship exists between the results obtained for the solubility studies and that obtained for the permeability studies. This was true for both drugs in the two different *in vitro* permeability models as an increase in permeation was observed for the 1:1 weight and 1:1 mol combinations compared to the single compounds. The increased permeation of sulfadoxine in the 70/30 and 30/70 combinations can be further investigated in a future project.

4.7 References

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CHAPTER 5

SUMMARY

5.1 Introduction

Drug bioavailability is influenced by drug release from the dosage form, drug dissolution and gastrointestinal epithelial permeability (Ochekpe *et al.*, 2012:59). Poorly aqueous soluble drugs will exhibit a slow dissolution rate, which will frequently be the rate-controlling step during absorption. As a result, high variability in pharmacokinetics and biological activities can occur within and between subjects, as evident from studies on drugs such as digoxin and indomethacin (Florence & Attwood, 1988:157). Sufficient drug permeation across the intestinal epithelium is necessary to obtain the required blood levels of a drug after oral administration (Hunter & Hirst, 1997:129). Membrane structure, solute-solvent interaction and a drug's physico-chemical properties dictates drug diffusion across a biological membrane (Bjarnason *et al.*, 1995:1566).

The Biopharmaceutical Classification System (BCS) is applied to classify drugs according to their intestinal epithelial membrane permeability and aqueous solubility properties. Its use, however, has been hindered due to a lack of solubility and permeability information of certain drugs. Drugs like sulfadoxine have not yet been classified in the BCS (Lindenberg *et al.*, 2004:265, 268-270). With regards to aqueous solubility, the information available refers to sulfadoxine and pyrimethamine as 'very slightly soluble' and 'practically insoluble', respectively (BP, 2015; Loutfy & Aboul-Enein, 1983:465). This may impact on their therapeutic effectiveness due to a relationship between aqueous solubility and bioavailability (Sinnaeve *et al.*, 2005:97).

5.2 Final conclusions

During this study, the solubility, dissolution and permeability of sulfadoxine and pyrimethamine as single compounds and in various combination ratios were investigated. Various solid-state characterization techniques were used to elucidate possible physico-chemical interactions between these two drugs. The solid-state investigations indicated a possible weak intermolecular interaction between sulfadoxine and pyrimethamine (through $^1\text{H-NMR}$). It is evident that this interaction or possible complex formation only occurs in solution as seen from the solubility and dissolution testing as well as DSC, isothermal microcalorimetry and vapour

sorption analysis. Results from XRPD indicated that no complex formation or interaction was detected with the physical powder mixtures and both DSC and XRPD, ruled out possible co-crystal formation. The DSC results portrayed single endotherms for only two combinations (1:1 mol and 1:1 weight combination ratios) which indicated formation of an eutectic mixture (Habib *et al.*, 2001:17).

Solubility studies were performed in various solvents, on each single compound and in different ratio combinations. The first set of solubility studies performed ranged in different concentration ratios of sulfadoxine and pyrimethamine and the solvent used was water. It is evident from the solubility results in water that both sulfadoxine and pyrimethamine displayed an increase in solubility in the different ratio combinations in comparison to the solubility of each single compound. The focus for the remainder of this study fell on the 1:1 mol and 1:1 weight ratio combinations, since these were the only two combinations that produced single endotherms as seen with the DSC results. For the next set of solubility studies, the single compounds were compared to the 1:1 weight combination ratio using a different solvent for each solubility study. Sulfadoxine and pyrimethamine displayed increased solubility when combined in most solvents (which varied in polarity and pH value), however, the most pronounced effects on solubility were obtained in water. In one solvent (0.1 N HCl), the inverse occurred, sulfadoxine and pyrimethamine single compounds displayed higher solubility as single compounds in comparison to the 1:1 weight combination. This could be due to charge transfer between these two drugs. Because of sulfadoxine's amphoteric nature, it is able to donate or receive protons, influencing the intermolecular interaction between sulfadoxine and pyrimethamine, to such an extent that in certain solvents increased solubility is achieved in combination whilst in others a decrease is obtained. Whilst this is the case, the pronounced increase in aqueous solubility for the different combinations necessitated further investigations regarding the dissolution of these two drugs in water.

The results obtained during the powder dissolution studies performed on the single compounds and three different combination ratios (500:25 and 1:1 weight ratios and 1:1 mol ratio) correlated well with the solubility results obtained in water. An increase in dissolution was obtained for sulfadoxine and pyrimethamine in combination compared to that of the single compounds.

The *in vitro* permeability of the single compounds and the different combination ratios were performed on two models (i.e. excised pig intestinal tissue for sulfadoxine and Caco-2 cell monolayers for pyrimethamine). The results obtained during the *in vitro* permeability studies proved that a relationship existed between the solubility and permeability of these two drugs. This was the case for each drug in each of the two different models. An increase in permeation

was obtained for the 1:1 mol and 1:1 weight combination ratios compared to the single compounds. Furthermore, an increase in permeation was also observed in the 70:30 and 30:70 weight combinations.

The focus of this study was to investigate the solubility and permeability of sulfadoxine and pyrimethamine single compounds and in different combinations (physical mixtures). The possibility of an intermolecular interaction is evident from the results obtained during this study, however, determining the exact mechanism of such interaction is challenging. Though the possibility of a eutectic mixture is evident from the DSC results, it was not the aim of this study to fully elucidate the mechanism of solubility enhancement.

5.3 Future recommendations

As mentioned, DSC results indicated the possibility of a eutectic mixture. The manufacturing of a eutectic mixture through methods such as the fusion or solvent evaporation method (Katariya & Patil, 2013:7) could be explored, after which solid state characterization is required to confirm a eutectic mixture. Upon confirmation of the eutectic mixture, a series of solubility, dissolution and permeability tests need to be performed to investigate the influence this eutectic mixture has on the solubility and permeation of both drugs.

Furthermore, the mechanism of the intermolecular interaction between these compounds in combination might be of significance in understanding the increase in solubility and permeation. Extensive chemical studies could possibly shed light on this matter. Further investigation on the isothermal calorimetry and vapour sorption analysis is needed to gain more information regarding the intermolecular rearrangement of these compounds in combination.

Though the focus of this study was primarily on the 1:1 mol and 1:1 weight ratio combinations, it was clear from the solubility results, that other weight combinations also produced increased solubility. This avenue, together with the increased permeation observed in other weight combinations, is for future exploration.

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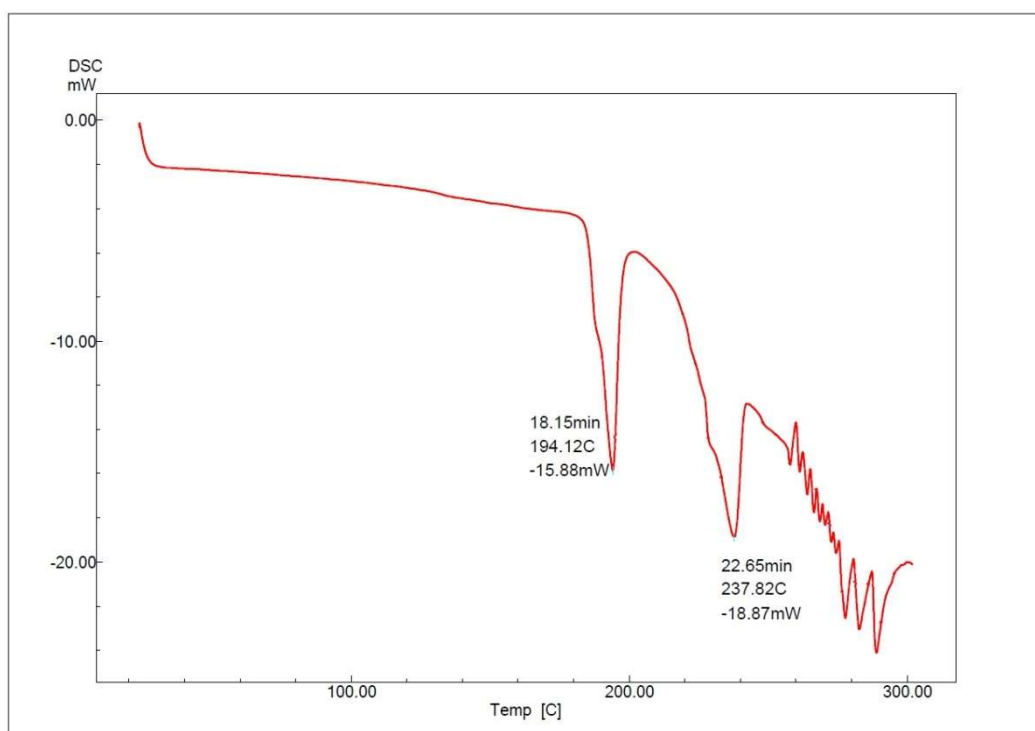


Figure A.1: DSC data for 30% sulfadoxine and 70% pyrimethamine combination.

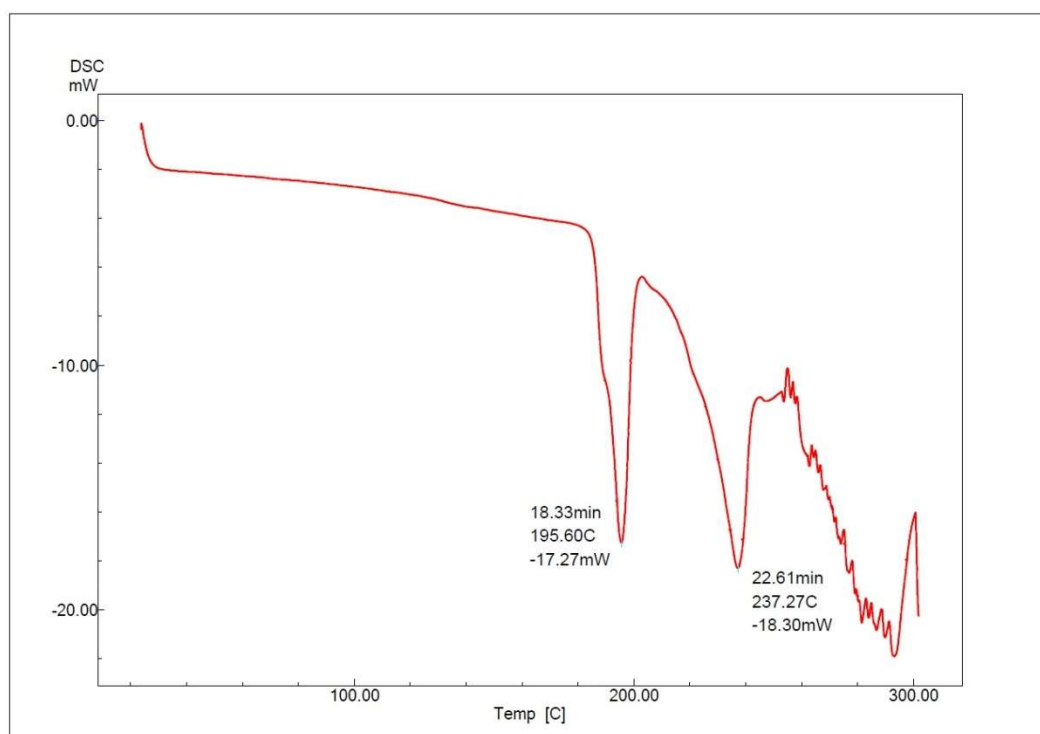


Figure A.2: DSC data for 40% sulfadoxine and 60% pyrimethamine combination.

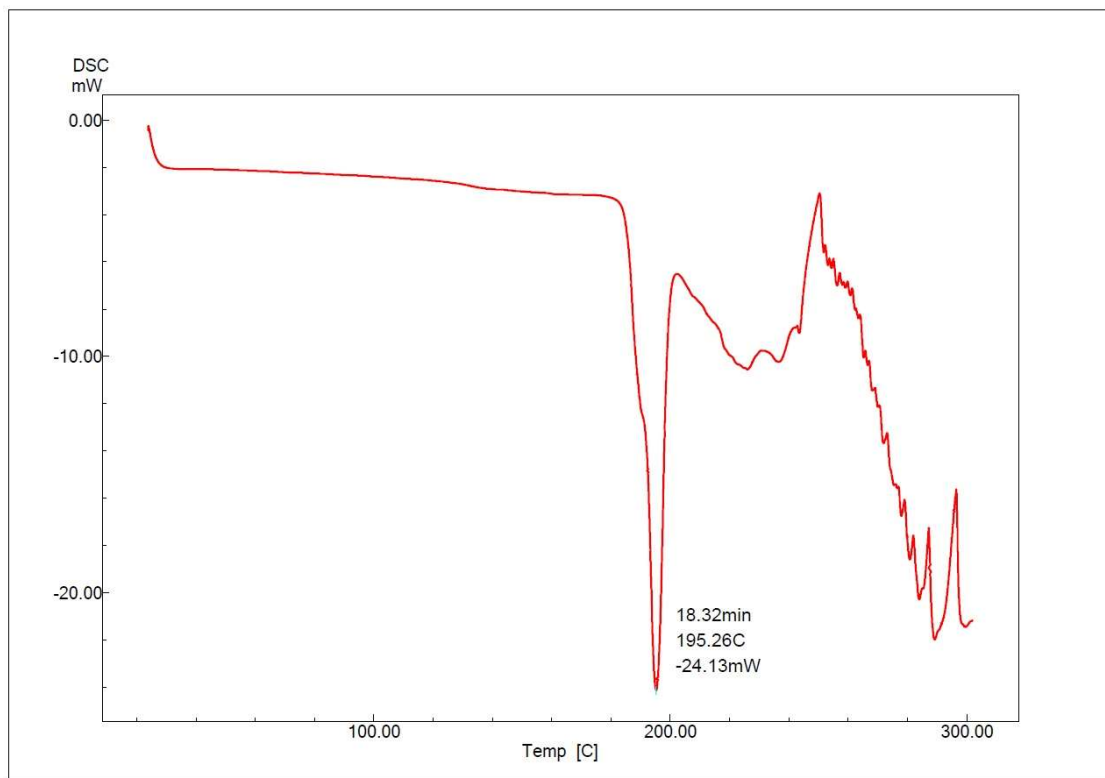


Figure A.3: DSC data for 60% sulfadoxine and 40% pyrimethamine combination.

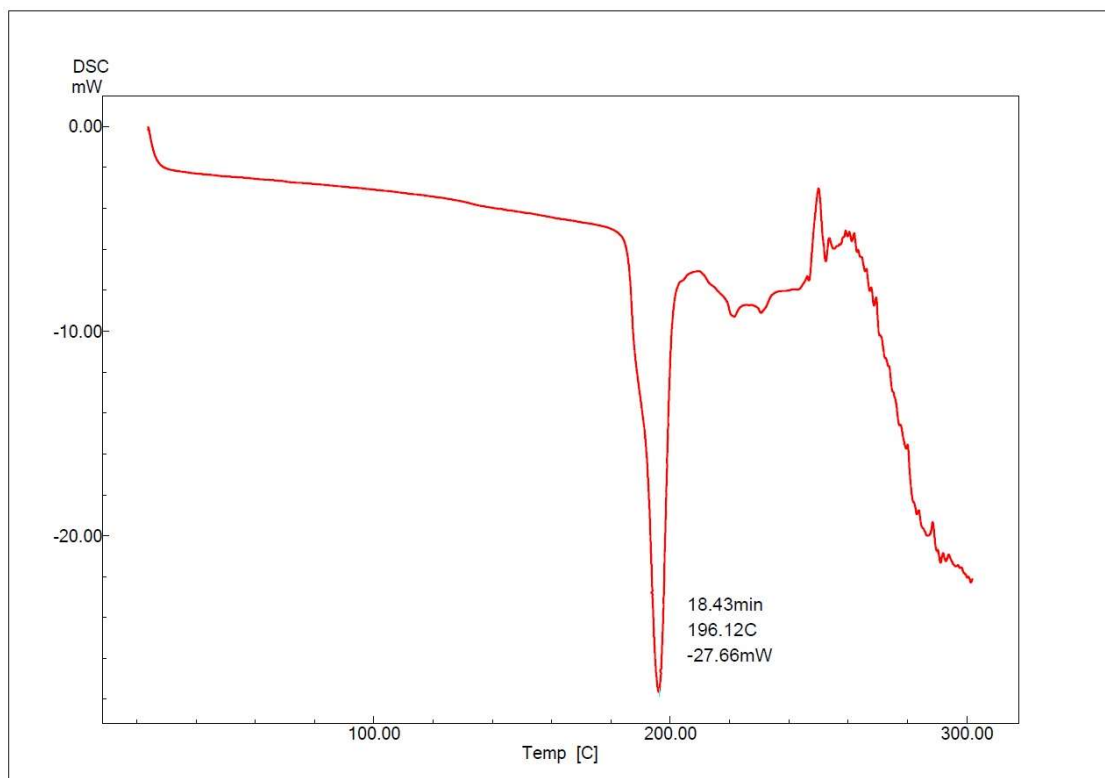


Figure A.4: DSC data for 70% sulfadoxine and 30% pyrimethamine combination.

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Solubility and permeability of sulfadoxine and pyrimethamine in different ratio combinations

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The novelty of this paper lies in the fact that although sulfadoxine and pyrimethamine as a fixed dose combination has been used for more than 30 years in the treatment of malaria, very little information is available on the solubility and permeability of these two drugs, when combined. The possible interaction/s between these two drugs in combination has never been reported before in literature.

Thanking you in anticipation,

Prof JC Wessels

Solubility and permeability of sulfadoxine and pyrimethamine in different ratio combinations

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Abstract

The combination of sulfadoxine and pyrimethamine has been used to treat malaria for many years and is currently still on the World Health Organization's (WHO) essential drug list. Relative little information is available about the solubilities of these two drugs, especially in combination. Furthermore, sulfadoxine has not yet been classified according to the Biopharmaceutics Classification System (BCS) due to incomplete solubility and permeability data. The solubility and intestinal epithelial permeability of the single compounds as well as different ratio combinations of sulfadoxine and pyrimethamine were investigated. The results displayed an increase in solubility when these two drugs were combined in 1:1 molar and 1:1 weight combinations. The *in vitro* permeability also increased when combinations of the two drugs were applied to intestinal epithelial membranes compared to that of the single compounds. Potential interactions between the two drugs in water were investigated using isothermal microcalorimetry and nuclear magnetic resonance spectroscopy. The results indicated that an intermolecular interaction occurred between sulfadoxine and pyrimethamine when combined in solution, which was associated with a decrease in terms of incompatibility.

Keywords: Sulfadoxine, pyrimethamine, solubility, permeability, combination, interaction, incompatibility

Introduction

Poor solubility of drug substances is one of the main reasons for poor bioavailability and remains one of the greatest challenges for formulation scientists [1]. Sulfadoxine and pyrimethamine are two anti-malarial drugs used in fixed-dose combinations (FDC's) primarily for the curative treatment of malaria. The formulation of the FDC product of the two drugs proved to be problematic due to poor reproducible and non-compliant dissolution results, mainly relating to pyrimethamine [2]. The solubility and dissolution of sulfadoxine and pyrimethamine after oral administration is an important factor in terms of anti-malarial effectiveness, since a low dissolution rate will impact on the bioavailability and subsequently effectiveness [3]. The absorption of poorly water-soluble drugs in general is limited by the dissolution rate of these drugs. This is typical for drugs in class II or IV of the Biopharmaceutics Classification System (BCS) [4].

Pyrimethamine (Figure 1a) is classified as a class II (low solubility and high permeability) or class IV (low solubility and low permeability) drug, while sulfadoxine (Figure 1b) has not been classified yet.

Insert Fig. 1 here

Various techniques are available to improve the solubility of a poorly water-soluble drug. These include physical modifications (solid solutions and solid dispersions), chemical modifications (salt formation and complexation) and miscellaneous methods (cosolvency and solubilizers) [5]. Complex formation can have an impact on, not only solubility, but several other pharmaceutical properties such as dissolution rate, permeability, absorption rate and bioavailability [6]. Sulfonamides have been successfully used in complexation or cocrystallization in a 1:1 molar ratio with drugs such as trimethoprim [7,8].

Materials

Both sulfadoxine and pyrimethamine were purchased from DB Fine Chemicals (Johannesburg, South Africa). All solvents and other reagents used during this study were either of chromatography or analytical grade.

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC), by Sigma Aldrich (Johannesburg, South Africa). The excised pig intestinal tissues were obtained from a local abattoir (Potchefstroom, South Africa). The use of animal tissue for the permeation studies was approved by the North-West University Research Ethics Regulatory Committee (reference number: NWU-00025-15-A5).

Methods

Equilibrium solubility studies

Equilibrium solubility studies were performed with sulfadoxine and pyrimethamine as single components as well as 1:1 weight and 1:1 molar combination ratios. A sufficient quantity of each drug was added to each test tube ($n = 6$) to ensure saturation of the resulting solutions. To each test tube, 10 ml of distilled water was pipetted. Parafilm[®] (Neenah, USA) was used to seal the test tubes and a screw cap was tightly fitted onto every test tube to prevent leakage. Subsequently, the test tubes were affixed to a rotating axis in a water bath, maintained at $37 \pm 2^\circ\text{C}$. The samples were rotated at 55 rpm for 24 h. From each test tube, a sample was collected, filtered (if necessary dilutions were made) and assayed by means of high performance liquid chromatography (HPLC).

Permeability studies

Two separate *in vitro* permeability models (i.e. excised pig intestinal tissues and Caco-2 cell monolayers) were employed to determine the permeability of sulfadoxine and pyrimethamine as single compounds and in the same ratio combinations that was tested in the solubility studies.

The most suitable model was employed for each drug as determined by preliminary permeation studies.

Excised pig intestinal tissues in Sweetana-Grass diffusion chamber apparatus

A piece of pig jejunum was collected from the abattoir directly after slaughtering of the pig and it was washed with Krebs Ringer Bicarbonate (KRB) buffer ($< 8^{\circ}\text{C}$). The clean section of the jejunum was transported in a container filled with cold KRB buffer. The tissue was prepared for use in the Sweetana-Grass diffusion chambers in the laboratory, where it was washed and the serosal layer removed. The jejunum was dissected along the mesenteric border to produce a flat sheet of tissue, washed again with KRB buffer and placed on a sheet of filtration paper (apical side facing upward). Small strips of tissue were mounted between two half cells of a Sweetana-Grass diffusion apparatus and the assembled chambers were mounted onto a heating plate. KRB buffer (37°C) was placed in the chambers (7 ml per chamber) and oxygenated for a period of 30 min for the tissue to acclimatise in this environment. The apical chambers were then filled with pre-heated experimental test solutions (37°C) and the basolateral chambers with fresh KRB buffer (7 ml at 37°C in each chamber). The experimental test solutions were prepared by adding approximately, accurately weighed 100 mg of sulfadoxine single compound or 200 mg of the different combinations to 12.5 ml of KRB in test tubes. All test solutions were ultrasonicated for 30 min to ensure saturation.

The trans-epithelial electrical resistance (TEER) was measured at the start and end of the permeation study to ensure tissue integrity was maintained throughout the permeation experiment. Every 20 min, over a 120 min period, a 200 μl sample was withdrawn from the basolateral chamber. Pre-heated KRB buffer was used to replenish the same volume withdrawn from each chamber. All samples were analysed by means of HPLC.

Caco-2 cell monolayers in Transwell membrane plates

The Caco-2 cells were cultured in high-glucose DMEM (Separations, Randburg, South Africa) supplemented with 1% penicillin/streptomycin (Separations, Randburg, South Africa), 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% amphotericin B (250 µg/ml) (The Scientific Group, Johannesburg, South Africa), 10% foetal bovine serum (Merck, Modderfontein, South Africa), and 1% 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa).

The Caco-2 cells were cultured at 37°C with 95% humidified air and 5% carbon dioxide in a Forma Steri-cycle CO₂ incubator (Life Technologies, Fairland, South Africa). The cells were inspected to ensure the absence of contamination and determine the percentage confluence. The growth medium (pre-warmed at 37°C) was replaced every second day under sterile conditions. The Caco-2 cells were sub-cultured by means of trypsinisation upon reaching 50% confluency [9]. The growth medium was removed and the cells rinsed twice with 10 ml of pre-warmed phosphate buffered saline solution (PBSS) (Sigma Aldrich, Johannesburg, South Africa) under sterile conditions. Trypsin-Versene (EDTA) mixture (3 ml) was added to the cell culture flask, distributed evenly and incubated for 5 min at 37°C. Upon complete cell detachment (determined by means of light microscope) 6 ml of pre-warmed growth medium (37°C) was added to the flask content to inactivate the trypsin mixture's action. The cell suspension was agitated, ensuring that all cells were rinsed from the bottom of the flask and the cells were maintained under normal cell culturing conditions prior to seeding.

The Caco-2 cells were seeded onto membranes in Transwell® 6-well plates (Corning Costar® Corporation, Tewksbury, USA) with a surface area of 4.67 cm² and a pore diameter of 0.4 µm. A cell suspension was obtained by trypsinisation with Trypsin-Versene. After cell detachment, 6 ml of growth medium (pre-warmed at 37°C) was added to the flask and the suspension agitated extensively to ensure de-agglomeration and complete detachment, resulting in a suspension consisting of single cells. The single cell suspension was transferred to a 50 ml

tube. To ensure a homogenous distribution of the cells, a Pasteur pipette was used to agitate the suspension. Cells were counted employing a haemocytometer using Trypan blue (Sigma Aldrich, Johannesburg, South Africa) as staining agent to monitor the percentage cell death. In a 2.5 ml Eppendorf tube, a volume of 15 μ l PBSS, 10 μ l cell suspension and 25 μ l Trypan blue were mixed and incubated for 3 min, after which a volume of 10 μ l of the mixture was placed on either side of the cover slip of the haemocytometer. Cells were counted on five of the nine squares on the haemocytometer's grid on each side by means of a light microscope. The average number of cells was calculated (per square), which was then multiplied with the dilution factor (5×10^4). This established the total number of cells per ml in the cell suspension. The suspension was diluted to a concentration of approximately 20 000 cells per ml.

Cell seeding onto Transwell[®] membranes occurred in a laminar flow hood under sterile conditions by pipetting 2.5 ml of the final cell suspension into each apical chamber of the membrane filter plate wells. The Caco-2 cells were grown for 21 to 24 days on the membranes to produce intact epithelial cell monolayers. The growth medium was replaced every second day under sterile conditions. Prior to the transport studies, the TEER of each cell monolayer was measured using a Millicell ERS II meter (Millipore, Billerica, Massachusetts, USA) to ensure that confluent monolayers were formed. A resistance of at least 150 Ω per well was required to commence with the experiment.

For the transport studies, the growth medium was removed from the basolateral chamber and replaced with 2.5 ml of pre-warmed (37°C) DMEM buffered with HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (Scientific group, Biochrom, Randburg, South Africa) and incubated at 37°C for 30 min, after which TEER measurements were performed. The growth medium was removed from the apical chambers and replaced with 2.5 ml of test solution.

For all test solutions, supersaturated solutions were prepared where approximately 100 mg of pyrimethamine single compound or 200 mg of the different combinations (the same as for the solubility studies) were weighed off in test tubes and 12.5 ml of DMEM added as solvent and pre-heated to 37°C.

A 200 µl sample was withdrawn from the basolateral chamber every 20 min over a period of 120 min. The chamber from which the sample was withdrawn was immediately replenished by the same volume of fresh pre-heated DMEM. To ensure that the monolayer integrity was maintained, TEER measurements were performed at the beginning and end of the permeation study. All samples were analysed by means of HPLC.

Isothermal microcalorimetry

A 2277 Thermal Activity Monitor (TAMIII) (TA Instruments, USA) was used as microcalorimeter equipped with an oil bath with a stability of ± 100 µK over 24 hours. The compatibility of sulfadoxine and pyrimethamine in different concentration ratios (same as for solubility and permeability studies) in distilled water was investigated at 37°C. For each single compound as well as the different ratios, approximately 50 mg of sample was accurately weighed into glass ampoules. In the instances where distilled water was added, 1 ml was accurately pipetted into each ampoule. The pipetted volume was subsequently weighed since the heat flow data is normalised to sample weight. Each ampoule was tightly sealed with a crimping cap. The ampoules were lowered into the microcalorimeter and a heat flow baseline was established prior to lowering the ampoules into the measuring position. After the ampoules were lowered into the measuring position, another baseline measurement was taken.

The heat flow signals obtained for each individual component as well as each combination were captured and subsequently analysed. The calorimetric output obtained for each individual component is used to calculate a theoretical, hypothetical response when in combination with

one another. Therefore, when an interaction or incompatibility occurs between the different components, the actual measured calorimetric output will differ from the theoretically calculated output. The difference between the actual measured heat flow output and the calculated heat flow output is then indicated as an interaction heat flow curve.

Nuclear magnetic resonance

To investigate the possibility of intermolecular interactions and possible molecular mobility changes between sulfadoxine and pyrimethamine in solution, nuclear magnetic resonance (NMR) studies were conducted. Saturated solutions were prepared for all samples in D₂O with the addition of a drop of DCl (Sigma-Aldrich, Steinheim, Germany) to each sample in order to enhance the solubility of pyrimethamine and sulfadoxine in the D₂O. Proton (¹H) NMR spectra were recorded on a Bruker advance 600 NMR spectrometer at 600.17 MHz. Chemical shifts are reported in parts per million (δ). Three samples were analysed namely sulfadoxine and pyrimethamine each alone and in a 1:1 molar combination.

HPLC analysis

All HPLC analyses throughout this study were performed using a Shimadzu (Kyoto, Japan) UFLC (LC-20AD) chromatographic system. The system consisted of a SPD-M20A UV/VIS Photodiode Array detector, an auto-sampler (SIL-20AC) with a sample temperature controller and a solvent delivery module (LC-20AD). The mobile phase consisted of 20 ml of acetic acid glacial (100 %) and 1 ml triethylamine dissolved in approximately 1600 ml of water, diluted to 2000 ml and the pH adjusted with sodium hydroxide (10 N) to 4.2. Of this solution 1600 ml was mixed with 400 ml of acetonitrile. The final solution was degassed and filtered. A Phenomenex Luna C₁₈ (250 mm x 4.6 mm) column with particle size 5 µm was used and a detection wavelength of 254 nm was used. The flow rate was 2 ml per minute and the injection volume 20 µl.

Results

Equilibrium solubility studies

Literature states that sulfadoxine are very slightly soluble in water while pyrimethamine are insoluble in water [10]. The equilibrium solubility obtained for each of the single compounds after 24 hours were $269 \pm 10 \mu\text{g/ml}$ for sulfadoxine and $29 \pm 3 \mu\text{g/ml}$ for pyrimethamine. It is clear that both drugs exhibited low solubility in distilled water. Pyrimethamine presents with a solubility below $100 \mu\text{g/ml}$ causing dissolution limitations to absorption [11]. The equilibrium solubility concentrations for sulfadoxine single compound and sulfadoxine in the two different combination ratios with pyrimethamine are depicted in Figure 2.

Insert Fig. 2 here

From the data presented in Figure 2 it is clear that the solubility of sulfadoxine increased about two-fold when in combination with pyrimethamine, which varied only slightly between the two combination ratios. Pyrimethamine exhibited an approximately nine-fold increase in solubility when combined with sulfadoxine in the 1:1 weight and 1:1 molar ratios as seen in Figure 3.

Insert Fig. 3 here

It is evident that when sulfadoxine and pyrimethamine are combined, each of the two compound's solubility is considerably enhanced in distilled water. Cairra [7] reported that sulfonamides have been used in co-crystal or complex formation through molecular association with other drugs in a 1:1 molar ratio. This molecular association can occur through hydrogen bonding, as was the case with sulfamethoxazole and trimethoprim where proton transfer occurred from sulfamethoxazole to trimethoprim's nitrogen atom. Both sulfamethoxazole and sulfadoxine are sulfonamides, while pyrimethamine and trimethoprim are benzylpyrimidines [12]. The possibility exists that a molecular association in the form of hydrogen bonding can

occur between sulfadoxine and pyrimethamine when these drugs are combined in water, resulting in increased solubility. Complex formation between two drugs can influence an array of their pharmaceutical and biopharmaceutical properties, such as solubility, permeability, bioavailability and biological activity [6].

Permeability studies

The intestinal epithelial permeability results obtained for sulfadoxine and pyrimethamine alone and in different combination ratios are displayed in Figures 4 and 5, respectively.

Insert Fig. 4 here

A considerable increase in sulfadoxine permeability across excised pig intestinal tissue was obtained for both combination ratios when compared to the single compound (Figure 4). The increase in permeation of sulfadoxine across the excised pig intestinal tissue when combined with pyrimethamine may be attributed to the increased solubility that caused a higher concentration gradient, which is the driving force behind passive diffusion.

Insert Fig. 5 here

For pyrimethamine (Figure 5), the 1:1 molar and 1:1 weight ratio combinations produced a substantial increase in permeability of this drug across the Caco-2 cell monolayers. As mentioned before, the increase in permeation of pyrimethamine across the Caco-2 cell monolayers when combined with sulfadoxine may be attributed to the increased solubility that caused a higher concentration gradient, which resulted in an increased passive diffusion across the cell layer.

Isothermal microcalorimetry

From the results it became evident that an increase in solubility and permeability occurred when sulfadoxine and pyrimethamine are combined in 1:1 molar and 1:1 weight ratios. For

this reason, microcalorimetry was used to investigate if a potential interaction occurred between these drugs when they are combined in the presence of water. In order to compare the microcalorimetric data, the physical mixtures without water were also tested. The heat flow values obtained for the physical mixtures without water were 1.82 mW/g and 0.41 mW/g for the 1:1 weight and 1:1 mol combinations, respectively. The microcalorimetry results obtained for the 1:1 weight and 1:1 molar combination ratios are displayed in Figures 6 and 7 respectively.

Insert Fig. 6 here

Insert Fig. 7 here

From the heat flow data obtained it became evident that when the two drugs are combined in the 1:1 weight and 1:1 mol combination ratios in the mere solid-state, mentionable incompatibility between the two was measured. From Figure 6 it can be deduced that the 1:1 weight combination with water still shows incompatibility. This being clear from the distinct slope of the interaction curve (magenta curve). With the physical mixture of 1:1 mol combination ratio, without the addition of distilled water, incompatibility was measured but showed to be significantly small. If distilled water is however added to the mixture, the incompatibility was minimised and to such an extent that the 1:1 mol combination ratio showed almost no incompatibility. In summary it can be concluded from the microcalorimetry data that the 1:1 mol ratio showed the least incompatibility between the two drugs when in a mere physical mixture. The two drugs interact with one another or can be classified as incompatible with one another however, it seems that if distilled water is added to the drug mixtures the incompatibility is being minimised.

It is possible that the solubility of these drugs in combination increases as the incompatibility between them decreases. Since the 1:1 mol combination showed the least incompatibility, this combination and the single compounds were focussed on for NMR analysis (Figure 8).

Insert Fig. 8 here

NMR data in Figure 8 showed that for sulfadoxine the two doublets at $\delta = 7.33 - 7.82$ ppm correspond to the protons in the benzenesulfonamide group ($J = 8.7 - 9.0$ Hz) and the singlets at $\delta = 3.41$ ppm and 3.72 ppm correspond to the protons on the methoxy groups connected at position 5 and 6 on the pyrimidinyl-ring. The singlet at $\delta = 7.88$ ppm corresponds to the proton at position 4 on the pyrimidinyl-ring. For pyrimethamine the two doublets at $\delta = 7.09 - 7.34$ ppm correspond to the protons of the chlorophenyl-ring ($J = 8.3 - 8.7$ Hz), the triplet at $\delta = 0.83 - 0.85$ ppm ($J = 7.5$ Hz) correspond to the CH_3 protons and the quartet at $\delta = 2.11 - 2.14$ ppm ($J = 7.5$ Hz) correspond to the CH_2 protons.

The NMR of sulfadoxine in the sulfadoxine-pyrimethamine (1:1 molar) ratio displays a downfield shift in the spectra. For sulfadoxine the singlets of the protons on the methoxy groups shift to $\delta = 3.50$ ppm and 3.79 ppm respectively and the two doublets of sulfadoxine shift to $\delta = 7.39 - 7.92$ ppm.

The shifts observed were small and was only evident in the sulfadoxine part of the NMR, indicating a possible weak intermolecular interaction between sulfadoxine and pyrimethamine.

Conclusion

The solubility and membrane permeability of sulfadoxine and pyrimethamine increased when these two drugs are combined in solution. An intermolecular interaction may be responsible for this increase in solubility as evident from the NMR data, while isothermal microcalorimetry showed a decrease in the incompatibility between sulfadoxine and pyrimethamine when

combined. The exact mechanism of this molecular interaction and the possibility of co-crystal or complex formation between sulfadoxine and pyrimethamine should be further investigated.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper and they gave their consent for publication of this manuscript.

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Figure Captions

Fig. 1: Chemical structures of (a) pyrimethamine and (b) sulfadoxine

Fig. 2: Equilibrium solubility concentrations for sulfadoxine alone and in two combination ratios with pyrimethamine in distilled water

Fig. 3: Equilibrium solubility concentrations for pyrimethamine alone and in two combination ratios with sulfadoxine in distilled water

Fig. 4: Accumulated concentration of sulfadoxine alone and in two combination ratios with pyrimethamine that permeated across excised pig intestinal tissues over a period of 120 min

Fig. 5: Accumulated concentration of pyrimethamine alone and in two combination ratios with sulfadoxine that permeated across Caco-2 cell monolayers over a period of 120 min

Fig. 6: The heat flow data obtained with the physical mixture of the 1:1 weight combination with 1 ml distilled water, measured at 37°C for a 22 hour period.

Fig. 7: The heat flow data obtained with the physical mixture of the 1:1 molar combination with 1 ml distilled water, measured at 37°C for a 22 hour period.

Fig. 8: ^1H NMR of sulfadoxine (a) and pyrimethamine (b) and of the 1:1 molar ratio combination (c).

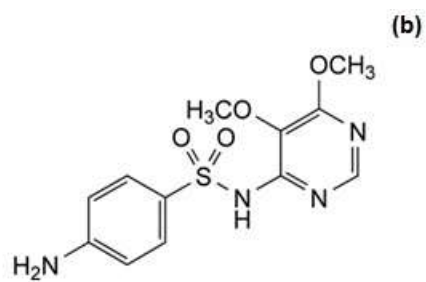
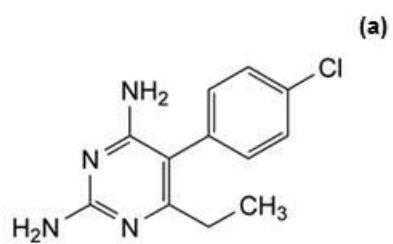


Fig. 1

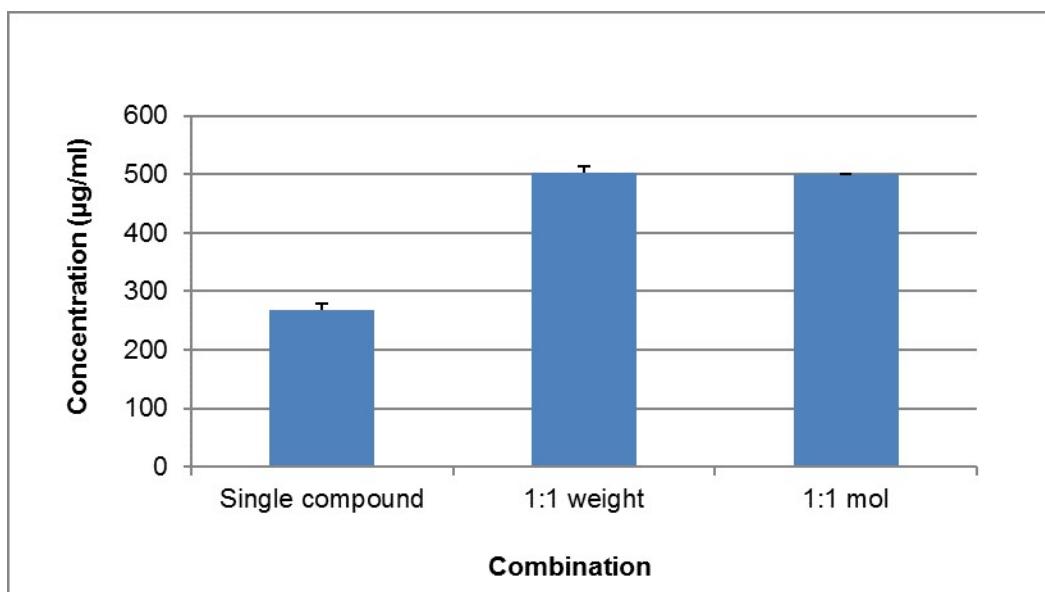


Fig. 2

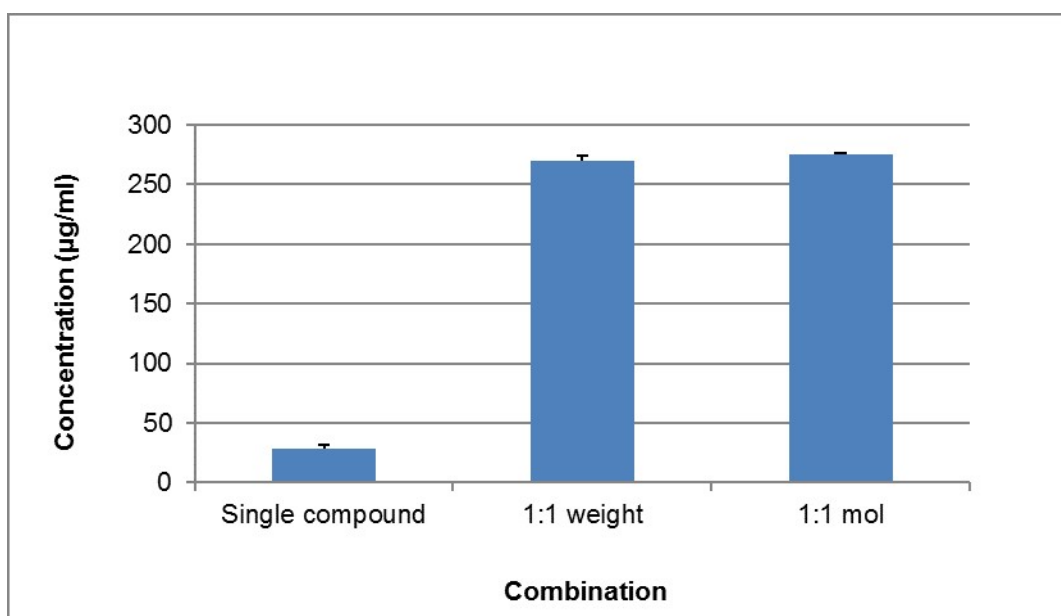


Fig. 3

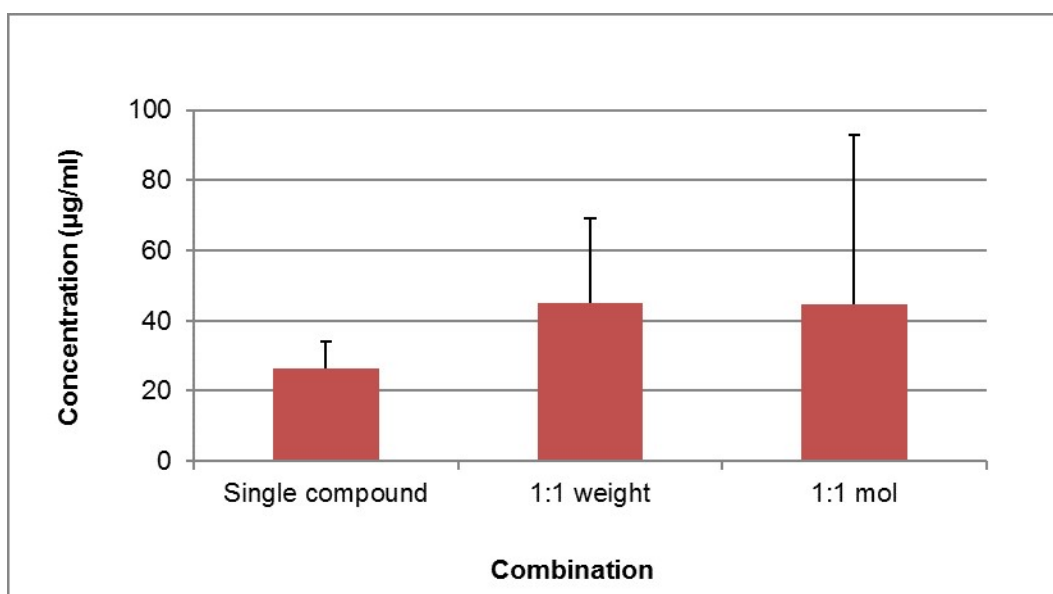


Fig. 4

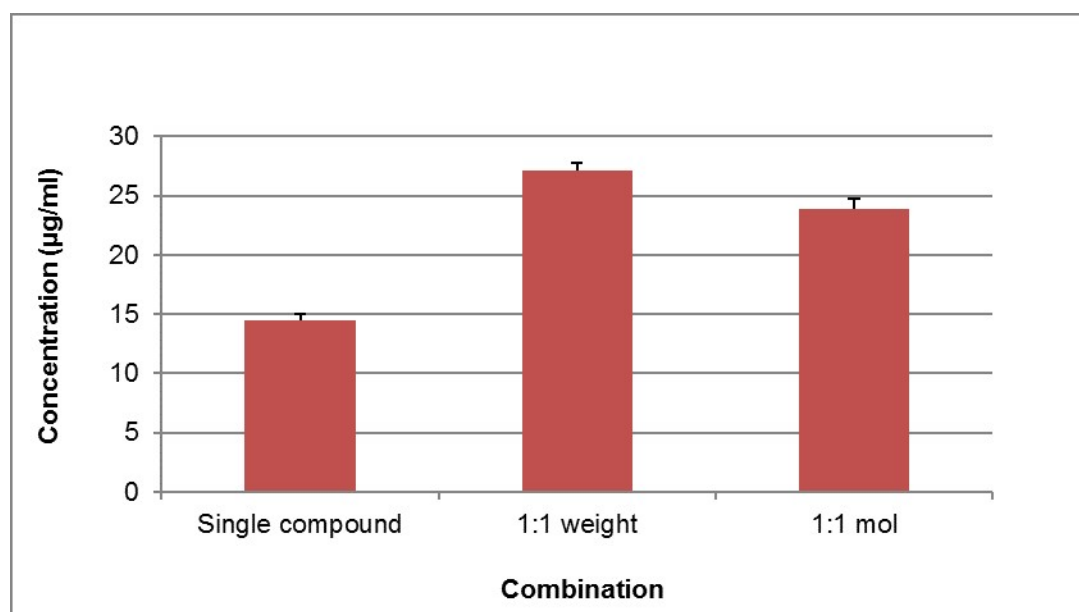


Fig. 5

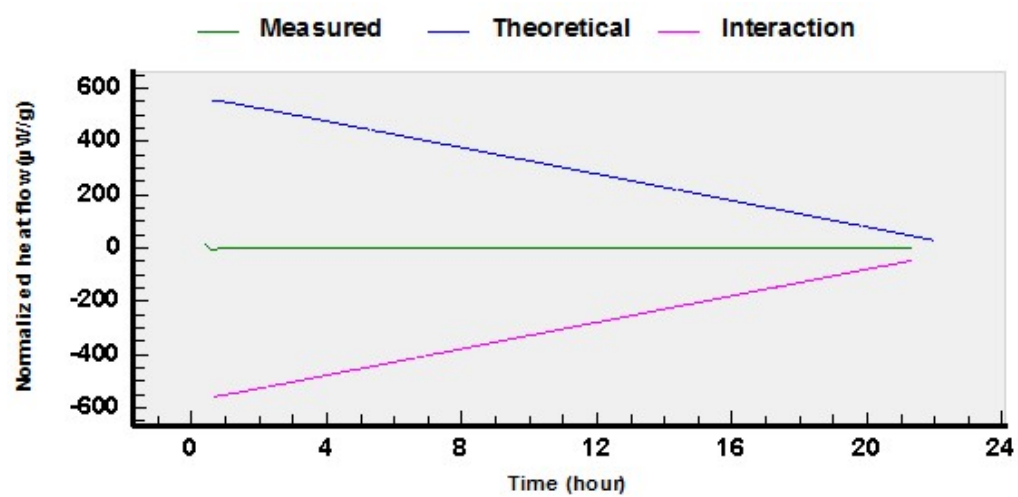


Fig. 6

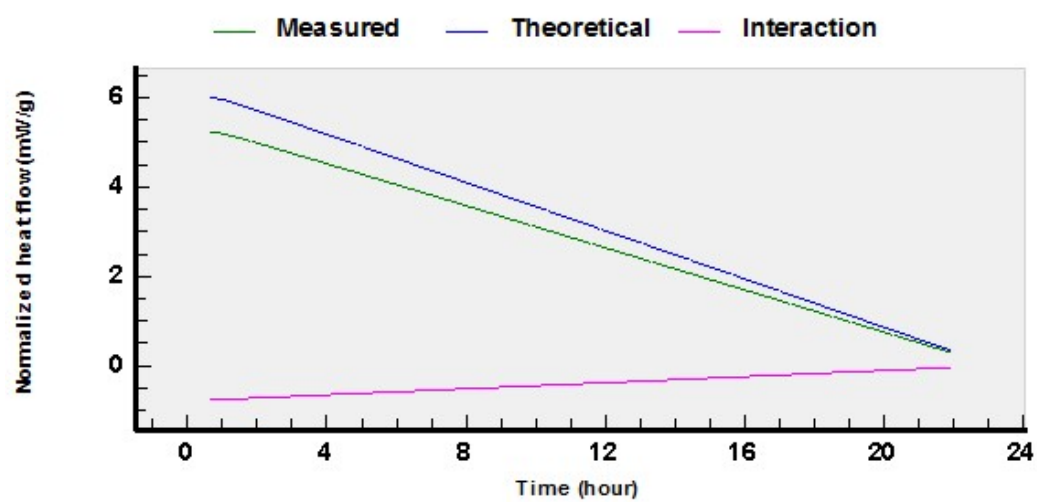


Fig. 7

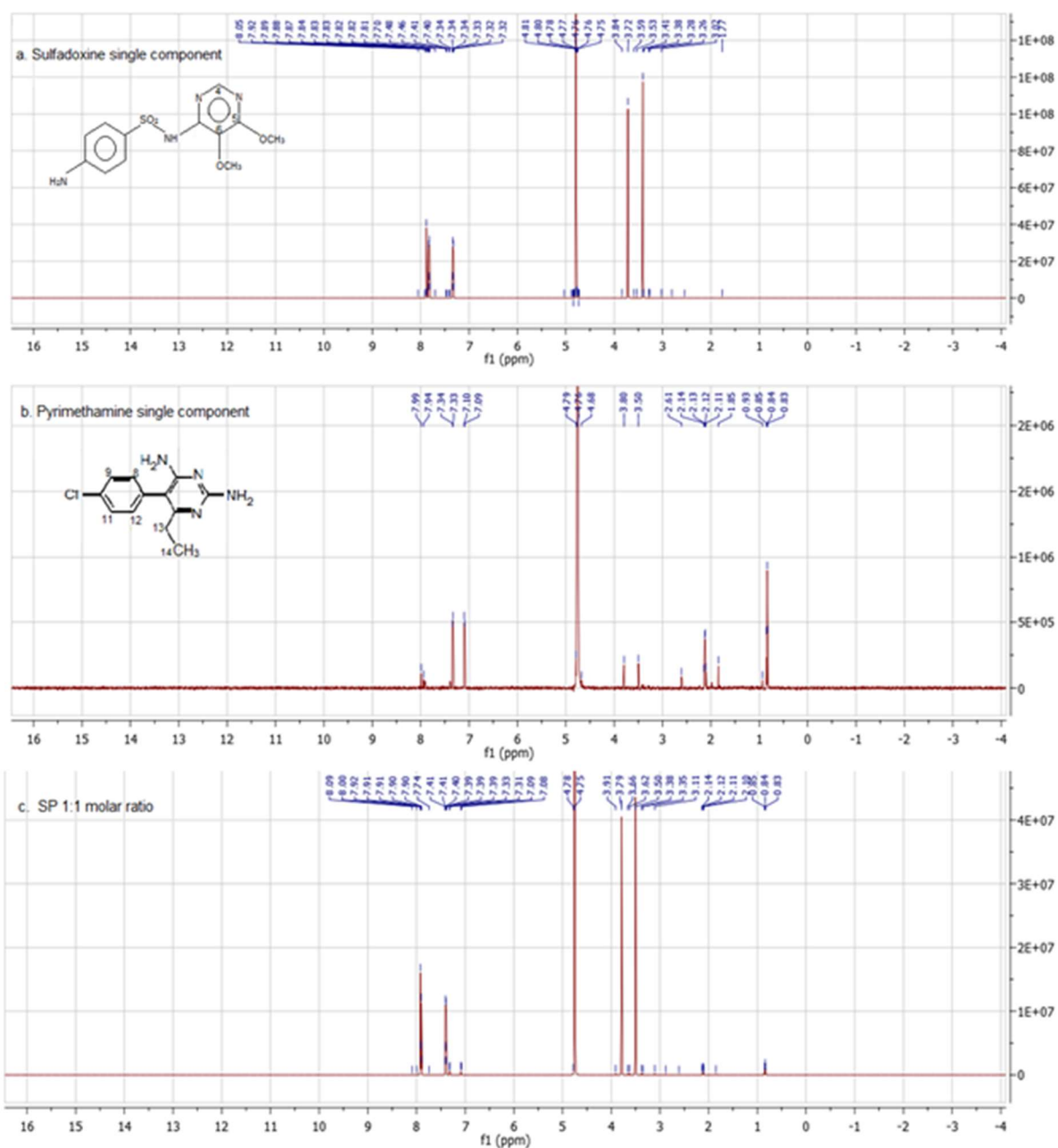


Fig.8