LC-MS/MS METHODS FOR THE QUANTIFICATION OF SULFASALAZINE AND SULFAPYRIDINE IN VARIOUS MATRICES: APPLICATION TO A PHARMACOKINETIC STUDY

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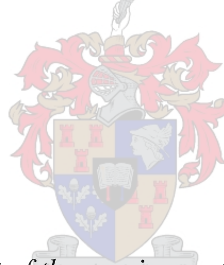

Thesis presented in partial fulfilment of the requirements for the degree of Master of Sciences (Pharmacology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

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Abstract

Introduction: An early phase clinical trial which took place at The Mercy Hospital for Women in Australia assessed the use of sulfasalazine as a treatment for preterm pre-eclampsia. This project consisted of the development and validation of a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method according to the Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines to simultaneously quantitate sulfasalazine and its metabolite, sulfapyridine, in placenta for pharmacokinetic analysis.

Methods: A Shimadzu 8040 mass spectrometer was operated in multiple reaction monitoring (MRM) mode to monitor the mass-to-charge (m/z) transition of the protonated precursor ions m/z 398.90 and m/z 250.07 to the product ions m/z 381.05 and m/z 156.00 for sulfasalazine and sulfapyridine, respectively. Sulfasalazine-d4 and sulfapyridine-d4 were used as internal standards. 100 µL of placental tissue homogenate was extracted using acetonitrile:methanol (90:10, v/v) and the supernatant was eluted through hydrophilic-lipophilic balanced cartridges. The extraction procedure was followed by liquid chromatographic separation using a Poroshell C_{18} column. Gradient elution using a mobile phase combination of water + 0.1% formic acid (A) and acetonitrile:methanol (90:10, v/v) + 0.1% formic acid (B) was used. Accuracy and precision were assessed over three consecutive, independent runs. The ratios of analyte peak area to internal standard peak area were plotted against the nominal concentrations to generate a calibration curve which fits a quadratic regression (weighted by 1/x, x= concentration) over the range 30-30 000 ng/mL for both sulfasalazine and sulfapyridine.

Results and Discussion: The average accuracy of calibration standards during inter-day validations ranged from 94.2-103.2% (%CV= 1.4-10.8) for sulfasalazine and 96.6-103.4% (%CV= 1.4-8.3) for sulfapyridine. The accuracy of quality controls ranged from 101.6-112.7% (%CV= 4.4-6.7) and 97.4-108.4% (%CV= 3.7-10.0) for sulfasalazine and sulfapyridine, respectively. Endogenous matrix components were shown to have no impact on the reproducibility of the method when placental tissue from six different sources were analysed. The average recovery of sulfasalazine and sulfapyridine from placental tissue homogenate was 121.5% and 119.6%, respectively. Autosampler stability experiments indicated that placental tissue homogenate extracts were stable on instrument for up to 48-hours at the method-defined temperature. Re-injection reproducibility experiments illustrated that the method remained accurate and precise for analysis of both analytes following a re-injection of a batch for up to 48 hours after the initial injection. Furthermore, sulfasalazine and sulfapyridine were found to be stable in placental tissue homogenate for 10 days when stored at -80 °C, for six hours when left on bench at room temperature, and when subjected to three-freeze thaw cycles. Upon analysis of patient samples (n= 9), the concentrations ranged from 491-4201 ng/g tissue for sulfasalazine and 637-26756 ng/g tissue for sulfapyridine, with two patient samples below the limit of quantitation (BLQ) of the assay for both analytes.

Conclusion: An LC-MS/MS method for the quantification of sulfasalazine and sulfapyridine in human placenta was successfully validated and applied to a clinical study to evaluate the efficacy of sulfasalazine as an intervention for pre-eclampsia.

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Opsomming

Inleiding: ŉ Vroeë-fase kliniese proef wat by The Mercy Hospital for Women in Australië gedoen is, het die gebruik van sulfasalasien as behandeling vir premature pre-eklampsie geassesseer. Hierdie projek het die ontwikkeling en bevestiging behels van ŉ vloeistofchromatografie-tandemmassaspektrometrie-metode (LC-MS/MS-) ingevolge die riglyne van die Food and Drug Administration (FDA) en die European Medicines Agency (EMA) om terselfdertyd sulfasalasien en die metaboliet daarvan, sulfapiridien, vir farmakokinetiese ontleding in die plasenta te kwantifiseer.

Metodes: ŉ Shimadzu 8040-massaspektrometer is in meervoudige reaksiemoniteringsmodus (MRM-) gebruik om die massa-tot-lading-oorgang (m/z-) van die geprotoneerde voorloper-ione m/z 398.90 en m/z 250.07 na die produk-ione m/z 381.05 en m/z 156.00 vir sulfasalasien en sulfapiridien, onderskeidelik, te moniteer. Sulfasalasien-d4 en sulfapiridien-d4 is as interne standaarde gebruik. 100 µL plasenta-weefselhomogenaat is met asetonitriel:metanol (90:10, v/v) onttrek en die bodrywende stof is met hidrofilies-lipofilies-gebalanseerde patrone geëlueer. Die ekstraksieprosedure is gevolg deur vloeistofchromatografiese skeiding met ŉ Poroshell C_{18}-kolom. Gradiënt-eluëring is gedoen met behulp van ŉ mobiele fasekombinasie van water + 0.1% metanoësuur (A) en asetonitriel:metanol (90:10, v/v) + 0.1% metanoësuur (B). Akkuraatheid en presisie is oor drie opeenvolgende, onafhanklike lopies geassesseer. Die verhoudings van die analiet-piekoppervlakte tot die standaard piekoppervlakte is aangestip teen die nominale konsentrasies om ŉ kalibreringskromme te lewer wat pas by ŉ kwadratiese regressie (beswaar met 1/x, x= konsentrasie) bó die reeks 30-30 000 ng/mL vir sulfasalasien en sulfapiridien.

Resultate en Bespreking: Die gemiddelde akkuraatheid van kalibreringstandaarde tydens interdaaglikse bevestigings het gewissel van 94.2 tot 103.2% (%CV= 1.4-10.8) vir sulfasalasien en van 96.6 tot 103.4% (%CV= 1.4-8.3) vir sulfapiridien. Die akkuraatheid van gehaltebeheermaatreëls het gewissel van 101.6 tot 112.7% (%CV= 4.4-6.7) en van 97.4 tot 108.4% (%CV= 3.7-10.0) vir sulfasalasien en sulfapiridien, onderskeidelik. Daar is getoon dat endogene matrikskomponente geen impak op die herhaalbaarheid van die metode het nie, deur plasentaweefsel uit ses verskillende bronne te ontleed. Die gemiddelde onttrekking van sulfasalasien en sulfapiridien uit die plasentaweefselhomogenaat was 121.5% en 119.6%, onderskeidelik. Stabiliteitsproewe met ŉ outomatiese monsternemer het aangedui dat uittreksels uit die plasentaweefselhomogenaat instrumentstabiel was vir tot en met 48 uur teen die temperatuur wat vir die metode omskryf is. Herinspuitingsherhaalbaarheidsproewe het getoon dat die metode akkuraat en presies vir ontleding van albei analiete bly, ná die herinspuiting van ŉ bondel tot en met 48 uur ná die aanvanklike inspuiting. Voorts is bevind dat sulfasalasien en sulfapiridien vir 10 dae lank stabiel in plasentaweefselhomogenaat bly as dit teen -80 °C geberg word, vir ses ure as dit teen kamertemperatuur op die werksbank gelaat word en ook as dit aan drie vries-ontdooi-siklusse onderwerp word. Met ontleding van die pasiëntmonsters (n= 9) het die konsentrasies gewissel van 491 tot 4201 ng/g weefsel vir sulfasalasien en 637 tot 26756 ng/g weefsel vir sulfapiridien, met twee pasiëntmonsters wat onder die kwantifiseringsperk (BLQ) van die toets vir albei analiete is.

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Gevolgtrekking: ’n LC-MS/MS-metode vir die kwantifisering van sulfasalasien en sulfapiridien in menslike plasenta is suksesvol bevestig en toegepas op ’n kliniese studie om die doeltreffendheid van sulfasalasien as ingryping vir pre-eklampsie te evalueer.

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Failure is inevitable when you're pushing the limits of knowledge

Erika Hamden

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This thesis is dedicated to my parents – Louis and Adrienne – who have been a constant source of support and encouragement throughout this milestone.

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Outline

A description of the work presented in each chapter is outlined below:

Chapter 1 provides a brief introduction and context for this MSc project, as well as the aims and objectives for this study.

A literature review providing a detailed overview of pre-eclampsia followed by clinical features and diagnosis of the disorder, identification of risk factors, and the study of its pathophysiology has been presented in **Chapter 2**. Possible clinical relevance of sulfasalazine as a treatment for preterm pre-eclampsia has been evaluated, together with other drugs and combination of drugs that have been assessed as a treatment for this disorder. Furthermore, the mechanism of action of sulfasalazine and its metabolites were examined.

This MSc project consisted of the development and validation of Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) methods for the detection and quantitation of sulfasalazine and sulfapyridine in various matrices. Therefore, the analytical techniques and processes involved in LC-MS/MS have been described in **Chapter 3**. Moreover, a summary and discussion of the analytical methods that have previously been reported for the simultaneous and individual quantification of sulfasalazine and sulfapyridine has been provided. Lastly, a brief description of LC-MS/MS validation criteria has been described.

Chapter 4 provides an overview of the LC-MS/MS infusion process, and the challenges encountered, particularly with sulfapyridine, during LC-MS/MS method development and validation for the quantification of sulfasalazine and sulfapyridine in maternal- and umbilical cord- plasma.

The experimental techniques used to develop and validate an LC-MS/MS method to quantify sulfasalazine and sulfapyridine in placental tissue homogenate have been presented in **Chapter 5**. The results of the method are discussed, and a chapter summary was provided.

The data obtained from the patient samples have been reported and discussed in **Chapter 6**.

Chapter 7 presents overall conclusions, study limitations, and future work.

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List of Abbreviations and Symbols

%: Percentage

%CV: Coefficient of variation

~: Approximately

°C: Degrees Celsius

2-ME: 2-Methoxyestradiol

5-ASA: 5-Aminosalicylic acid

ACN: Acetonitrile

APCI: Atmospheric pressure chemical ionisation

API: Atmospheric pressure ionisation

APPI: Atmospheric pressure photoionisation

AT_1 receptor: Angiotensin II type-1 receptor

AT_1AAs: Angiotensin II type-1 receptor autoantibodies

BLQ: Below limit of quantitation

CAD: Collision-activated dissociation

CID: Collision-induced dissociation

C_{max}: Maximum plasma concentration

COMT: Catechol-O-methyltransferase

DMSO: Dimethyl sulfoxide

EGFR: Epidermal growth factor receptor

EMA: European Medicines Agency

EP: European Pharmacopoeia

ESI: Electrospray ionisation

ET-1: Endothelin-1

FDA: United States Food and Drug Administration

g: Gram

GFR: Glomerular filtration rate

H_2O: Water

HELLP syndrome: Haemolysis, Elevated Liver enzymes, Low Platelets

HIC: Hydrophobic interaction chromatography

HIF-1α: Hypoxia-inducible factor-1α

HILIC: Hydrophilic interaction chromatography

HLB cartridges: Hydrophilic-lipophilic balanced cartridges

HO-1: Haem oxygenase-1

HPLC: High performance liquid chromatography

IBD: Inflammatory bowel disease

IEC: Ion-exchange chromatography

IL-6: Interleukin-6

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ISTD: Internal standard

LC: Liquid chromatography

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LLE: Liquid-liquid extraction

LLOQ: Lower limit of quantification

LOD: Limit of detection

LOQ: Limit of quantification

m/z: Mass-to-charge ratio

M: Molar

MeOH: Methanol

mg/mL: Milligram per millilitre

mg: Milligrams

miRNA: MicroRNA

Mitochondrial ETC: Mitochondrial electron transport chain:

mL: Millilitre

mm Hg: Millimetres mercury

mM: Millimolar

M_{mi}: Monoisotopic mass

MMP14: Matrix metalloproteinase-14

MMP9: Membrane metalloproteinase 9

MRM: Multiple reaction monitoring

NF-κB: Nuclear factor kappa B

ng/g: Nanogram per gram

ng/mL: Nanogram per millilitre

ng: Nanogram

NK cells: Natural killer cells

NO: Nitric oxide

NPC: Normal-phase chromatography

pH: Potential of hydrogen

pKa: Acid dissociation constant

PlGF: Placental growth factor

PP: Protein precipitation

QCs: Quality controls

ROS: Reactive oxygen species

RPC: Reversed-phase chromatography

R_t: Retention time

RUPP: Reduced uterine perfusion pressure

SEC: Size-exclusion chromatography

sENG: Soluble endoglin

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sFlt-1: Soluble fms-like tyrosine kinase

SOP: Standard operating procedure

SP: Sulfapyridine

SP-d4: Sulfapyridine-d4

SPE: Solid phase extraction

SS: Stock solution

SSZ: Sulfasalazine

SSZ-d4: Sulfasalazine-d4

STDEV: Standard deviation

STDs: Calibration standards

TCA: Trichloroacetic acid

TGF-β: Transforming growth factor- β

TIC: Total ion count

TLC: Thin-layer chromatography

TNF-α: Tumour necrosis factor-α

ULOQ: Upper limit of quantification

UV: Ultraviolet

VCAM-1: Vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

WS: Working stock solution

µg/mL: Microgram per millilitre

µg: Microgram

µL: Microlitre

µM: Micromolar

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CHAPTER 1

Introduction

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1.1 Background

Pre-eclampsia is a common, yet serious complication occurring during pregnancy and is the leading cause of maternal, foetal, and neonatal death and disability [1][2]. Currently, there are no medical treatments available for pre-eclampsia, thus delivering the pregnancy is the only option to stop disease progression [1][2]. However, when delivery occurs at an early gestation, newborns may be subjected to serious risks associated with prematurity [1][2][3][4][5][6][7][8][9]. A treatment that stabilises the disease, allowing for the safe prolongation of pregnancy, would be a major advance in the field of Obstetrics and Gynaecology. Recent literature describes several *in vitro* experiments that were conducted to investigate the hypothesis that sulfasalazine, a prodrug initially developed for the treatment of rheumatoid arthritis and inflammatory bowel disease (IBD), has the potential to mitigate key features of pre-eclampsia due to its anti-inflammatory and antioxidant properties [1][2][10]. Furthermore, sulfasalazine is considered safe for use during pregnancy and continues to be administered to pregnant patients diagnosed with IBD or rheumatoid arthritis [1][2].

Based on the promising results obtained from the *in vitro* experiments, an early phase clinical trial has taken place at The Mercy Hospital for Women in Australia to assess the use of sulfasalazine as a treatment for preterm pre-eclampsia (ACTRN12617000226303) [11]. Ten patients with preterm (between 24-34 weeks gestation) pre-eclampsia were recruited from this hospital and treated with 1.5 g sulfasalazine twice daily, 12 hours apart, until delivery. The primary objective of the early phase clinical trial was to determine the safety and pharmacokinetic profile of sulfasalazine administered to pregnant patients diagnosed with preterm pre-eclampsia. The secondary objective was to determine the effects of sulfasalazine on the clinical and biochemical markers of pre-eclampsia, and to compare the length of gestation prolongation to a historical cohort.

As part of this MSc project, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) methods were developed to simultaneously quantify sulfasalazine and its main metabolite, sulfapyridine, in various biological matrices. By using these established analytical methods to measure the concentration of sulfasalazine and sulfapyridine in samples obtained from patients treated with the investigational drug during the clinical trial, the pharmacokinetic profile was evaluated. Overall, this collaborative study contributed towards improving maternal and perinatal outcomes globally.

1.2 Aims and Objectives

The overall aim of this project was to aid in the pharmacokinetic analysis of sulfasalazine and sulfapyridine in various matrices with application to a clinical study that evaluated the efficacy of sulfasalazine as an intervention for preterm pre-eclampsia.

Objectives of this project were as follows:

1. To develop and validate sensitive LC-MS/MS methods according to the United States Food and Drug Administration (FDA) [12][13] and the European Medicines Agency (EMA) [12][14] guidelines for the simultaneous quantitation of sulfasalazine and sulfapyridine in maternal- and umbilical cord- plasma and placenta.

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2. To determine suitable, reproducible extraction methods of sulfasalazine and sulfapyridine from the various matrices.
3. To ensure sensitivity, specificity, suitability, and robustness of the analytical methods.
4. To perform pre-validation experiments as well as inter- and intra-day validations in terms of accuracy, precision, sensitivity, specificity, and quantitation limit.
5. To describe the pharmacokinetics of each compound when administered at the above-mentioned dosing strategy. This will provide information on the changes in blood levels of the drug in maternal- and umbilical cord- plasma over time.
6. To establish if the drug sufficiently distributes to the site of action (i.e., the placenta) and at what concentration the drug and its metabolite are present.

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CHAPTER 2

Sulfasalazine as a Treatment for Preterm Pre-eclampsia

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2.1 Introduction

Pre-eclampsia, one of the "great obstetrical syndromes" [15][16], is a complication unique to pregnancy and is often described as a hypertensive disorder originating in the placenta [1][3]. This disorder may be defined as either "term pre-eclampsia", occurring after the 34th week of gestation (late onset), or "preterm pre-eclampsia" which occurs before 34 weeks' gestation (early onset) [4]. Jointly, term and preterm pre-eclampsia are known to affect 5-8% of pregnancies worldwide [1][17], resulting in 500 000 stillbirths or neonatal deaths and 60 000 maternal deaths, annually [1]. Pre-eclampsia is especially problematic in developing countries because the rate of maternal mortality and preterm births are 20 times higher in comparison to the rates in developed countries [18]. However, due to a lack of understanding of the pathological mechanisms responsible for pre-eclampsia, developing medicinal treatments for this disorder has proven to be challenging [19][20].

The only definitive treatment for pre-eclampsia is delivery of the foetus and placenta as symptoms typically resolve within 48-72 hours after delivery [1][2][3][4][5][6][7][8][9][21]. As a result, clinicians are often compelled to deliver the baby earlier to prevent disease progression and maternal morbidity (e.g., serious injury to maternal organs). However, in the case of preterm pre-eclampsia, newborns may be subjected to serious complications associated with prematurity, such as severe disability, cerebral palsy, chronic lung disease, retinopathy of prematurity, intracerebral bleeding, and mortality [1][2][3][4][5][6][7][8][9][22]. Thus, the risks associated with preterm pre-eclamptic deliveries are much higher as opposed to delivery following late onset pre-eclampsia, demonstrating that a treatment for preterm pre-eclampsia will result in a significant improvement within the field of Obstetrics and Gynaecology.

Only a few candidate drugs proposed for the treatment of pre-eclampsia have been tested in human clinical trials [22]. Symptomatic treatment of pre-eclampsia involves the use of steroids (to improve foetal lung maturity), antihypertensive drugs, magnesium as a prophylaxis of seizures, and bed rest [3]. However, despite improvements in perinatal care, the overall frequency in the occurrence of pre-eclampsia has not decreased [20][23][24]. Outcomes for both the mother and foetus may be improved by ensuring early detection, diagnosis, monitoring, and appropriate management and care [3][6][20]. Therefore, developing or repurposing a medical treatment that delays the disease process will allow for pregnancies to continue to a gestation where foetal outcomes are drastically improved, reducing the burden on hospitals caused by iatrogenic premature delivery [2][9][22][25][26].

2.2 Clinical Features and Diagnosis

Pre-eclampsia may present as either a maternal or foetal syndrome [20]. Maternal syndrome includes hypertension and proteinuria with or without other multisystem malfunctions [20]. Maternal complications associated with pre-eclampsia include a severe pre-eclampsia variant known as HELLP syndrome (Haemolysis, Elevated Liver enzymes, and Low Platelets) (10-20%) [3][6][21][27][28], pulmonary oedema (2-5%), acute renal failure (1-5%), abruptio placentae (1-4%), eclampsia (<1%), liver failure or haemorrhage (<1%), stroke (rare), death (rare) and long-term cardiovascular morbidity [3][20]. Foetal syndrome may be described as abnormal oxygenation, reduced amniotic fluid, and foetal growth restriction [20]. Several complications are

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associated with the foetus during pre-eclampsia such as preterm delivery (15-67%), foetal growth restriction (1-25%), perinatal death (1-2%), hypoxia-neurological injury (<1%), and long-term cardiovascular morbidity as a result of low birthweight [3][20]. **Table 2.1** summarises the clinical manifestations of pre-eclampsia and the complications related to both maternal and foetal syndrome. The severity of complications associated with pre-eclampsia will be dependent on the severity of disease processes, how far along the pregnancy is at the time of diagnosis, delivery, quality of management, and the presence or absence of pre-existing associated medical conditions [20].

Table 2.1 - A summary of the manifestations of pre-eclampsia and the complications associated with both maternal and foetal syndrome of the disease.

Maternal Syndrome		**Foetal Syndrome**	
Manifests as hypertension and proteinuria [20]		Manifests as abnormal oxygenation, reduced amniotic fluid, and foetal growth restriction [20]	
Complication [3][20]	**Frequency** [3][20]	**Complication** [3][20]	**Frequency** [3][20]
HELLP Syndrome	10-20%	Preterm delivery	15-67%
Pulmonary oedema	2-5%	Foetal growth restriction	1-25%
Acute renal failure	1-5%	Perinatal death	1-2%
Abruptio placenta	1-4%	Hypoxia-neurological injury	<1%
Eclampsia	<1%		
Liver failure or haemorrhage	<1%		
Stroke	Rare		
Death	Rare		

After 20 weeks' gestation, pre-eclampsia may be diagnosed by the onset of hypertension and proteinuria [3][15][17][20][21][27][29][30]. Hypertension, expressed in millimetres mercury (mm Hg), may be defined as a systolic blood pressure of 140 mm Hg or a diastolic blood pressure of 90 mm Hg that occurs twice, 4-6 hours apart, in women with a history of normal blood pressure [3][15][17][20][21][27][29][30]. It is currently a matter of debate whether or not proteinuria, the excretion of more than 300 mg of protein within 24 hours, is required to be present for diagnosis because pre-eclampsia may present prior to the development of serious glomerular capillary endotheliosis [3][4][15][17][20][21][27][29][30]. In the absence of proteinuria, pre-eclampsia may be diagnosed if hypertension is accompanied by visual or cerebral disturbances which persist, renal insufficiency, pulmonary oedema, abnormal or elevated liver enzymes, and epigastric or right upper-quadrant pain with nausea, vomiting, or thrombocytopenia [4][20]. Pre-eclampsia may be considered life-threatening in the case of multiorgan involvement such as seizures, pulmonary oedema, oliguria (<500 mL/day), thrombocytopenia (platelet count of <100 000/μL), abnormal liver enzymes combined with continuous epigastric or right upper-quadrant pain, or intense central nervous system symptoms (e.g. headaches, changed mental state, blurry vision or blindness) which persist [3][20][30].

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2.3 Risk Factors

Given that healthy pregnancies are already a state of systemic inflammation, pre-eclampsia is merely an exaggeration of normal maternal inflammatory responses occurring during pregnancy, rather than a separate entity [20][31]. Thus, pregnant women will be pre-disposed to pre-eclampsia by factors that further enhance the maternal inflammatory responses [20][32]. For example, recent studies have shown that rheumatic disease or infections such as periodontal disease, chlamydia, urinary tract infections, and cytomegalovirus are linked to the development of pre-eclampsia [20][27][33][34]. Medical disorders such as chronic hypertension, renal disease, gestational diabetes mellitus, and pre-existing hypercoagulable states may also increase the likelihood of pre-eclampsia [3][6][20][21][27][29][35]. Furthermore, obesity and insulin resistance elevate a woman's risk of developing pre-eclampsia, however the exact mechanisms by which these factors give rise to the disorder are unknown [3][6][20][21][27]. Counterintuitively, the risk for pre-eclampsia is reduced by 50% in a dose-dependent manner if the mother smokes during the entire pregnancy [3][20][21][27][29]. Evidence has revealed that exposure to cigarette smoke and carbon monoxide formed during smoking may positively affect endothelial function and angiogenic markers, thus lowering the incidence of pre-eclampsia [3][20][21][27][29]. However, smoking during pregnancy is not advocated because of other detrimental effects on the mother and foetus [29][36].

If a woman has been diagnosed with pre-eclampsia in previous pregnancies, the possibility of developing this disorder in subsequent pregnancies will be higher [3][20][27]. Conditions such as multifoetal gestations and hydatidiform mole are both related to increased placental mass and are associated with a higher probability of developing pre-eclampsia [3][4][6][21][35][37]. Furthermore, genetics or maternal susceptibility genes (i.e., the diagnosis of pre-eclampsia in a first-degree relative) may increase a woman's risk of developing pre-eclampsia by 2- to 4- fold [3][20][21][27][29][35]. It has been reported that extremes of maternal age (very young women or women >40 years of age) may contribute to an elevated risk of developing pre-eclampsia [6][20][29], however, additional literature suggests that pre-eclampsia is considered a disorder of first pregnancy, thus will often occur in young women [4][38].

Dekker *et al*. (2011) describe pre-eclampsia as "a couple's disease with maternal and foetal manifestations" [39]. Several studies have established the importance of paternal factors, known as the "dangerous father" hypothesis, whereby men who have previously fathered a pregnancy complicated by pre-eclampsia are twice as likely of doing so again with a new partner, regardless of whether or not the women has previously been diagnosed with this disorder [4][20][21][27][39]. An additional theory, known as the "primipaternity" hypothesis, may be described as an event whereby the maternal immune system develops tolerance to paternal alloantigens after sperm and/or seminal fluid exposure [4][20][27][29][38][39]. The primipaternity hypothesis explains why the possibility of developing pre-eclampsia is higher in women who have had a different partner since their previous pregnancy, those utilizing barrier methods of birth control, and those undergoing assisted reproduction that involves artificial insemination [4][20][27][29][38][39].

To summarise, pre-eclampsia may be defined as heterogeneous. It is evident that there are several risk factors involved in developing this disorder, and each risk factor or combination of risk factors could present with varying pathogeneses.

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2.4 Pathophysiology of Pre-eclampsia

A brief overview of the pathophysiology of pre-eclampsia has been presented in **Figure 2.1**, a schematic diagram that describes several factors which may result in pre-eclampsia. The figure also illustrates the overall response of the placenta to dysfunction and ischaemia. The pathogenesis of pre-eclampsia may be described by a two-stage model. The first stage may be initiated by poor placentation occurring early on during pregnancy. This has been confirmed by various studies that show a relationship between decreased placental blood flow prior to the 20th week of gestation and an increased risk of developing pre-eclampsia [5][35][37][39]. During the second stage, the maternal endothelium responds to atypical placentation. [5][21][35][37][39].

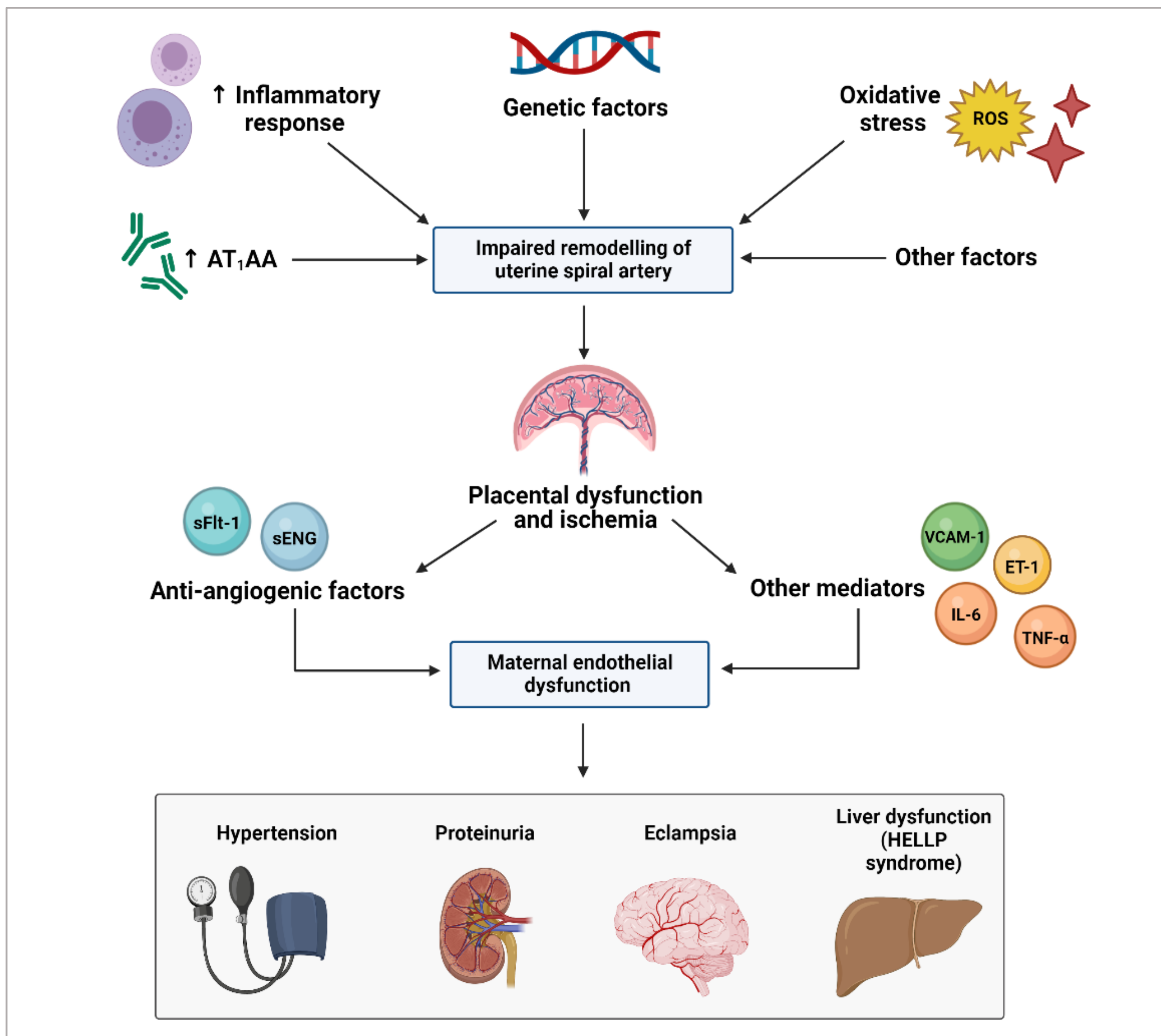

Figure 2.1 – A schematic diagram to illustrate the pathogenesis of pre-eclampsia. Figure created with BioRender.com [40].

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2.4.1 Placental Vascular Development

Following a pre-eclamptic diagnosis, delivering the placenta improves the condition and alleviates disease symptoms, thus implicating this organ in the development of the disease [5][28][41]. It is well-established that unique vascular remodelling is associated with the successful formation and arrangement of the placenta (i.e., placentation) during pregnancy [16][42]. If placental vascular development is disturbed, serious complications such as pre-eclampsia and foetal growth restrictions may occur [5][43][44].

During a typical pregnancy, spiral arteries in the myometrium of the uterus undergo a physiological transformation, resulting in increased uterine blood flow that supports foetal growth and ensures adequate perfusion of the placental intervillous space [4]. Cytotrophoblasts, a subset of placental cells, lose their epithelial markers (E-cadherin and $\alpha_6\beta_3$ integrin) and acquire endothelial markers (vascular endothelial-cadherin and $\alpha_v\beta_3$ integrin), allowing the conversion from an epithelial phenotype to an invasive endothelial phenotype [16][21][41][43]. Cytotrophoblasts are then able to invade the arterial wall of the myometrium and decidua early on in normal placental development [4][17][21][43][41]. An enzyme known as membrane metalloproteinase-9 (MMP-9) is responsible for demolishing the tunica media of maternal spiral arteries, allowing the vasculature to transform from narrow diameter-high resistance vessels to large diameter-low resistance vessels [4][17][42][43].

During pre-eclampsia, cytotrophoblasts fail to adopt an invasive endothelial phenotype, negatively affecting endovascular invasion [16][41][43]. Spiral arteries remain narrow with high-resistance, causing a reduction in placental size and restricted utero-placental blood flow that does not meet the needs of the developing foetus [3][4][5][21][41][42][45]. Consequently, placental ischaemia and hypoxia develops [3][4][5][21][41][42][45], resulting in the elevation of hypoxia-inducible factor 1α (HIF-1α), an indicator of cellular oxygen deficiency [4][6][46]. Furthermore, a particular group of microRNAs (miRNAs), namely miRNA-24, miRNA-26a, miRNA-103, and miRNA-181a, have been reported to be elevated in pre-eclampsia via a crucial involvement of HIF in response to low oxygen [8].

2.4.2 Maternal Endothelial Dysfunction

Although pre-eclampsia originates in the placenta, the maternal endothelium is greatly affected. Studies have indicated that pre-eclampsia is associated with elevated levels of vasoconstrictors such as endothelin-1 (ET-1) and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) [11][25][47]. Moreover, an increase in TNF-α will result in the upregulation of vascular cell adhesion molecule-1 (VCAM-1), a biomarker of endothelial injury [25]. Other unbalanced endothelial injury markers that occur in pre-eclampsia include soluble tissue factor, soluble E-selectin, and platelet-derived growth factor (PDGR) [21]. An elevation in factors which impact vascular integrity will cause significant physiological disturbances such as end-organ ischaemia and subsequently, oedema, convulsions, cerebral ischaemia, liver failure, proteinuria, and fluid build-up in the abdomen and between the layers of the pleura outside the lungs [43]. Therefore, pre-eclampsia is associated with endothelial dysfunction because compromised blood vessels lose their typical, fundamental homeostatic functions and develop new pathological functions, resulting in the inability of endothelial cells to regulate vasodilation and vasoconstriction [3][11][21].

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2.4.3 Anti-angiogenic Factors

Utero-placental ischaemia occurs due to poor placental vascular development and causes the production of anti-angiogenic factors such as soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sENG) [3][4][43]. sFlt-1 and sENG are released into maternal circulation and are responsible for the clinical presentation of the disorder [3][4][43]. Literature indicates that pre-eclampsia is associated with an upregulation in the expression of anti-angiogenic factors, and diminished expression of pro-angiogenic factors such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) [3][21][5]. Thus, possible therapies should be aimed at re-establishing a balance between the biological activities of pro-angiogenic factors relative to anti-angiogenic factors [3].

2.4.3.1 Soluble fms-like Tyrosine Kinase

When vascular endothelial growth factor receptor-1 (VEGFR-1), also known as Flt-1, is alternatively spliced, sFlt-1 is produced [3][21]. sFlt-1 is the soluble form of VEGFR-1 and binds to VEGF-A, VEGF-B, and PlGF with high affinity [48]. sFlt-1 consists of the extracellular ligand-binding domain of Flt-1, however it lacks the transmembrane and intracellular signal domain [3][37][42][49]. When sFlt-1 is released into circulation, it binds to pro-angiogenic factors such as VEGF and PlGF, blocking the ability of these molecules to interact with their endogenous full-length receptors [3][28][42]. This may result in endothelial dysfunction and will negatively affect the production of new blood vessels [3]. Furthermore, the sequestration of VEGF by sFlt-1 results in decreased VEGF-mediated nitric oxide (NO) production and, consequently, the stimulation of systemic hypertension [17]. Following delivery of the placenta and foetus, sFlt-1 levels return to normal, and hypertension and proteinuria will improve [3]. Several studies have reported that, in rodent models, overexpression of sFlt-1 causes hypertension, proteinuria, and glomerular endotheliosis, a syndrome resembling that of pre-eclampsia in humans, thus implicating sFlt-1 in the disease pathology [3][21][28][50]. Furthermore, using microarray chips, gene expression profiling of placental tissue from women with and without pre-eclampsia revealed that mRNA expression for sFlt-1 is dramatically upregulated in pre-eclamptic patients [42].

2.4.3.2 Soluble Endoglin

Endothelial cells and syncytiotrophoblasts express endoglin, a cell surface co-receptor of transforming growth factor-β (TFG-β) [21][28]. sENG, a truncated form of endoglin, has been reported to be up-regulated in pre-eclampsia [3][21]. sENG binds to and antagonises TGF-β, a cytokine that contributes towards the production of blood vessels [48]. Subsequently, NO levels are decreased and mechanisms of homeostasis that are essential for the maintenance of healthy vasculature are disrupted [48]. sENG levels have been reported to be elevated in reduced uterine perfusion pressure (RUPP) rat models of pre-eclampsia [3][21]. Additionally, studies performed in pregnant rats have indicated that vascular damage that induces a severe pre-eclampsia-like disorder is mediated by sFlt-1 and amplified by sENG, illustrating that these anti-angiogenic factors may act together [3][21][48]. Moreover, due to similarities in the gestational patterns of sFlt-1 and sENG, it has been proposed that these factors are potentially controlled by a common upstream pathway [3].

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2.4.4 Podocyturia and Glomerular Endotheliosis

Pre-eclampsia is associated with renal dysfunction that occurs as a result of a deficiency in podocyte-specific VEGF [17]. VEGF ensures the proper phenotype and function of glomerular endothelial cells, and is a rapid and efficient inducer of vascular permeability [51][52] However, sFlt-1 prevents VEGF from binding to its endothelial cell and podocyte receptors, which damages the glomerular filtration barrier, and ultimately results in glomerular endotheliosis, podocyte injury, kidney dysfunction and proteinuria [17][21]. The glomerular filtration barrier consists of an endothelial layer, the glomerular basement membrane, and podocytes [17]. Podocytes control permeability of the glomerular barrier thus limiting entry of proteins into the urine [17]. However, podocyturia, the occurrence of live podocytes in the urine, signifies ongoing podocytic damage associated with pre-eclampsia, and may occur as a result of decreased podocyte nephrin expression [17][37]. Furthermore, glomerular endotheliosis, a major element of a pre-eclamptic kidney, is identifiable by endothelial swelling with evident loss of endothelial fenestrae and noticeable glomerular capillary narrowing [17][21][37][53]. Although glomerular endotheliosis is characteristic of pre-eclampsia, Strevens *et al.* (2003) reported that slight glomerular endotheliosis may also occur in pregnancies uncomplicated by pre-eclampsia, primarily in a subset of individuals with gestational hypertension [21][53]. These results indicate that endothelial dysfunction present in pre-eclamptic patients may be an exaggeration of standard physiological processes that occur towards the end of a term pregnancy [21].

2.4.5 Catechol-O-Methyltransferase and 2-Methoxyestradiol

2-Methoxyestradiol (2-ME), a natural metabolite of oestradiol, has been suggested to increase trophoblast invasiveness and is generated in the placenta by catechol-O-methyltransferase (COMT) [3][16][17][54][55]. Therefore, during pre-eclampsia, incomplete cytotrophoblast invasion may occur because of reduced COMT and/or 2-ME expression [54]. In a study conducted by Kanasaki *et al*. (2008) it was shown that administration of exogenous 2-ME reversed the pre-eclamptic phenotype in COMT knockout mice, illustrating the potential role of 2-ME as a therapeutic agent [54]. Moreover, the same study performed Western blot analyses of six human placentas acquired from normal term gestations and six human placentas obtained from pre-eclamptic term gestations and established that COMT expression is reduced during pre-eclampsia [54]. However, in contradicting evidence, a study performed by Palmer *et al*. (2011) reported that insignificant differences were present in human placental COMT expression in severe pre-eclampsia when compared to term or preterm normotensive cohorts, suggesting that severe pre-eclampsia may not be associated with reduced placental expression of COMT [55]. Discrepancies in the results between these two studies may have occurred due to differences in sample size and gestational time between the two studies. Kanasaki *et al* performed the human placental analyses of term gestations in a limited cohort (normal pregnant women, n=6; pre-eclamptic women, n=6), whereas Palmer *et al.* performed human placental analyses in early-onset pre-eclampsia in a much larger cohort (normal term pregnancies, n=14; pre-term pregnancies not complicated by pre-eclampsia, n=8; pregnancies complicated by severe early-onset pre-eclampsia, n= 22). Therefore, the role of COMT and 2-ME in pre-eclampsia needs to be examined further but is beyond the scope of this study.

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2.4.6 Immunology

Alteration in the production of inflammatory cytokines and activation of components from the innate immune system, such as natural killer (NK) cells, neutrophils, monocytes, and dendritic cells, have been implicated in the pathophysiology of pre-eclampsia [17]. NK cells modulate immune tolerance, induce angiogenic factors, contribute towards vascular remodelling, and account for 70% of local lymphocytes at the maternal-foetal interface during the first trimester of pregnancy [21][42]. Studies involving NK cell-deficient mice have shown manifestations of defective decidual vessel remodelling, reinforcing the important role of NK cells in the maintenance and support of the pregnancy [42]. Moreover, recent genetic studies have suggested that human leukocyte antigens (HLAs) may influence susceptibility to pre-eclampsia [21]. Invading cytotrophoblasts express rare and distinctive combinations of three class I molecules, namely HLA-G, HLA-E, and HLA-C [55]. HLA-C, a major ligand for killer immunoglobulin-like receptors (KIR) on NK cells, shows considerable polymorphism which is dependent on the father's contribution [55]. Because KIR interact with specific foetal trophoblast cell markers, certain combinations of HLA-C and KIRs are associated with pre-eclampsia as they do not allow for optimum trophoblast invasion [35][55]. These findings illustrate involvement of the maternal immune system in controlling placentation and depth of cytotrophoblast invasion during pregnancy [55].

2.4.7 Oxidative Stress

Oxidative stress may arise when the production of reactive oxygen species (ROS) such as superoxide, hydroxyl radical, and hydrogen peroxide overwhelm the central antioxidant defence mechanisms in tissues [4][5][56][57][58]. In contrast to a normal pregnancy, the smooth muscles in the spiral arteries remain responsive to external signals during a pre-eclamptic pregnancy [5]. Therefore, the risk of sporadic blood flow and inconsistent oxygenation of the intervillous space is magnified, resulting in intermittent hypoxia and reoxygenation [4][5][31][59][56]. Subsequently, the production of uric acid, superoxide, NADPH and hydrogen peroxide is elevated [4][5][31][59][56]. Moreover, during pre-eclampsia the blood vessels remain narrow and transport blood to the placenta at a high velocity [5]. This causes cytotrophoblast shedding whereby the resulting fragments activate the immune system, contributing towards endothelial cell injury [5][17]. It has been suggested that a combination of vitamin C and E may be effective in preventing pre-eclampsia, as they act in synergy to prevent lipid peroxidation [60]. Furthermore, vitamin E has anti-inflammatory properties that may be particularly efficacious at treating this disorder. The complex role of oxidative stress in pre-eclampsia warrants further investigation of antioxidants as potential interventions, however this is beyond the scope of the study.

2.4.8 Haem Oxygenase

Free haem, a pro-oxidant molecule which is a vital source of free radicals in humans, is produced via the breakdown of circulating red blood cells [4]. Haem oxygenase (HO) is an inducible cytoprotective antioxidant enzyme which provides protection against free haem by converting it into biliverdin and then bilirubin, causing a subsequent release of carbon monoxide and free iron [4][61][62]. This reaction is of utmost importance because biliverdin and bilirubin possess potent antioxidant properties, and carbon monoxide promotes angiogenesis, induces vasodilation and has anti-apoptotic properties [4][61]. There are three protein variants of HO, namely

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HO-1, HO-2, and HO-3 [4]. HO-1 is upregulated by nuclear factor erythroid 2-related factor 2 (Nrf2) [58][62]. When Nrf2 is activated, it will translocate to the nucleus and upregulate antioxidant genes such as HO-1 [62]. It has also been suggested that HO-1 reduces sFlt-1 expression, thus it is generally accepted that pre-eclampsia is associated with reduced HO-1 activity [3][4][62]. This statement may be supported by a study illustrating that HO-1-deficient mice display defective vasculogenesis, hypertension and elevated circulating levels of sFlt-1 [3]. However, in humans, evidence of lowered HO-1 expression in pre-eclampsia is limited to only two studies, one of which has a limited sample size [62][63] and the other does not account for gestational age [62][64]. Moreover, functional studies on molecular regulation were conducted by Cudmore *et al*. (2007) in primary human placental and endothelial tissues, providing experimental data indicating that HO-1 negatively modulates the production of anti-angiogenic factors [65]. Conflicting evidence by Tong *et al*. (2015) suggests that there is no variability in mRNA expression or protein levels of HO-1 in pre-eclamptic placentas in comparison with gestationally matched controls [62]. Additionally, silencing of HO-1 or Nrf-2 in primary cytotrophoblasts did not alter sFlt-1 secretion after 24 or 48 hours [62]. However, the same study reported that silencing of HO-1 selectively enhanced mRNA expression of sFlt-1 il3 (predominantly expressed in endothelial cells) but did not increase mRNA expression of sFlt-1 e15a (largely expressed in the placenta) [62]. It is evident that the role of HO-1 in pre-eclampsia is unclear and requires further research.

2.4.9 Endoplasmic Reticulum Stress

The endoplasmic reticulum is in control of cellular protein synthesis and folding, and also plays a critical role in detecting cellular stress [66]. When nutrient and oxygen transport are not adequate enough to completely process proteins, endoplasmic reticulum stress and a characteristic "unfolded protein response" (UPR) occurs whereby protein synthesis is reduced and ROS formation is increased [5][66]. It has been reported that increased endoplasmic stress and evidence of the UPR have been observed in pre-eclampsia [67].

2.4.10 Mitochondrial Electron Transport Chain

In an experiment performed by Brownfoot *et al*. (2016), it was reported that mitochondrial electron transport chain (ETC) activity is elevated in the placenta of preterm pre-eclamptic patients in comparison to gestationally-matched control subjects [25]. Consistent with these findings, experiments which were performed revealed that the mitochondrial ETC positively regulates the secretion of sFlt-1 [25]. This study examined the use of metformin, an inhibitor of mitochondrial ETC activity, as a potential treatment for pre-eclampsia [25]. Metformin appeared to reduce sFlt-1 and sENG from primary human tissues, possibly by inhibition of the mitochondrial ETC [25]. Furthermore, in a study performed by Hastie *et al*. (2019) it was reported that signalling pathways of both the mitochondria and the epidermal growth factor receptor (EGFR) control sFlt-1 secretion [68].

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2.4.11 Angiotensin II Type-1 Receptor Autoantibodies

Research using animal models has suggested that the angiotensin receptor and agonistic autoantibodies are involved in the sequence of events leading to pre-eclampsia [5]. Angiotensin II plays a vasoactive role on blood vessels (i.e., arteries and veins) as it is responsible for constriction of smooth muscle, heartbeat elevation, and increased blood pressure [69]. During a normal pregnancy, the vasculature becomes less responsive to angiotensin II, whereas in pre-eclampsia, women experience increased sensitivity to the effects of angiotensin II, a change which may be identified as early as the 24th week of gestation [4][70]. The potential underlying cause of enhanced response to angiotensin II may include defective immune responses, genetic predisposition, and triggers from the environment [4].

It has been suggested that angiotensin II type-1 receptor autoantibodies (AT_1AAs) are present at elevated levels in pre-eclamptic sera compared to the serum of non-pregnant women or women who had normal pregnancies [4][70][71]. The angiotensin II type-1 (AT_1) receptor is activated when AT_1AAs bind to these receptors with high affinity, resulting in elevated quantities of intracellular calcium, activation of TNF-α pathways and intracellular MAP/ERK kinase [7]. AT_1AAs stimulate inflammatory factors, ROS production, and hypertensive mechanisms [7][70]. Moreover, literature has suggested that because sFlt-1 is downstream of AT_1 receptor activation, the upregulation of sFlt-1 and sENG may be mediated by AT_1AA [3][7]. When AT_1AAs bind to the AT_1 receptor, NADPH oxidase is activated and the activity of calcineurin and protein kinase C is increased [70]. This causes activation of transcription factors such as activating protein-1 (AP-1), nuclear factor kappa B (NF-κB), and nuclear factor activating T-cell (NF-AT) which are then translocated to the nucleus whereby an upregulation of target gene expression such as IL-6, tissue factor, ET-1, plasminogen activating inhibitor-1 (PAI-1), sFlt-1, sENG, and oxidative stress occurs [70]. The activation of AT_1 receptors by AT_1AAs on a human trophoblast cell line produces PAI-1 via the NF-AT pathway, which may account for the increase in intracellular calcium reported in pre-eclampsia. It has been suggested that if this occurs in the smooth muscle it will offer explanation for the constrictive elements of the disease [70].

A study performed by Amarel *et al*. (2018), used the RUPP rat model to illustrate that AT_1AAs isolated from both human pre-eclamptic patients and RUPP rats produce a pre-eclamptic-like phenotype when administered to normal, pregnant rodents [7]. In AT_1AA-injected mice it has been shown that a subsequent TNF-α blockade diminishes the vital features of pre-eclampsia such as hypertension and circulating sFlt-1 and sENG, demonstrating the chief role of TNF-α production in gestational hypertension [70]. It has also been shown that IL-6 infusion into pregnant rats causes increased renin activity and hypertension, diminished renal function, and stimulation of AT_1AA, whereas blood pressure and AT_1AA production remained unaffected when IL-6 was infused into non-pregnant rats [70]. The activation of AT_1 receptors by AT_1AAs may be blocked by AT_1 receptor antagonists such as losartan, which may cause a decrease in blood pressure and oxidative stress, and reduced levels of ET-1 and sFlt-1 [7][44][70]. However, losartan causes foetal toxicity and thus cannot be used during pregnancy [7].

Furthermore, peripheral blood from pre-eclamptic patients indicate elevated levels of a subset of B-cells, specifically $CD19^+CD5^+$, in comparison to patients who have normal pregnancies, suggesting the role of

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$CD19^+CD5^+$ in the production of AT_1AAs during pre-eclampsia [4]. Rituximab administration to RUPP rats causes depleted B cells and a reduction in AT_1AA, however this drug may have detrimental effects in the foetus because it crosses the placental barrier, causing newborn B-cell depletion [7]. It is evident that AT_1AA may contribute towards the pathogenesis of pre-eclampsia, and further research should be performed to explore its role.

2.5 Treatments for Pre-eclampsia

It is necessary to discover drugs that will reduce the disease process and allow for pre-term pre-eclamptic pregnancies to continue safely to a gestation where foetal outcomes are drastically improved [9][22][26]. Literature reports that for every additional day a pregnancy between 24-34 weeks gestation is extended, a consequent non-linear gain of 1% in foetal survival may occur [72]. An attractive possibility for discovering novel therapeutics is to repurpose drugs already known to be safe for use during pregnancy [11]. Furthermore, innovative targeted delivery systems such as nanoparticle delivery systems, originally developed and used by the oncology field, are being adapted to the obstetrics field to safely and directly administer therapeutics to the placenta and/or endothelium, combating safety concerns for the developing foetus [11].

2.5.1 Pravastatin

Statins, primarily used to lower cholesterol and to treat cardiovascular disease, are 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors [11][19]. It has been shown that statins have the ability to revert angiogenic imbalance through their pleiotropic effects and to restore endothelial dysfunction in animal models of pre-eclampsia [73][74]. It has also been reported that statins up-regulate haem oxygenase-1 (HO-1) (an antioxidant enzyme), decrease oxidative stress, reduce inflammation, and decrease expression of EGFR [19][68][74][75]. As opposed to other lipophilic statins, pravastatin is soluble in water, slowly crosses the placenta, and may have fewer adverse effects for the foetus [74].

Brownfoot *et al*. (2015) performed experiments using primary human tissues to assess whether pravastatin has the ability to eliminate endothelial dysfunction and to decrease secretion of sFlt-1 and sENG [19]. Results from this study indicated that pravastatin decreased endothelial cell, cytotrophoblast cell, and placental explant secretion of sFlt-1, but increased secretion of sENG from endothelial cells [19]. Furthermore, pravastatin caused an upsurge in endothelial cell migration and invasion and resulted in decreased markers of endothelial dysfunction on endothelial cells [19]. Following this study, Brownfoot *et al*. (2016) proceeded with further studies to determine the effects of simvastatin, rosuvastatin and pravastatin on the secretion of antiangiogenic factors and it was found that all statins decreased endothelial cell, trophoblast, and placental secretion of sFlt-1 but increased endothelial cell secretion of sENG [75].

A pilot randomised controlled trial was conducted by Costantine *et al.* (2016) to assess the safety and pharmacokinetics of pravastatin administered as a preventative treatment to pregnant women (12-16 weeks' gestation) who are at high risk of developing pre-eclampsia [73]. Data from the study indicated that none of the 10 subjects developed pre-eclampsia in the pravastatin group whereas four subjects out of 10 in the placebo group developed pre-eclampsia [73]. The results were preliminary but no identifiable safety risks were linked

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to pravastatin, justifying the use of this drug in a larger clinical trial at a higher dose [73]. Moreover, Ahmed *et al*. (2019) conducted a randomised, blinded, placebo-controlled trial (StAmP trial) to assess the effects of pravastatin on sFlt-1 levels in plasma during pre-eclampsia [76]. It was found that pravastatin did not improve PlGF levels or maternal sFlt-1 plasma levels following diagnosis of preterm pre-eclampsia and did not prolong gestation [76]. It is evident that pravastatin yielded promising data during pre-clinical laboratory studies, however both clinical trials discussed above were underpowered as the sample sizes were not large enough to answer the research question.

2.5.2 Metformin

During pre-clinical laboratory studies performed by Brownfoot *et al*. (2016), metformin, an oral drug administered to decrease blood glucose, was identified as a potential candidate for the treatment of preterm pre-eclampsia due to its ability to improve endothelial dysfunction and decrease the secretion of sFlt-1 and sENG [25][77]. Metformin is an inhibitor of HIF-1α and is safe for use during pregnancy [25]. Ischaemia/hypoxia occurs as a result of pre-eclampsia, and is associated with an upregulation of HIF-1α, prompting the exploration of HIF-1α inhibitors as a treatment for this disorder [25]. Based on the positive pre-clinical trial data obtained from Brownfoot *et al*. (2016) [25], Cluver *et al*. (2021) proceeded with a randomised, double blind, placebo controlled trial to investigate the effect of extended-release metformin on gestation prolongation in women diagnosed with pre-term pre-eclampsia [77]. Data from this trial indicated that pregnancy gestation was prolonged by 7.6 days following administration of 3 grams extended release metformin (1 gram three times daily) from time of randomisation to delivery in women diagnosed with preterm pre-eclampsia (26-32 weeks' gestation) [77]. Furthermore, it is possible that metformin decreased the length of hospital admissions in the neonatal nursery after birth [77]. In the future, multicentre trials should be performed in other countries to assess whether metformin is effective in other populations and health systems, given that the study performed by Cluver *et al*. was a single centre study and several women were diagnosed with conditions such as chronic hypertension, obesity, or HIV [77]. Overall, this novel research positively contributes towards the field of Obstetrics and Gynaecology because no previous clinical trials have evaluated the use of metformin to treat preterm pre-eclampsia.

2.5.3 Metformin and Sulfasalazine

Research has demonstrated that, independently, metformin and sulfasalazine have the ability to decrease secretion of antiangiogenic factors [1][25]. Brownfoot *et al*. (2020) conducted functional experiments using primary human placenta to assess the effect of a low dose metformin-sulfasalazine combination on sFlt-1 and sENG secretion, as well as on PlGF and VEGF-α expression [47]. The aim of this study was to determine whether the combination treatment may be more effective than either drug alone [47]. This study illustrated that low dose combination treatment with metformin and sulfasalazine decreased sFlt-1 and sENG secretion from cytotrophoblasts, increased VEGF-α expression, and diminished markers of endothelial dysfunction by lowering TNF-α-induced ET-1 mRNA expression [47]. The expression of PlGF was also increased, however it was not more significant than what was observed with sulfasalazine treatment alone [47]. Lastly, a lack of change was observed in VCAM expression which may have been attributed to the use of a dose that was not

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suitable enough to exert an effect, or as a result of an unsuitable time course of treatment [47]. The data from this study indicated that combination therapy has potential to treat pre-eclampsia, however future studies should investigate higher doses.

2.5.4 Esomeprazole

Esomeprazole, a proton pump inhibitor used to treat severe gastric reflux, is known to have antioxidant properties and is safe for use during pregnancy, even if administered during the first trimester [2]. It has been reported that esomeprazole decreases EGFR expression and has been shown to reduce markers of endothelial dysfunction (i.e., VCAM and ET-1) within *in vitro* and *ex vivo* models [2][68][78]. Research also suggests that esomeprazole is vasoactive *in vivo*, demonstrating its capability of preventing hypertension in a pre-eclamptic mouse model [2][78]. In a study conducted by Saleh *et al*., (2017) it was illustrated that administration of proton pump inhibitors during pregnancy reduced circulating levels of sFlt-1, sENG, and ET-1 [79]. However, a large epidemiological study reported that proton pump inhibitors did not reduce the overall incidence of pre-eclampsia [2][79]. Furthermore, a randomised control Phase II clinical trial was conducted to determine the efficacy of 40 mg esomeprazole administered daily to women diagnosed with preterm pre-eclampsia [2][22]. The study indicated that there were no benefits for the use of esomeprazole as a lone agent for the treatment of preterm pre-eclampsia, as it did not extend gestation or diminish concentrations of circulating sFlt-1 [2][22]. It has been proposed that perhaps a higher dose of esomeprazole may be necessary for a clinical effect to occur, and this should be examined in future clinical trials [2][22][11].

2.5.5 Esomeprazole and Sulfasalazine

Of importance, it has been demonstrated that, *in vivo,* a combination of low dose esomeprazole-sulfasalazine has greater potential to treat pre-eclampsia than either drug alone [2]. Results from this study indicate that esomeprazole-sulfasalazine additively decreases sFlt-1 secretion and mRNA expression of VCAM and ET-1, however it does not result in decreased ET-1 protein secretion or additive reduction of sENG [2]. These results suggest that it may be worth initiating further animal studies and clinical trials for the combination treatment of pre-eclampsia [2].

2.5.6 Sulfasalazine

Sulfasalazine is a prodrug that was initially developed as an antirheumatic agent, however it is mainly used for the treatment of inflammatory bowel disease (IBD) [10]. This drug has been investigated as a potential treatment for pre-eclampsia as it is safe to use during pregnancy, crosses the placental barrier, and has antioxidant and anti-inflammatory properties. However, sulfasalazine may inhibit folate synthesis, thus folic acid intake should be increased to 2 mg/day during treatment to minimise the baby's risk of developing neural tube defects [80][81].

Literature indicates that sulfasalazine reduces mRNA expression of two predominant sFlt-1 mRNA variants, specifically sFlt-1 il3 (widely expressed in numerous tissues and endothelium) and sFlt-1 e15a (highly expressed in the placenta), and decreases the secretion of sENG via the inhibition of matrix metalloproteinase-14 (MMP14), an enzyme which, under typical conditions, cleaves membrane-bound endoglin to create circulating sENG [1][25]. Sulfasalazine also upregulates HO-1 by producing ROS, which activates translocation

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of Nrf2, a transcription factor that binds to the antioxidant response element of the HO-1 promoter region [58]. Furthermore, sulfasalazine inhibits NF-κB, a transcription factor responsible for mediation of the immune response [58][82]. Under normal conditions in the cytoplasm, NF-κB remains inactive while bound to IκB (an inhibitor protein) [82]. However, upon activation of IκB, NF-κB degrades rapidly and free dimers translocate to the nucleus where they activate target genes, increasing expression of inflammatory markers [82]. Following sulfasalazine administration, IκB degradation is blocked and translocation of NF-κB into the nucleus is inhibited, consequently decreasing mRNA expression of inflammatory markers [82]. Contrasting literature reveals that cytotrophoblast secretion of sFlt-1 and sENG remains unmodified after silencing or overexpressing genes which are involved in the NF-κB pathway, suggesting non-involvement of anti-angiogenic factors in the NF-κB pathway [1]. This same study reported that sulfasalazine has the ability to lower cytotrophoblast sFlt-1 secretion despite silencing of HO-1, indicating that the drug's mechanism of action may not involve the NF-κB pathway or HO-1 when used as a treatment for pre-eclampsia [1]. Lastly, sulfasalazine targets the EGFR pathway by reducing mRNA expression of EGFR and by decreasing protein expression of EGFR key adaptor molecules, known as ERK1/2 and STAT3 [2][68]. sFlt-1 is regulated by the mitochondria and EGFR signalling pathways, hence decreased mRNA expression of EGFR as a result of sulfasalazine will be closely mirrored by a reduction in sFlt-1 secretion [25][68].

Brownfoot *et al*. (2019) conducted functional experiments using primary human pregnancy tissues to observe the effects of sulfasalazine on anti-angiogenic factors and PlGF secretion [1]. This study also examined the ability of sulfasalazine to decrease vital markers of endothelial dysfunction and to promote dilation of whole blood vessels [1]. Results from the study indicate that sulfasalazine reduces secretion of sFlt-1 and sENG and upregulates PlGF secretion from primary human placental tissues [1]. Moreover, in various *in vitro*/*ex vivo* assays, sulfasalazine was able to mitigate endothelial dysfunction, improve migration and proliferation of endothelial cells, and promote dilation of blood vessels and angiogenic sprouting from whole blood vessel rings [1]. Furthermore, TNF-α-induced VCAM-1 expression in human umbilical vein endothelial cells was reduced after treatment with sulfasalazine [1]. **Figure 2.2** illustrates key results obtained from the study [1]. Additionally, **Table 2.2** summarises treatments which have been or are currently being tested to treat pre-eclampsia and a brief proposed mechanism of action for each drug or combination of drugs has been provided.

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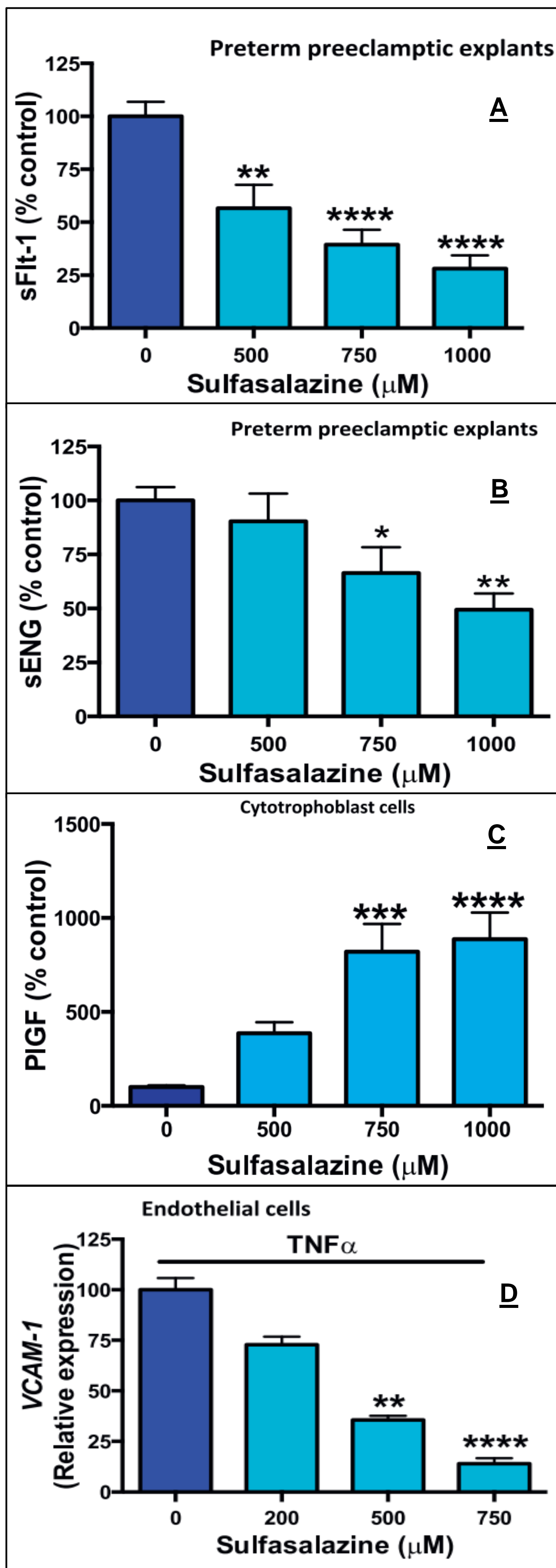

Figure 2.2 - Effect of sulfasalazine on **(A)** sFlt-1 and **(B)** sENG secretion; **(C)** PlGF secretion from cytotrophoblast cells; and **(D)** VCAM-1 transcription.

(A) Effect of sulfasalazine on sFlt-1 secretion in preterm pre-eclamptic explants:

Sulfasalazine dose-dependently reduces sFlt-1 secretion from preterm pre-eclamptic placental explants [1].

(B) Effect of sulfasalazine on sENG secretion in preterm pre-eclamptic explants:

Sulfasalazine reduces sENG secretion from preterm pre-eclamptic placental explants [1].

(C) Effect of sulfasalazine on PlGF secretion from cytotrophoblast cells:

Sulfasalazine dose-dependently increases PlGF protein secretion from cytotrophoblast cells [1].

(D) Effect of sulfasalazine on endothelial cell VCAM-1 transcription:

Sulfasalazine dose-dependently reduces TNF-α-induced VCAM-1 mRNA expression [1].

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Table 2.2 - Potential treatments which have been or are currently being tested for use as a treatment for pre-eclampsia. A brief mechanism of action has been proposed for each drug or combination of drugs.

Treatments	Mechanism of Action	References
Pravastatin	Reverts angiogenic imbalance and restores endothelial dysfunction (animal models). Upregulates HO-1, decreases oxidative stress, decreases inflammation, and decreases expression of EGFR.	[19], [68], [73], [74], [75]
Metformin	Inhibits HIF-1α, improves endothelial dysfunction and decreases secretion of sFlt-1 and sENG (pre-clinical studies). Prolongs gestation by 7.6 days and decreases length of hospital admissions of neonates (clinical trial).	[25], [77]
Metformin and Sulfasalazine	Decreases sFlt-1 and sENG secretion from cytotrophoblasts Increases VEGF-α expression Reduces TNF-α-induced ET-1 mRNA expression	[47]
Esomeprazole	Reduces EGFR expression. Decreases expression of endothelial dysfunction markers (VCAM-1 and ET-1). Vasoactive properties in vivo.	[2], [68], [78]
Sulfasalazine and Esomeprazole	Additively reduce sFlt-1 secretion and mRNA expression of VCAM and ET-1.	[2]
Sulfasalazine	Reduces mRNA expression of sFlt-1 il13 and sFlt-1 e15a. Decreases the secretion of sENG by the inhibiting MMP14. Upregulates HO-1 and inhibits NF-κB. Decreases mRNA expression of EGFR. Reduces protein expression of EGFR key adaptor molecules Upregulates PlGF secretion, mitigates endothelial dysfunction, improves migration and proliferation of endothelial cells, and promotes dilation of blood vessels and angiogenic sprouting from whole blood vessel rings. Dose-dependently decreases TNF-α-induced mRNA expression of VCAM-1.	[1], [2], [25], [58], [68], [82]

2.5.7 Other Drugs

More recently, drugs such as sofalcone, resveratrol, sildenafil citrate, and melatonin have been examined for the prevention and treatment of pre-eclampsia [11]. Both sofalcone and resveratrol are naturally occurring molecules that have antioxidant properties, suggesting their ability to combat oxidative stress associated with pre-eclampsia [11]. Additionally, these drugs increase placental levels of HO-1, diminish secretion of sFlt-1, and decrease markers of endothelial dysfunction [11]. Drugs such as NO, magnesium sulphate, aspirin, and corticosteroids have previously been administered to women diagnosed with pre-eclampsia, however these drugs do not delay the progression of pre-eclampsia and are considered as purely symptomatic treatments. Both NO and anti-platelet agents such as aspirin have vasodilatory effects, which rescue hypertension, a

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common clinical feature in pre-eclampsia [11][71][72][74]. However, limitations associated with NO use include tolerance due to side effects (e.g., headaches) [72]. In addition to vasodilation, aspirin corrects the prostacyclin and thromboxane imbalance present from the 13th week of gestation in women with a high risk of developing pre-eclampsia [11][71][74]. Moreover, corticosteroids are used to correct the development of foetal lungs and is particularly important in newborns as it may decrease respiratory deficiency and discomfort in the newborn and may improve foetal outcome [71]. Lastly, magnesium sulphate may be used to inhibit seizures or recurrent seizure episodes in eclampsia [74]. This drug has been shown to decrease the rate of seizures by 52% when compared to diazepam, and 67% when compared with phenytoin [74]. Other methods and treatments suggested to prevent or treat pre-eclampsia include magnesium or zinc supplementation, diet and exercise, protein or salt restriction, fish-oil supplementation, antioxidant vitamins for the treatment of oxidative stress, antihypertensive medications, calcium supplementation (as calcium deficiency has been associated with pre-eclampsia), and heparin. However, these treatments are either not recommended or insufficient evidence is available to recommend as a treatment [20][74].

2.6 Pharmacokinetics of Sulfasalazine and its Metabolites

Sulfasalazine, an inactive parent drug, is composed of sulfapyridine and 5-aminosalicylic acid (5-ASA) (also known as mesalamine or mesalazine) linked by an azo bond [10][83]. This linkage minimises absorption of the drug in the upper gastrointestinal tract [10] and as a result, roughly 10-30% of sulfasalazine is absorbed from the small intestine [82][83][84] and enterohepatic recycling occurs to a large extent [84]. Sulfasalazine is cleaved at the azo bond in the large intestine by distal ileum and colonic bacteria (azoreductases) to release the active constituents, sulfapyridine and 5-ASA [10][82][84][85]. Sulfapyridine is readily absorbed (>90%) from the large intestine [84][83], whereas 5-ASA is poorly absorbed (20-30%) from the large intestine and the remainder is excreted in the faeces [10][82][83][84][85]. 5-ASA is the useful, active component for treatment of IBD [10][83], whereas sulfasalazine and sulfapyridine are effective in treating rheumatoid arthritis [83][86]. Sulfasalazine that has been systemically absorbed is metabolised by the liver to sulfapyridine and 5-ASA [84]. A slight fraction of administered sulfasalazine is excreted unchanged in the urine [84][85]. The major route of metabolism of sulfapyridine occurs via acetylation by polymorphic N-acetyltransferase 2 (NAT2) in the liver to form N-acetyl-sulfapyridine, which is assumed to be inactive and is excreted in the urine [84][85][86]. Furthermore, sulfapyridine and N-acetyl-sulfapyridine undergo hydroxylation and glucuronidation [84][85]. The hydroxylated metabolites (5-hydroxy-sulfapyridine and N-acetyl-hydroxy-sulfapyridine) and the glucuronide conjugates are excreted in the urine [84]. 70-80% of 5-ASA is eliminated unchanged in the urine, although 5-ASA also undergoes pre-systemic and systemic acetylation by N-acetyltransferase 1 (NAT1) in the liver to form acetyl-5-mesalazine, which is inactive and excreted in the urine [84][85][86]. Lastly, in plasma, both sulfasalazine and sulfapyridine are bound to albumin, however sulfasalazine is extensively bound (>99%), whereas sulfapyridine is bound to a lesser extent (70%) [84].

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2.7 Chapter Conclusion

There has been significant progression in the knowledge about pre-eclampsia and its pathogenesis. Literature suggests that the interaction of reduced placental perfusion with modified maternal factors such as genetic, environmental, and behavioural factors, may be responsible for clinical pre-eclampsia [35]. Currently, no definitive treatments are available for women diagnosed with pre-eclampsia other than delivering the foetus and placenta, which may result in significant risks of preterm gestation complications associated with prematurity [1][3][4][6][7][8]. There are several treatments that have been tested or are currently being tested to treat preterm pre-eclampsia. However, sulfasalazine, a prodrug that has anti-inflammatory and antioxidant properties, offers a safe medical treatment for pre-eclampsia [1][2]. Sulfasalazine has the potential to diminish placental secretion of sFlt-1 and sENG and may improve maternal endothelial dysfunction present in pre-eclampsia [1]. Thus, sulfasalazine may stabilise the disease, allowing for the pregnancy to progress to a gestation that is safer for delivery of the baby, attenuating preterm gestation complications [1]. Sulfasalazine offers promising potential as a treatment or prevention for preterm pre-eclampsia and warrants investigation in clinical trials [1].

Additional research is necessary to further determine the relevance of anti-angiogenic factors and other serum markers in the pathological process of pre-eclampsia. It is important that future research defines the regulation of placental vascular development and the expression of angiogenic and anti-angiogenic factors in normal versus diseased pregnancies. Moreover, it is necessary to explore processes responsible for variability in maternal immune response. This will ensure the classification and use of reliable markers for early identification of pre-eclampsia and for the prediction of disease severity. Further research focusing on the role of circulating anti-angiogenic factors and other contributing factors in the development of pre-eclampsia will have exciting clinical implications which are prone to alter detection and treatment of this disorder in the near future.

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CHAPTER 3

Analytical Methods for the Detection of Drugs and Their Metabolites in Physiological Fluids

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3.1 Introduction

LC-MS/MS plays a large role in small-molecule drug discovery and development, and has several applications such as mass measurement, structure elucidation, and trace quantitation [87]. LC-MS/MS is the "gold standard" for bioanalytical testing of patient samples in pharmacokinetic studies, owing to its high sensitivity, selectivity, robustness, and linearity for a large number of sample extracts [87][88][89]. The determination of drug and metabolite levels in biological matrices allows for the optimisation of pharmacotherapy, aids in understanding therapeutic and toxic effects of the drug(s), and provides the basis for pharmacokinetic studies and studies focusing on patient compliance, bioavailability, genetics, organ function, and the influence of co-medication [90]. This chapter highlights the power of combining the high resolving power of liquid chromatography with the superior mass detection capability of mass spectrometry in quantitative and qualitative analyses.

3.2 Liquid Chromatography

Liquid Chromatography is a technique dependent upon an interplay of polarity of the analyte(s) of interest with the stationary phase and mobile phases, which enables the components within a mixture to be separated, identified, and purified [91][92]. The stationary phase is composed of a hydrophobic functional group chemically bonded to a silica support in a column, whereas the mobile phase, which is composed of a liquid, flows along or through the stationary phase in a definite direction [91][92][93][94]. High performance liquid chromatography (HPLC) is a modern form of liquid chromatography whereby the mobile phase is pumped at high pressure through small-particle columns, allowing for high-resolution separations [91][94]. The primary separation modes of HPLC include normal-phase chromatography, reverse-phase chromatography, ion-exchange chromatography, and size-exclusion chromatography [91]. Other separation modes include affinity chromatography, chiral chromatography, hydrophilic interaction liquid chromatography, hydrophobic interaction chromatography, electrochromatography, supercritical fluid chromatography, and thin-layer chromatography [91].

Reverse-phase chromatography is used in more than 70% of all HPLC analyses and is suitable for the analysis of analytes which range from polar to non-polar [91][95]. Separation is dependent on the analyte's partition coefficient between a non-polar stationary phase (e.g., hydrophobic C_{18} column) and a polar mobile phase, which consists of two components [91]. Mobile phase A is the aqueous, polar component, and mobile phase B, referred to as the organic modifier (e.g., methanol or acetonitrile), is used to vary the retention of analytes by reducing the overall polarity of the mobile phase [96]. When using LC-MS/MS, volatile additives such as formic acid or acetic acid, or salts such as ammonium acetate or ammonium formate may be added to the mobile phase to act as a buffer for pH control [95]. The use of non-volatile buffers and inorganic additives should be avoided during LC-MS/MS as they may possibly contaminate the MS and result in a large degree of ion suppression [95]. During reverse-phase chromatography, polar analytes elute first while non-polar analytes elute later due to their strong interaction with the hydrophobic C_{18} groups that form a "liquid-like" layer around the solid silica support of the column [91].

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3.3 Detection using Mass Spectrometry

There are several detectors that may be coupled with HPLC, such as ultraviolet, diode array, or mass spectrometer detectors [94][97]. Mass spectrometry is a technique that is highly selective and sensitive, and when coupled with HPLC, provides both quantitative and qualitative data [94]. A standard mass spectrometer consists of three major components, namely an ionisation source, the mass analyser and a detection system [87].

The separated species that elute in the mobile phase are sprayed into the atmospheric pressure ionisation (API) source of the mass spectrometer whereby they will be exposed to a set temperature, gas flow, and voltage to ensure that the mobile phase solvent is eliminated, and the analytes are ionised [87][95][97][98]. There are three main modes of ionisation/API techniques used to generate ions for mass spectrometry [99], namely electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), and atmospheric pressure photoionisation (APPI) [95][98]. ESI, the most dominant ionisation source, is used in 80-90% of LC-MS applications [87]. This soft ionisation technique relies on the application of high voltage at the capillary tip which assists in the transfer of ions from solution into the gaseous phase [95][98]. Three important processes occur to transfer sample ions from the HPLC eluent into the gas phase: droplet formation, desolvation, and gas phase ion formation [98]. Droplet formation occurs when the HPLC eluent is introduced into the electrospray source [100]. The capillary tip is charged and will produce charged droplets through electrophoretic charge separation [87][98][100]. **Figure 3.1** illustrates both (A) positive ion electrospray mode and (B) negative ion electrospray mode. In positive ion mode, the capillary tip acts as the positive electrode (i.e., anode) whereas the sampling aperture plate is the negative electrode (i.e., cathode) [98]. Positive ions within the eluent solution will be repelled from the inner walls of the capillary needle and will form a droplet of positive ions [98]. In negative ion mode , the opposite situation occurs whereby the capillary tip acts as the cathode and the sampling aperture plate will act as the anode, resulting in formation of a droplet that consists of predominantly negative ions [98]. Positive ion mode is most suitable for analytes such as bases that form cations in solution, and negative ion mode is suitable when analytes such as acids form anions in solution [98].

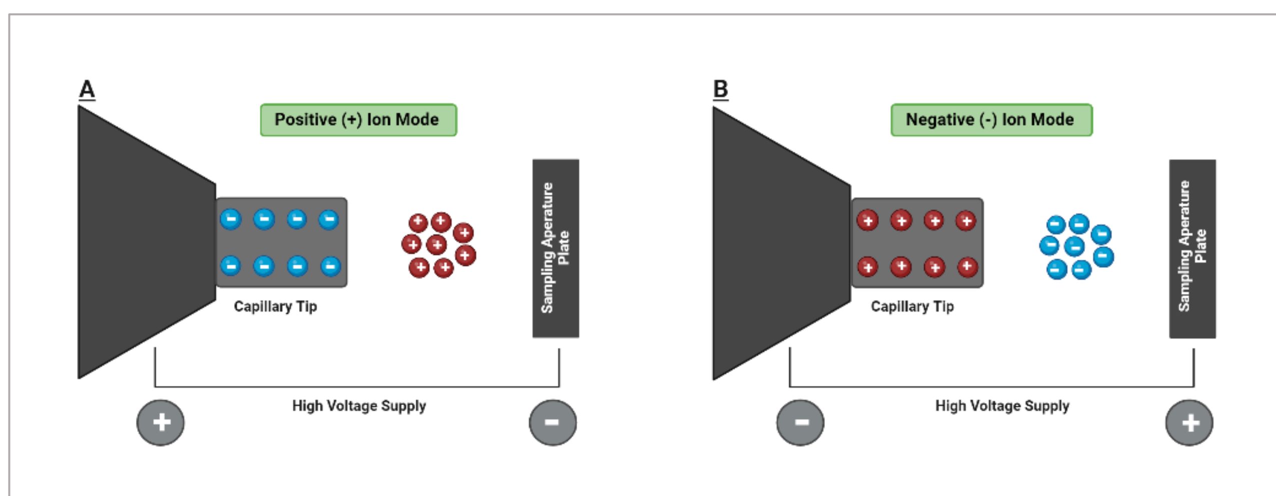

Figure 3.1 – A schematic diagram to represent an ESI source. (A) Positive ion mode whereby the capillary acts as the anode; (B) Negative ion mode whereby the capillary tip acts as the cathode. Figure has been adapted from Chromacademy.com [96] and created with BioRender.com [40].

Following the formation of droplets, a process known as desolvation occurs, whereby a counter flow of heated nitrogen drying gas evaporates the droplets, resulting in the formation of gas phase ions [100]. The skimmer cone will preferentially sample gas phase ions into the mass analyser. Popular analyser types include quadrupoles, time-of-flight, ion trap, and magnetic sector analysers [98]. Triple quadrupole LC-MS/MS, the technique used in this project, consists of a collision cell between two mass analysing devices [95][98]. Because of the triple quadrupole configuration, different scans such as precursor ion, product ion, constant neutral loss, single ion monitoring, or multiple reaction monitoring (MRM) scans may be performed [95][97]. For a series of quantitation of analytes, MRM has been reported be the most appropriate acquisition method [87][94][95]. During an MRM scan, gas phase ions will enter quadrupole 1, and will be subjected to an electric field that separates ions according to their mass-to-charge (m/z) ratio [98]. Once the precursor ion has been selected and stabilised in quadrupole 1, it will move into the collision cell (quadrupole 2) and collide with inert gas ions such as nitrogen or argon, causing them to fragment [98]. Next, the desirable product ions will be selected in quadrupole 3 and accelerated into the detector which counts the ions that emerge from the mass analyser and amplifies the signal generated by each ion [98]. Widely used detector types include electron multipliers, dynodes, photodiodes, and multi-channel plates [98]. The signal will then be interpreted by specialised software which will convert data into a typical chromatogram and a mass spectrum for each peak specific to that particular compound [98]. Both mass analysis and detection are carried out under a high vacuum [98]. Overall, mass spectrometry is a highly selective and specific technique owing to the unique fragmentation pattern generated for each analyte. The LC-MS/MS process is schematically illustrated in **Figure 3.2**.

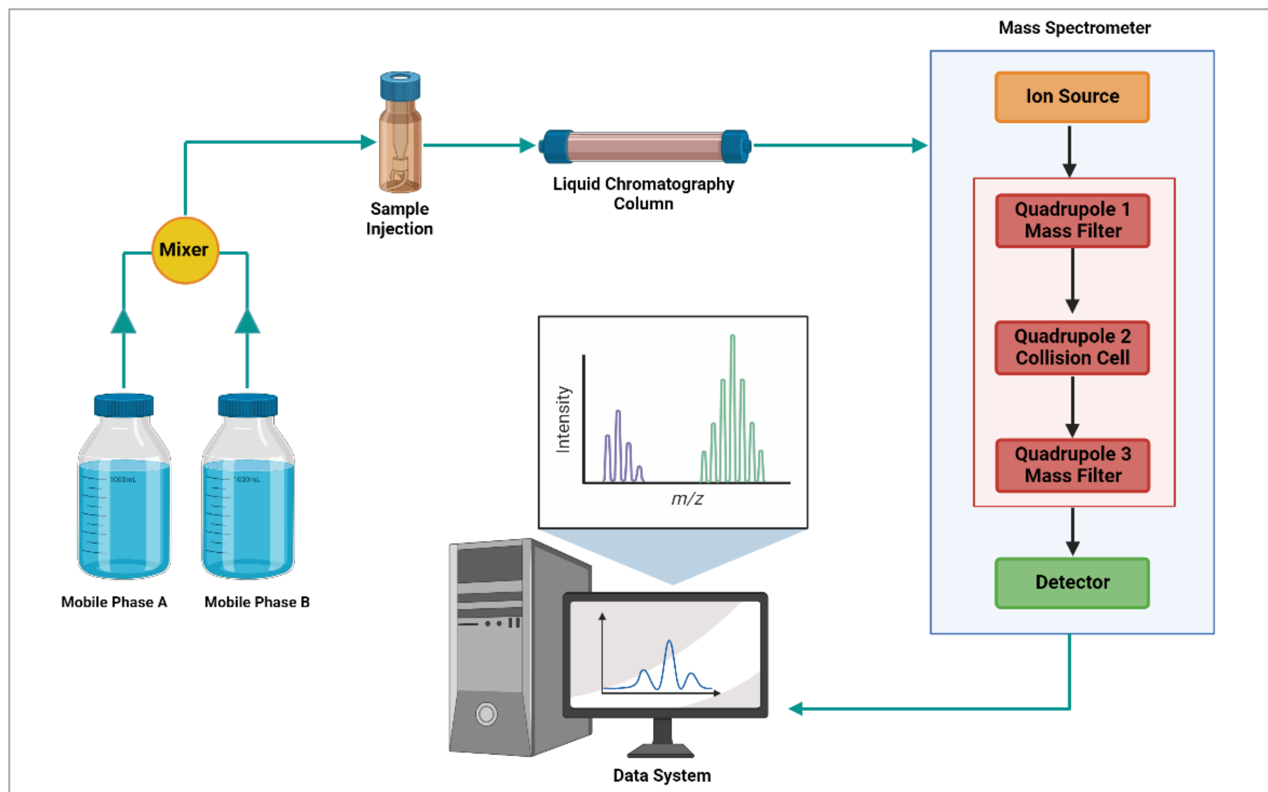

Figure 3.2 – A diagram to illustrate the LC-MS/MS process. Figure created with BioRender.com [40].

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3.4 Sample Preparation Techniques

Samples collected for the analysis of pharmaceuticals are usually present in complex matrices where the analytes or target compounds are present at low concentrations relative to other sample constituents such as proteins, salts, acids, bases, and organic compounds [99]. Therefore, it is necessary to carry out sample preparation techniques to concentrate the target analytes or to isolate them from species that may interfere with the analysis [90][94][101]. It has been reported that during the analytical process, over 80% of analysis time is spent on sample preparation steps [90]. Consequently, the choice of an appropriate sample preparation method directly influences the reliability, accuracy, sensitivity, selectivity, and robustness of the analysis, and also impacts assay throughput, analysis cost, and data quality [90][95][99][101]. To successfully accomplish the process of sample preparation, it is important for a scientist to understand the detection technology, the biological matrix, and analyte chemistries [101]. The following sample preparation techniques, which will be discussed briefly, produce sample extracts or solutions that are compatible with the LC-MS system [99].

3.4.1 Protein Precipitation

Protein precipitation is a straightforward sample preparation technique used in the bioanalysis of samples that have a relatively high abundance of proteins (e.g. plasma, whole blood, serum) to generate extracts that are partially clean for LC-MS quantitation [95][99]. Typically, protocols involve the addition of an organic solvent (e.g., methanol, acetonitrile, acetone), ammonium sulphate, or trichloroacetic acid (TCA) to the biological sample [99]. Because proteins are not soluble in organic solvents or neutral detergents, the addition of such agents alters the pH of the environment which interferes with intra-molecular hydrophobic interactions, thus decreasing protein hydration [101]. Ultimately, this causes proteins to denature, aggregate, and precipitate out of solution [101]. After the addition of a protein precipitation reagent, the sample mixture may be refrigerated to improve the efficiency of protein removal, and is followed by centrifugation to draw the protein precipitate to the bottom of the sample vial, leaving other components in the liquid layer [99][101]. The "protein-free" liquid is removed and may either be analysed directly, or evaporated to dryness and reconstituted in a more suitable solvent prior to analysis on LC-MS [99]. Common protein denaturation methods such as aggregation by salt, acid, and heat usually trap the analytes in the aggregate [101]. Thus, water miscible organic solvents are more commonly used as precipitation reagents to prevent analyte loss [101].

3.4.2 Liquid-Liquid Extraction

A liquid-liquid extraction (LLE) is based on the solubility of an analyte between two immiscible solvents, whereby the target analyte passes from the solvent of origin into a solvent in which the analyte is more soluble and the polarities are more compatible [99]. The immiscible liquids are separated by centrifugation, and the organic layer containing the analyte(s) of interest is removed, taking care not to withdraw the solvent too close to the solvent partition [99]. The organic solvent may then be evaporated to dryness and reconstituted into an appropriate solvent for LC-MS/MS analysis [89][99]. To increase analyte recovery, the extraction may be repeated [99]. Furthermore, to encourage the dissociation and movement of the analyte(s) from one phase to another, the pH of the solvents may be manipulated [99]. Thus, factors such as analyte solubility, pKa, and pH of the solution may affect the recovery and selectivity of analytes from solution [89]. Although LLE may result

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in cleaner extracts, it usually requires large volumes of solvents, which is costly [90]. Furthermore, the procedure is not amenable to automation because of the requirement of several disjointed steps [89].

3.4.3 Solid Phase Extraction

Solid phase extraction (SPE) relies on the partitioning of compounds between a liquid phase and a solid stationary phase sorbent contained in a cartridge whereby the intermolecular forces between the phases influence elution and retention [90][99]. Many sorbent chemistries (i.e. cartridge types) are available for the strategic and selective retention of target analytes or sample interferences [95][99]. Among the available sorbents such as mixed phase, silica-based reverse phase, ion-exchange phase, and polymer-containing hydrophilic moieties, C_{18} cartridges are the most popular for drug analysis [90][101]. Briefly, in SPE, the sample is percolated through a solid phase to retain analytes of interest, which are later eluted and recovered [90]. This technique enables the extraction of virtually all compounds from organic or aqueous matrices and offers selectivity and higher recoveries [90]. Furthermore, SPE uses relatively low quantities of solvents, allows for the preparation of multiple samples in parallel, and may be readily automated [89][90]. SPE protocols may be generic or can be optimised if an improvement in sample clean-up is desired [95]. A typical SPE protocol involves several steps [99]. Firstly, the sample needs to be pre-treated (e.g., dilutions, deprotonation, pH adjustments, and/or particulate removal via filtration or centrifugation) to ensure that it is compatible with the SPE procedure [99]. The column undergoes wetting and conditioning to activate the chromatographic sorbent, which ensures that the stationary phase will interact properly with the sample [99]. The pre-treated sample is then loaded onto the conditioned cartridge such that the compound is retained on the stationary sorbent [99]. The column undergoes a rinsing step which allows for undesired contaminants to be washed off the sorbent while target analytes remain retained [99]. An elution step is performed with a suitable solvent which disrupts interactions between the analyte and the stationary sorbent, allowing for the recovery of the desired analytes from the cartridges [99]. Finally, the eluted sample may be evaporated to dryness and reconstituted in a more appropriate solvent [99].

3.5 The Role of Internal Standards

An internal standard may be described as either (a) an isotopically labelled analogue of the analyte of interest, or (b) a structural analogue of the analyte of interest [95]. Internal standards may be added to samples during the extraction step to compensate for possible variability that may occur during sample preparation or on-instrument [95][101]. Stable isotopically labelled internal standards such as H^2 (D, deuterium), C^{13}, N^{15}, or O^{17} are suitable because they display similar sample preparation, retention, and ionisation properties as the analyte of interest [95]. However, isotopically labelled internal standards are often not available and may be very expensive, in which case structural analogues of the analytes of interest may be used [95].

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3.6 Literature Survey

3.6.1 Compound Summary

A compound summary of sulfasalazine and sulfapyridine is provided in **Table 3.1** below. This table outlines the solubility of the compounds as per the certificate of analysis obtained from Toronto Research Chemicals (TRC). Furthermore, the monoisotopic masses (M_{mi}) are indicated along with the product ions that have previously been reported for these compounds in the studies conducted by Gu *et al.* [83] and Choi *et al.* [102].

Table 3.1 - Relevant information regarding the solubility, molecular formulas, monoisotopic masses, and previously reported product ions for sulfasalazine and sulfapyridine.

Compound	Solubility	Molecular Formula	Monoisotopic Mass (M_{mi})	Previously Reported Product Ions $[M+H]^+$ as per Gu *et al.* [83]	Previously Reported Product Ions $[M+H]^+$ as per Choi *et al.* [102]
Sulfasalazine	DMSO, Methanol (sparingly)	$C_{18}H_{14}N_4O_5S$	398.0685	398.90 > 381.1; 317.2; 212.9; 223.5; 241.3; 286.8; 333.0	399.07 > 381.06; 223.05; 317.10; 119.01; 94.05; 147.02
Sulfapyridine	DMSO (slightly), Methanol (slightly)	$C_{11}H_{11}N_3O_2S$	249.0572	250.00 > 156.2; 183.9; 108.1; 157.2	250.06 > 108.04; 156.01; 184.08; 92.04

3.6.2 Previously Reported Analytical Techniques

Analytical techniques that have previously been developed for the individual or simultaneous quantitation of sulfasalazine and sulfapyridine in plasma are discussed below and summarised in **Table 3.2**. An extensive literature search was performed, however no LC-MS/MS or HPLC methods have been developed for the individual or simultaneous quantitation of sulfasalazine and sulfapyridine in placenta, thus development and validation of these methods are novel.

3.6.2.1 Simultaneous Quantitation of Sulfasalazine and Sulfapyridine in Plasma

A method published by Gu *et al.* (2011) [83] described the simultaneous quantitation of sulfasalazine and its two main metabolites, sulfapyridine and 5-ASA, in human plasma using a validated LC-MS/MS method. An API-3000 LC-MS/MS was operated in MRM mode using ESI. Separation was achieved using a XBP Phenyl column (2.1 x 100 mm, 5 µm). Gradient elution was performed using the following mobile phase combination: water with 2 mM ammonium acetate and 0.2% formic acid (A), and methanol with 2 mM ammonium acetate and 0.2% formic acid (B). Protein precipitation was achieved using 300 µL methanol (containing 50 ng/mL dimenhydrinate as an internal standard) to extract the compounds from 100 µL of plasma. A volume of 100 µL supernatant was removed and diluted with 100 µL water. The range of concentrations 10 -10 000 ng/mL ($r > 0.99$) for sulfasalazine and 10-1000 ng/mL ($r > 0.99$) for sulfapyridine and 5-ASA were used to establish a linear response function. This method was successfully used to determine the pharmacokinetics of sulfasalazine, sulfapyridine, and 5-ASA from 10 healthy volunteers who were administered a single dose of 250 mg sulfasalazine as an intervention for rheumatoid arthritis.

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A Liquid Chromatography-Time-of-Flight-Mass spectrometry (LC-TOF-MS) method was developed and published by Choi *et al.* (2021) [102] to identify sulfasalazine and sulfapyridine in mouse plasma. Chromatographic separation was performed on a reverse-phase C_{18} column (Phenomenex Kinetex XB-C18 column; 2.1 x 50 mm) using gradient elution. Mobile Phase A consisted of water and 0.1% formic acid, and mobile phase B was composed of acetonitrile and 0.1% formic acid. The calibration curve had an average correlation co-efficient of >0.99 over the concentration range of 9.15 - 6670 ng/mL. A protein precipitation extraction was used whereby 100 µL of acetonitrile containing internal standard (verapamil) was added to 20 µL of plasma. The samples were vortexed for 30 seconds and centrifuged at 13 000 x g for 5 minutes. The supernatant was removed and diluted three-fold with water before analysis.

The publications from Gu *et al.* [83] and Choi *et al.* [102] were used as a guide, with laboratory-specific adaptations, to develop and validate an LC-MS/MS method to quantitate sulfasalazine and sulfapyridine in maternal- and umbilical cord- plasma.

3.6.2.2 Individual Quantitation of Sulfasalazine and Sulfapyridine in Plasma

There have also been reports of methods developed for the individual determination of sulfasalazine and sulfapyridine in plasma, however these publications are outdated and only served as a guide where relevant.

An HPLC method was developed by Astbury *et al.* (1987) [103] to quantitate sulfapyridine and its acetyl metabolites in plasma using sulfamethoxazole (SMX) as an internal standard. For the extraction procedure, 200 µL methanol was added to 500 µL plasma. All samples were mixed with 1 mL of 1 molar (M) sodium acetate buffer (pH 4.7) and extracted into 8 mL dichloromethane. The organic layer was decanted, evaporated under nitrogen, and reconstituted in 250 µL mobile phase composed of methanol with 0.05 M phosphate buffer (pH 7.4) (25:75). Separation was achieved using a Spherisorb ODS analytical column (3.2 x 250 mm, 10 µm) and a Whatman CO:Pell ODS guard column (4.6 x 75 mm). The sulfapyridine and acetylated sulfapyridine concentration range was 0.5 - 20 µg/mL for single-dose pharmacokinetic studies or 2.0 - 40 µg/mL for steady-state concentrations. The assay was used to evaluate plasma samples of 45 patients with rheumatoid arthritis receiving long-term sulfasalazine (2 g/day) or sulfapyridine (1.25 g/day) therapy and for single-dose pharmacokinetic studies in eight rheumatoid arthritis studies following the administration of 2 g sulfasalazine.

Astbury *et al.* (1988) [104] also developed an HPLC method to quantitate sulfasalazine in plasma using a concentration range of 1000 - 20 000 ng/mL. As part of the extraction procedure, 100 µL methanol was added to 500 µL plasma and 100 µL of piroxicam solution was used as an internal standard. All samples were mixed with 1 mL of 1 M hydrochloric acid and extracted with 4 mL ethyl acetate. The organic layer was decanted, evaporated under nitrogen, and reconstituted in 500 µL mobile phase composed of acetonitrile and 0.05 M phosphate buffer (pH 7.9) (20:80, v/v). Separation was achieved using a LiChrosorb RP 18 analytical column (3.2 x 150 mm, 5 µm) coupled with a Whatman CO:Pell ODS guard column (4.6 x 75 mm). The assay was used to determine sulfasalazine plasma concentrations in patients with rheumatoid arthritis treated with single- and multiple-dose oral administration of sulfasalazine (2g/day).

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Table 3.2 - Summary of internal standards, columns, and solvents reported in the literature for the analysis of sulfasalazine and sulfapyridine in plasma using HPLC and LC-MS/MS methods.

Dose and Analyte	Analytical Method, Matrix and ISTD	Sample Preparation	LC Columns	Mobile Phases	Range (ng/mL)
Dose: single oral dose of 250 mg SSZ **Analyte:** SSZ, SP, and 5-ASA [83]	LC-MS/MS 100 µL human plasma **ISTD:** DHN at 50 ng/mL)	Protein precipitation of 100 µL plasma with 300 µL methanol and 50 ng/mL of ISTD. 100 µL supernatant was mixed with 100 µL water.	XBP Phenyl column (2.1 x 100 mm, 5 µm)	**Mobile Phase A:** H_2O, 2 mM ammonium acetate, 0.2% formic acid **Mobile Phase B:** Methanol, 2 mM ammonium acetate, 0.2% formic acid	**SSZ:** 10-10000 ng/mL **SP, 5-ASA:** 10-1000 ng/mL
Dose: N/A **Analyte:** SSZ, SP [102]	LC-TOF-MS 20 µL human plasma **ISTD:** Verapamil	Protein precipitation of 20 µL plasma with 100 µL methanol and 50 ng/mL of ISTD. Supernatant was removed and underwent a 3-fold dilution with water.	Phenomenex Kinetex XB-C18 column (2.1 x 50 mm)	**Mobile Phase A:** H_2O + 0.1% formic acid **Mobile Phase B:** Acetonitrile + 0.1% formic acid	**SSZ:** 9.15-6670 ng/mL
Dose: single oral dose of 2 g SSZ **Analyte:** SP, acetylated SP [103]	HPLC 500 µL human plasma **ISTD:** SMX	200 µL methanol added to 500 µL plasma with 100 µL SMX ISTD. Samples were mixed with 1 mL of 1 M sodium acetate buffer (pH 4.7) and extracted into 8 mL dichloromethane. Organic layer was evaporated under N_2 and reconstituted in 250 µL mobile phase.	Spherisorb ODS analytical column (3.2 x 250 mm, 10 µm) and a Whatman CO:Pell ODS guard column (4.6 x 75 mm)	Methanol and 0.05 M phosphate buffer (pH 7.4) (25:75, v/v)	**Single-dose PK studies:** 500-20000 ng/mL **Steady state:** 2000-40000 ng/mL
Dose: single- and multiple- dose oral SSZ 2 g daily **Analyte:** SSZ [104]	HPLC 500 µL human plasma **ISTD:** Piroxicam	100 µL methanol and 100 µL ISTD was added to 500 µL plasma. All samples were mixed with 1 mL hydrochloric acid (1 M) and extracted with 4 mL ethyl acetate. Organic layer was evaporated under N_2 and reconstituted in 500 µL mobile phase.	LiChrosorb RP 18 analytical column (3.2 x 150 mm, 5 µm) coupled with a Whatman CO:Pell ODS guard column (4.6 x 75mm).	Acetonitrile and 0.05 M phosphate buffer (pH 7.9) (20:80, v/v)	**SSZ:** 1000-20000 ng/mL

SSZ: Sulfasalazine; SP: Sulfapyridine; 5-ASA: 5-Aminosalicylic acid; ISTD: Internal Standard; SMX: Sulfamethoxazole; DHN: Dimenhydrinate; N_2: Nitrogen; M: Molar

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3.7 Bioanalytical Method Validation

3.7.1 Overview of the Method Development and Validation Process

There are several processes involved in the development and validation of an LC-MS/MS method for the quantitation of analytes in biological matrices prior to using the method for patient sample analysis. Firstly, a standard solution containing the analyte of interest (e.g., reference standard or internal standard) is introduced into the ionisation source of the mass spectrometer in a process known as 'infusion' [99]. This will be followed by the development of an MRM method which should provide optimum mass spectrometry conditions for the detection of the compound(s) of interest. Secondly, a chromatographic method must be developed to separate the analyte(s) of interest from endogenous interferences and to obtain baseline chromatographic separation [99]. The method with a mobile phase combination and an elution time which is most suitable and provides optimum separation in minimum run time will be selected. Thirdly, a suitable extraction method should be developed to extract the analyte(s) of interest from the biological matrix, to remove contaminants or endogenous components which may cause ion suppression or enhancement, and/or to increase the sensitivity of the assay [99]. The type of extraction method used depends on the biological matrix and how compatible the analytes of interest are with the extraction method. It is important to note that each step in the method development process requires optimisation [99][105].

To determine analyte concentrations within patient samples, a calibration curve needs to be constructed such that the unknown samples may be compared with a set of standards of known concentration [99]. To generate a calibration curve that corresponds with the dosing strategy of the study, thorough research needs to be conducted to investigate the pharmacokinetics of the analytes of interest and their expected concentrations within the relevant matrix [105]. This will aid in determining the concentration range of calibration standards (STDs) and quality controls (QCs) to achieve a calibration curve for each analyte [105]. A calibration curve is constructed from a set of no less than six STDs that span the entire concentration range, a double blank sample (sample matrix without analyte or internal standard), a blank sample (sample matrix without analyte and extracted with internal standard), and QCs which allow for the integrity and validity of the results in an individual run to be assessed [12][13][105]. QCs are prepared at concentrations that fall within the range of the calibration curve, specifically at high, medium, low, and lowest limit of quantitation (LLOQ) concentrations [106]. STDs, QCs, blanks, and double blanks are prepared in the same biological matrix as the samples in the intended study, however if the matrix is rare they may be prepared in a surrogate matrix [14][105][107]. For each STD, the instrument plots a ratio of the analyte peak area to internal standard peak area, measured against the nominal concentrations to generate a calibration curve [105]. The simplest model that describes the concentration-response relationship should be chosen [105]. Once the bioanalytical method has demonstrated acceptable accuracy and precision during method development, a full validation may be performed.

The objective of validating a bioanalytical method is to demonstrate that the method is suitable for its intended purpose, and to ensure that data generated for pre-clinical and clinical drug trials are of high quality, reliable, accurate, precise, and reproducible [12]. The FDA [12][13] and EMA [12][14] outline a set of guidelines for accuracy, precision, selectivity, sensitivity, reproducibility, and stability of a method for the quantitative measurement

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of analytes in biological fluids (e.g., plasma, serum, urine, etc.). However, these regulatory authorities have not published specific guidance for LC-MS bioanalysis in tissue samples [108]. Because of the variety and complexity of solid tissue matrices, Gao and Williams (2013) [108] proposed the implementation of a "fit-for-purpose" qualification strategy for tissue analysis with wider acceptance criteria. Although working with tissue is challenging and has several limitations, for the purposes of this MSc, the FDA and EMA guidelines were closely adhered to for placenta bioanalysis because of its application to a clinical study.

Method validation consists of intra- and inter-day validations which are performed over three days, and includes experiments such as sensitivity and specificity, carry-over, matrix effects, recovery, process efficiency, re-injection stability, autosampler stability, fresh versus frozen stability, freeze-thaw stability, benchtop stability, long-term storage stability, the effect of haemolysis, whole blood stability, dilution integrity, and others. Once method validation has been performed, the LC-MS/MS method may be used for the evaluation of patient study samples. Study samples with concentrations below the LLOQ of the assay should be reported as below limit of quantitation (BLQ), whereas samples with concentrations above the upper limit of quantitation (ULOQ) should be diluted and re-analysed, or the standard curve should be extended and re-validated [105].

3.7.2 Description and Criteria of Validation Experiments

3.7.2.1 Accuracy and Precision of a Validation Batch

The purpose of performing intra- and inter- day validations is to illustrate that the method is accurate, precise, and reproducible [12]. STDs in duplicate and QCs in six-fold are prepared and extracted in three separate batches on three separate days. Intra-day validations are based on the accuracy and precision of the three individual batches, whereas inter-day validations use the combined average accuracy and precision results of the three batches [109].

Acceptance Criteria: All STDs and QCs must have an accuracy of between 85-115% of the nominal concentration, except for the LLOQ which may have an accuracy of between 80-120% [12][13][14][106]. The precision or co-efficient of variation (%CV) at each level must be less than 15%, except for the LLOQ which may be less than 20% [12][13][14][106]. Furthermore, at each concentration level, 50% of STDs and QCs must pass, and within a batch, 75% of STDs and 67% of QCs must pass [12][105].

3.7.2.2 Accuracy of Stock Solutions

Stock solutions are prepared at a known concentration by weighing out an appropriate amount of high purity reference standard or internal standard and adding a known volume of solvent in which the compound is soluble [106]. Stock solutions are required to be accurate because they are used to prepare a series of dilutions, referred to as working solutions, which are then used to prepare STDs and QCs. Thus, prior to the preparation of working solutions, stock solutions are prepared by two different analysts and compared for accuracy.

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Acceptance Criteria: The percentage difference in average peak area (HPLC or LC-MS), or the percentage difference in average absorbance (spectrophotometric analysis) between two stock solutions prepared by two different analysts must be less than 5.0%.

3.7.2.3 Stock Solution and Working Solution Stability

The purpose of evaluating the stability of stock solutions and working solutions is to determine whether the analytes are stable at the various temperature conditions to which the solutions are expected to be exposed during handling and throughout the preparation of STDs and QCs [12][14].

Acceptance Criteria: Stock solutions and working solutions are stable at the various temperature conditions if the % CV between the replicates at each condition is less than 15%, and the percentage difference in average peak area (HPLC or LC-MS) or average absorbance is less than 15% from the reference stock solution [105].

3.7.2.4 Sensitivity and Specificity

Sensitivity is defined as the lowest analyte concentration in the matrix (i.e., the LLOQ) that can be measured with acceptable accuracy and precision, and directly relates to the effectiveness of gas phase ion production [105]. Specificity is the ability of the method to unequivocally assess the analyte in the presence of other endogenous components (e.g., impurities or degradation products) and endogenous matrix constituents [14]. Similarly, selectivity is the extent to which the method can determine a particular compound in the analysed matrices without interferences from matrix components [14]. Thus, the purpose of a specificity/selectivity experiment is to ensure that the analytical method is able to quantitate and differentiate between analytes in the presence of other endogenous matrix components in the sample [110].

Acceptance Criteria: When determining sensitivity, the %CV between the six LLOQ replicates should be less than 20%, and the accuracy should be within 20% of the nominal concentration [105]. Moreover, the mean analyte signal-to-noise ratio for all six LLOQ's must be greater than five [105]. For a method to be reported as specific, blank extracted samples should have no peaks or peak areas of less than 15% of the LLOQ [105].

3.7.2.5 Carry-Over Assessment

Carry-over is caused by a residual amount of analyte that remains in the analytical system after an injection has been completed [105]. If carryover is present, it should be eliminated during method development because it can affect the accuracy and precision of the assay [105]. However, if carryover cannot be eliminated, the impact of carryover on the accuracy of study sample concentrations should be assessed during method validation [105].

Acceptance Criteria: The acceptance criteria for assessing carry-over in a blank sample state that if a peak is observed for the analyte with internal standard present, the peak area should not exceed 20% of the peak area obtained at the LLOQ [105]. Similarly, the acceptance criteria for assessing carry-over in the double blank sample states that if a peak is observed for the analyte, the peak area should be less than 20% of the LLOQ peak area [105]. Furthermore, if a peak is observed for the internal standard, it should have a peak area of less than 5% of the peak observed for the internal standard at the working concentration [105].

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3.7.2.6 Matrix Effects

Matrix effects may be defined as the difference in mass spectrometric response for an analyte in standard solution and the response of the same analyte in a biological matrix [105]. Co-eluting matrix components may result in ion enhancement or suppression, which can impact ionisation of the target analytes. Therefore, matrix effects should be determined experimentally using a method developed by Matuszewski *et al*. (2006) [111] whereby sample extracts spiked with the analyte post-extraction are compared to pure solutions containing equivalent amounts of analyte [111][112]. For a method to be reproducible, reliable, and robust, it is of utmost importance to mitigate possible matrix effects by using an appropriate internal standard to compensate for any matrix interferences, or by improving chromatographic separation and ensuring that any substances present in the sample, other than the analytes of interest, are effectively removed during extraction [113].

Acceptance Criteria: To illustrate that the method is robust and not susceptible to matrix effects, the peak area ratios of the analyte to internal standard for QC H, M, and L in each source are used to generate regressions for each individual matrix [14]. The % CV between the replicates from six different matrix sources should not exceed 15% at each concentration, and the average regression slope precision across the six matrices should be less than 5.0% [14][111][112].

3.7.2.7 Recovery

This experiment is performed to determine the average percentage recovery of the analytes from the matrix after extraction [106]. It is not necessary for the recovery of an analyte or internal standard to be 100%, but it should be consistent and reproducible [105][106]. For the determination of recovery at QC H, M, and L, the average peak area of extracted samples should be compared with the average peak area of blank extracts spiked with the analyte post-extraction (i.e., representing 100% recovery) [12]. The average recovery across QC H, M, and L is also determined [12].

Acceptance Criteria: The %CV of the six replicates at QC H, M, and L should not exceed 15% for the samples extracted as per the final extraction method, as well as for the samples spiked post-extraction [105]. When determining the average recovery, the recovery reproducibility (i.e., average %CV) between the concentrations should not exceed 15% [105].

3.7.2.8 Process Efficiency

This experiment is performed to assess the effect of both recovery and matrix effects on an analyte's response [113]. Process efficiency is assessed by comparing the ratios of analyte peak area to internal standard peak area of samples extracted as per the final extraction method to that of unextracted, neat samples spiked with the analytes to represent 100% efficiency [109].

Acceptance Criteria: The %CV of the six replicates at QC H, M, and L should not exceed 15% for the samples extracted as per the final extraction method, as well as for the samples spiked post-extraction [105]. When determining the average process efficiency, the process efficiency reproducibility (i.e., average %CV) between the concentrations should not exceed 15% [105].

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3.7.2.9 Re-injection Stability

Re-injection reproducibility is performed to evaluate whether a batch passes or fails as a whole following a 24- to 72-hour re-injection. The results obtained indicate whether a batch may be re-injected after instrument disruption or malfunction [114].

Acceptance Criteria: The entire batch is subjected to the same criteria as for a validation batch, thus determining whether a batch passes or fails as a whole after a 24- to 72-hour re-injection [107].

3.7.2.10 Autosampler Stability

The same set of results used to assess re-injection stability is used to determine autosampler stability. The purpose of performing an autosampler stability experiment is to evaluate the stability of analytes post-extraction after 24- to 72-hours in the autosampler at the method-defined temperature [14]. Because instrument availability is often a challenge, it is not always possible to run samples on the day of extraction. Therefore, it was important to determine for how long post-extracted samples are stable for in the autosampler without compromising the accuracy and precision of the method [12]. The experiment also gives an indication of how well the internal standard compensates for any changes. This will provide information about whether or not a batch may be re-injected in part [107].

Acceptance Criteria: The percentage difference in the ratio of the average peak area to internal standard peak area between the initial injection and the subsequent re-injection(s) at the QC high and low concentrations should not exceed 15% [105][107].

3.7.2.11 Fresh vs Frozen Stability

This experiment is performed to determine whether the process of freezing the analytes in the respective matrix has an impact on analyte stability. One of the three validation batches must compare freshly prepared (i.e., not frozen) STDs to QCs that have been frozen for at least 12-24 hours [105]. The data obtained from these experiments may also be used to demonstrate stability of the analyte in matrix at the specified storage condition (°C) for a specified storage period (hours). Additionally, all stability assessments (freeze/thaw, benchtop, and long-term stability) need to be included in the validation batch whereby STDs are freshly prepared [12].

Acceptance Criteria: All STDs and QCs need to meet the requirements as per a validation batch [105].

3.7.2.12 Freeze-Thaw Stability

The purpose of conducting a freeze-thaw stability experiment is to account for stability during the re-analysis of study samples [12]. Stability is tested for after a minimum of three freeze-thaw cycles whereby QCs at high and low concentrations are frozen for at least 12-24 hours between cycles and thawed at room temperature for a sufficient amount of time [12][105].

Acceptance Criteria: The individual and average concentration of the QCs must be within 15% of the nominal concentration and the % CV between the replicates at each concentration should not exceed 15% [105][107].

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3.7.2.13 Benchtop Stability

The stability of the analytes in matrix needs to be determined for QC H and L under the laboratory conditions in which the study samples are expected to be exposed to before and during an extraction (i.e., on-bench at room temperature or on-bench on ice) for a set amount of time (i.e., typically the amount of time required for preparation and extraction) [12][105].

Acceptance Criteria: The individual and average concentration of the QCs must be within 15% of the nominal concentration and the % CV between the replicates at each concentration should not exceed 15% [105][107].

3.7.2.14 Long-Term Storage Stability

Long-term stability needs to be determined for QC H and L over a period of time equal to or exceeding the amount of time between the date of the first sample collection and the date of the last sample analysis [12][105]. Furthermore, the storage temperature studied should be the same as those used to store the study samples [105]. However, in the case that this is not possible, stability in the matrix should be determined for as long as possible [105].

Acceptance Criteria: The individual and average concentration of the QCs must be within 15% of the nominal concentration and the % CV between the replicates at each concentration should not exceed 15% [105][107].

3.7.2.15 Effect of Haemolysis

After a sample of blood has been collected from a patient, the whole blood is centrifuged to separate the red blood cells, white blood cells, and plasma [109]. The red blood cells accumulate at the bottom of the tube, the white blood cells will aggregate in the middle, and the plasma remains on the top, allowing for this layer to be pipetted off easily and transferred to a clean tube for analysis. However, during the process of phlebotomy, the red blood cells may burst (known as haemolysis) and the contents will partition into the plasma, as a result of several factors: an incorrect needle size or tube was used, excessive tourniquet, rough sample handling, or excessive centrifuge speed [109]. Other factors such as extreme temperature exposure, delayed processing and prolonged storage of the samples may also cause haemolysis [115]. Since it is known that haemolysis can influence the detection and quantification of analytes due to the presence of increased potassium levels and haemoglobin, it is important to evaluate the influence of 2% haemolysis by comparing the analytical results of haemolysed versus unhaemolysed samples [109][116].

Acceptance Criteria: The average peak areas between the unhaemolysed and haemolysed samples for QC H and L should not differ by more than 15% [107]. Furthermore, the % CV between the replicates at the high and low concentrations must not exceed 15% [107]. This indicates that haemolysis has no effect on the quantitation of the analyte and that the internal standard sufficiently compensates for haemolysis.

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3.7.2.16 Whole Blood Stability

The purpose of this experiment is to determine how soon after blood collection the sample needs to be processed and stored. Analytes may begin degrading in whole blood from the moment they have been drawn or they may adsorb to cellular components during the time period between collection and sample processing [105][107]. Whole blood stability is determined by comparing analytical results from blood processed directly after collection versus blood processed several hours (1-2 hours) after collection at QC high and low concentrations.

Acceptance Criteria: The average peak areas between QC H and L samples processed immediately after collection and samples processed within 1-2 hours after collection should not differ by more than 15% at each concentration [105]. Furthermore, the %CV between the replicates at each concentration should not exceed 15% [105].

3.7.2.17 Dilution Integrity

During the analysis of unknown patient samples, concentrations of sulfasalazine and/or sulfapyridine may be reported as greater than the upper limit of quantitation (ULOQ) in the respective matrix. Thus, the result may not be reported with confidence because it lies outside of the validated range. The purpose of performing a dilution integrity experiment is to evaluate if samples reported as above the ULOQ may be diluted to within the validation range and extracted with accuracy and precision [14][105].

Acceptance Criteria: The accuracy of the diluted samples needs to be within 15% of the nominal concentration and the %CV between the replicates should not exceed 15% [14][105][107].

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CHAPTER 4

LC-MS/MS Method Development and Troubleshooting

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4.1 Introduction

This chapter describes the infusion process of sulfasalazine and sulfapyridine into the ionisation source of the mass spectrometer, as well as the challenges that arose during method development, particularly with sulfapyridine. Furthermore, a summary of the results obtained during LC-MS/MS method development and validation for the quantitation of sulfasalazine and sulfapyridine in maternal- and umbilical cord- plasma has been provided. Overall, this chapter aims to describe the processes that occurred during the discovery of what was thought to be an adduct ion, but rather, revealed to be a defective reference standard.

4.2 Materials and Methods

4.2.1 Chemicals and Solvents

Sulfasalazine, sulfapyridine, sulfasalazine-d4, and sulfapyridine-d4 were obtained from Toronto Research Chemicals (TRC) (Ontario, Canada). An additional sulfapyridine European Pharmacopoeia (EP) reference standard was purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and formic acid were purchased from Fisher Scientific (Massachusetts, United States). Methanol and acetonitrile were purchased from ROMIL Pure Chemistry (Cambridge, United Kingdom). Acetone was purchased from KIMIX Chemical & Lab Supplies (Cape Town, South Africa). LC-MS/MS-grade Millipore water was produced in-house using a Synergy UV Water Purification System (Ultrapure Type 1 water) with a Biopak Polisher from Merck Millipore (Darmstadt, Germany).

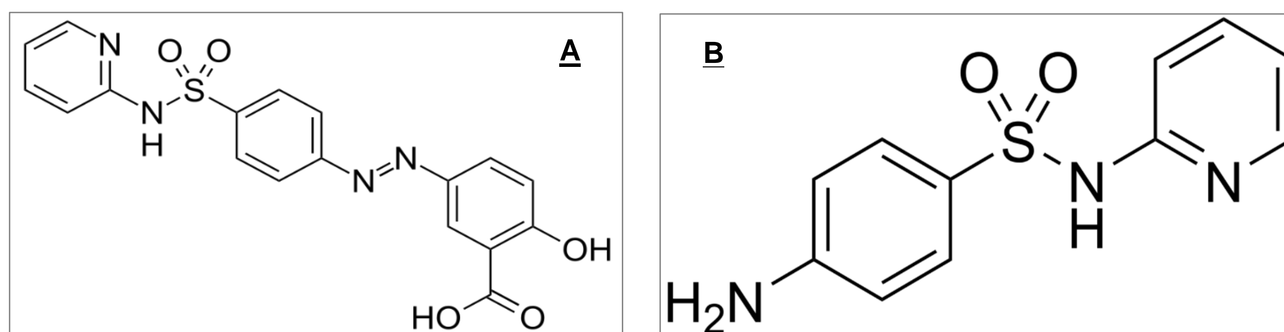

Figure 4.1 - Chemical structures of the analyte (A) Sulfasalazine ($C_{18}H_{14}N_4O_5S$) and its metabolite, (B) Sulfapyridine ($C_{11}H_{11}N_3O_2S$).

4.2.2 Instruments

Method development and validation was performed on a Shimadzu LCMS-8040 triple quadrupole mass spectrometer connected to a Shimadzu Prominence liquid chromatography system (Kyoto, Japan). The system was composed of LC-20AD XR pumps for solvent delivery, a Nexera SIL-20AC XR autosampler, a CTO-20A column oven, and an electrospray ionisation source. LabSolutions version 5.109 was used as the analysis software. Separation was achieved on a Poroshell 120 EC-C18 column (3.0 x 100 mm; 2.7 µm) manufactured by Agilent Technologies (California, United States). A Shimadzu High-Performance Liquid Chromatogram (Model LC-2050C 3D) (Kyoto, Japan) was utilised with version 5.106 of the LabSolutions analysis software. A Beckman DU® Series 600 Spectrophotometer (California, United States) was used. An SPE Ware CEREX System 48-II Positive Pressure Processor (California, United States) was utilised with Waters Corporation

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Oasis PRiME HLB 3cc (60 mg) extraction cartridges or Waters Corporation Sep-Pak ® Vac 3cc (200 mg) C18 cartridges (Massachusetts, United States). A Stuart sample Concentrator (SBHCONC/1) and Block Heater (SBH130D/3) was used (Staffordshire, United Kingdom). A Boeco Semi-Micro and Analytical Balance (BXX22) was used (Hamburg, Germany). A Bead Mill Homogeniser (Bead Ruptor Elite; Model NE486LL/A) and metal beads (2.4 mm) manufactured by Omni International (Kennesaw, Georgia) were utilised for tissue homogenisation.

4.2.3 Ethics

Ethics exemption was obtained from the Stellenbosch University Health Research Ethics Committee (SU HREC) for the use of placental tissue as a matrix for analytical method development (Reference No: X21/03/006; Project ID: 21742). The ethics letter has been attached in **Appendix A**. Leftover placental tissue samples due for bio-disposal from placenta harvested in a biobank were obtained from Prof Catherine Cluver in the Department of Obstetrics and Gynaecology, Stellenbosch University. Prof Catherine Cluver has obtained ethics approval for the storage and use of placenta from their biobank (Reference No: N17/05/048; Project ID: 4354). Dr Fiona Brownfoot, a Clinician-Scientists and Obstetrician at the University of Melbourne and the Mercy Hospital for Women in Australia, obtained ethics approval from the Mercy Health Human Research Ethics Committee to conduct the early phase clinical trial (R16/65). Lastly, ethics exemption was obtained from SU HREC for the use of plasma for method development and validation.

4.3 LC-MS/MS Infusion of Reference Standards and Internal Standards

An infusion may be described as an introduction of the analyte(s) of interest into the ionisation source of the mass spectrometer [99]. Literature describes three common methods which may be implemented to perform an infusion, and the type of method used is highly dependent on the make and model of the mass spectrometer [99]. The first method involves directly infusing the standard solution into the ionisation source using a syringe pump at a flow rate of between 10-20 µL/minute [99]. The second method entails infusing the standard solution into the ionisation source from a syringe pump mixed with a second stream of mobile phase from the liquid chromatography (LC) system [99]. The last procedure is known as flow injection analysis (FIA), whereby a standard solution containing the analyte of interest will be injected multiple times at a fixed volume from the autosampler in the LC system and directed into the ionisation source [99]. After each injection, the instrument parameters will be systemically changed, such that the parameters providing the best response will be selected as optimal [99]. In this project, the FIA procedure was used. An advantage of FIA is that optimisation may be achieved in realistic conditions such as flow rate and LC mobile phase [99]. Additionally, FIA has a low risk of contaminating the ionisation source [99]. The ionisation mode and product ion mass-to-charge (m/z) ratio achieved from the infusion of sulfasalazine, sulfapyridine, sulfasalazine-d4, and sulfapyridine-d4 are presented in **Table 4.1**. During the infusion process, the flow rate of the pumps was set at 0.200 mL/min, all compounds were prepared at a concentration of 1 µg/mL and were injected from the autosampler at a volume of 5 µL. The Monoisotopic mass (M_{mi}) of each analyte was determined using an online resource, specifically "Molecular Mass Calculator by Christopher Gohlke" (https://www.lfd.uci.edu/~gohlke/molmass/) which required inputting the molecular formula of the compounds.

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Table 4.1 - The mode of ionisation for the infusion of sulfasalazine, sulfapyridine, and the relevant internal standards are presented in the table. The m/z of the protonated precursor ion and product ions are also displayed.

Compound	Ionisation Mode	Protonated Precursor Ion > Product Ion m/z
Sulfasalazine $M_{mi} = 398.07$	Positive	398.90 > 381.05; 119.10; 223.10; 94.10
Sulfasalazine-d4 $M_{mi} = 402.09$	Positive	403.00 > 384.95; 223.15; 119.00; 97.95
Sulfapyridine $M_{mi} = 249.06$ $M_{mi} + 32.03 = 281.09$	Positive	282.95 > 158.00; 107.95; 64.90
Sulfapyridine-d4 $M_{mi} = 253.08$	Positive	254.00 > 159.95; 96.00; 112.10; 188.15

4.4 Molecular Ion Adduct Formation

In an attempt to infuse sulfapyridine into the ionisation source of the mass spectrometer, several challenges arose. Initially, a 1 µg/mL sample of sulfapyridine was prepared in DMSO, and the infusion was performed using various mobile phase combinations. However, the infusion of sulfapyridine was unsuccessful as no compound was detected at the protonated precursor ion $[M+H]^+$ mass of 250.00. It was thought that perhaps the lack of detection was attributed to partial insolubility of the analyte, thus the solvent in which sulfapyridine was prepared was changed to methanol. However, the infusion remained unsuccessful.

The next step was to assess the pKa of sulfapyridine and to determine the pH range in which ionisation will be optimal. The pKa value may be defined as the pH corresponding to the point at which two forms of the analyte, namely ionised and non-ionised, are present in equal concentrations, and also refers to the tendency of a molecular ion to keep a proton (H^+) at its ionisation centres [96]. Thus, the stronger an acid, the smaller the pKa will be, and conversely, the stronger a base, the larger the pKa will be [96]. Ideally, during an infusion, the pH of the mobile phase should be optimised to the pH at which the compound is approximately (~) 100% ionised [96]. The 2-pH rule states that two pH units away from an analyte's pKa will result in a change in extent of ionisation of ~ 100% [96]. More specifically, an acid will be ~100% ionised at 2 pH units above the pKa of the analyte, and ~100% non-ionised at 2 pH units below the pKa. The opposite is true of a base, whereby 2 pH units above the pKa of the base results in ~100% non-ionisation and 2 pH units below the pKa will result in ~100% ionisation. **Figure 4.2** illustrates the concentration of each ionised and non-ionised form of sulfapyridine over the full pH range and **Table 4.2** summarises the pKa and pH values of the analyte.

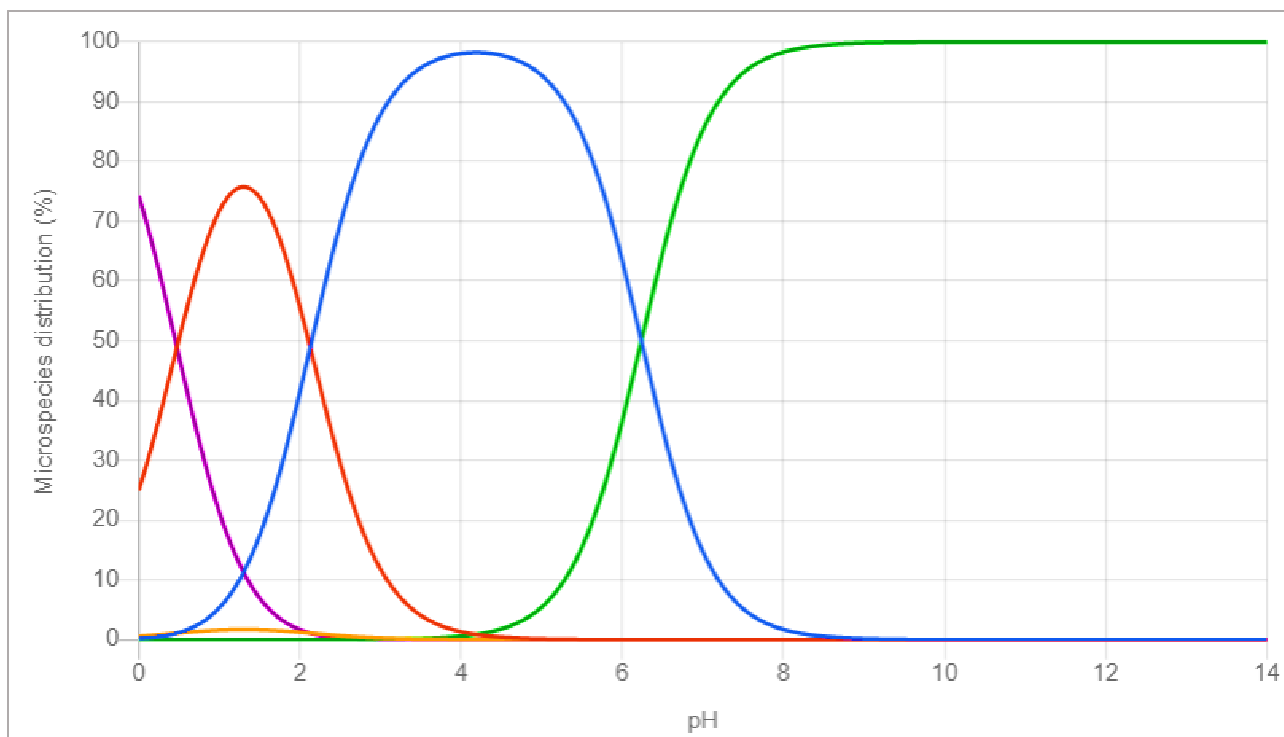

Figure 4.2 - A speciation (concentration) plot of each ionised and non-ionised form of sulfapyridine shown over the full pH range. The figure has been obtained from Chemicalize.com [131]. Each of the colourful lines represents a moiety of sulfapyridine.

The pKa of each ionisable functional group of sulfapyridine is 2.14 and 6.24, whereby approximately 50% of the analyte is in its ionised form. When following the 2-pH rule, 2 pH units above the pKa of 2.14 is a pH of 4.2, which results in 98.3% (~100%) ionisation of the analyte. Lowering or even raising the pH beyond this point will result in a reduction of ionisation. Moreover, 2 pH units above the pKa of 6.24 is a pH of 8.3 which results in 99.1% (~100%) ionisation of the analyte. However, raising the pH further will not significantly affect the degree of ionisation.

Table 4.2 - A summary of the pKa values of sulfapyridine at approximately 50% ionisation and 100% ionisation over the pH range 0-14.

pH at ~50% Ionisation	pH at ~100% Ionisation
2.14 (50.4%)	4.2 (98.3%)
6.24 (52.1%)	8.3 (99.1%)

Values that are absent are those that fall outside the pH range of 0-14.

Thus, the optimum pH for ~100% ionisation of sulfapyridine was determined to be either 4.2 or 8.3. However, although a column is not used during the infusion process, working at a pH above 8.0 will be too high when chromatographic method development is performed because most standard silica-based phases are stable within a pH range of 2.0-8.0. Thus, the pH of 4.2 was chosen as optimal, and aqueous mobile phases within the pH range of 4.0-5.0 were prepared in increments of 0.2, such that the following pH range was obtained: 4.0, 4.2, 4.4, 4.6, 4.8, and 5.0. The infusions were attempted using these pH ranges which is, in theory, the

optimal pH for ionisation of sulfapyridine. Despite attempts at improving conditions for optimal ionisation, the infusion of sulfapyridine still remained unsuccessful.

The final step in troubleshooting involved performing a full mass spectrometry scan of sulfapyridine, as seen in **Figure 4.3** below. This scan allowed for the instrument to collect information over a wide range of masses (m/z 200-300) revealing a predominant protonated m/z of 281.09 for sulfapyridine (1 µg/mL), which was present at an intensity of 500 000 counts per second (cps), as indicated by the arrow. However, it is evident that nothing was detected at a m/z of 250.00 Da, as illustrated by the square in the diagram below.

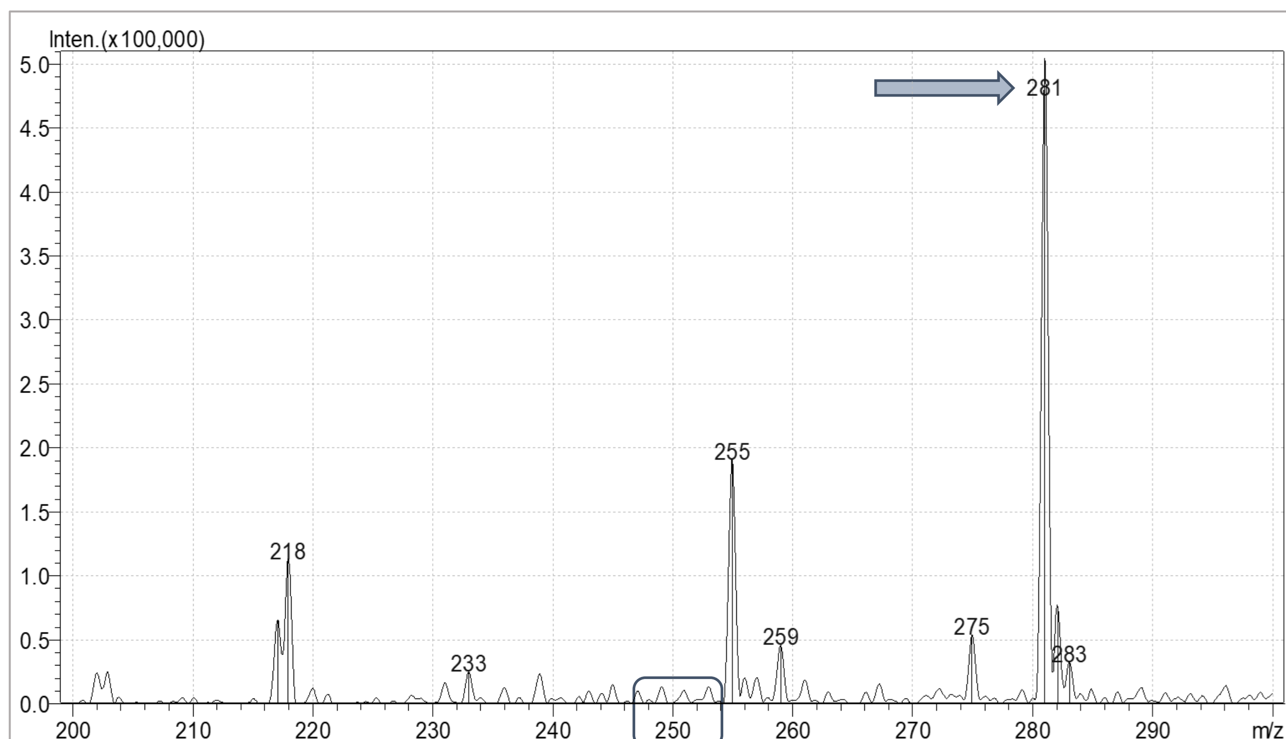

Figure 4.3 - Results obtained from the LabSolutions software after a full scan of sulfapyridine was performed. The graph represents the intensities of the m/z ratios detected by LC-MS/MS.

Because sulfapyridine was detected at a m/z of 281.09 Da when a full mass spectrometry scan was performed, using **Table 4.3** below which illustrates the protonated precursor mass of commonly observed molecular ion adducts, it was thought that the mass identified by the scan was indicative of a methanol adduct $[M + MeOH + H]^+$ = 249.06 + 32.03 + 1.01 = 282.09 Da. However, when the compound was infused it was detected at a protonated precursor mass of 282.95 Da with the following product ions: 158.00; 107.95; and 64.90. The precursor mass of 282.95 Da does not represent the mass observed in the scan (281.09 Da) or the mass of a sulfapyridine methanol adduct (282.09 Da). However, it was decided to move forward with method development and validation using the infused precursor mass of 282.95 Da because extraction and chromatographic separation illustrated good peak shape, reproducible peak areas, and the detection was consistent. Moreover, the product ions determined in this study corresponded, for the most part, to those determined by Choi *et al*. (108.04; 156.01; 184.08; and 92.04) [102] and Gu *et al*. (156.2; 183.9; 108.1; and 157.2) [83].

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It is important to note, however, that the molecular ion "adduct" was incorrectly identified and the reference standard was later deemed defective. Further explanations regarding this oversight have been provided in this chapter under the Heading "**4.5.3 Determination of a Defective Reference Standard**".

Table 4.3 - Typical molecular ion adducts and their associated protonated precursor ions observed in positive ESI mass spectra for sulfapyridine [117][118].

Ion	Ion Mass (M = 249.06 Da)	Charge	Result
[M+2H]2+	M/2 + 1.01	2+	125.54
[M+H]+	M + 1.01	1+	250.06
[M+MeOH+H]+	**M + 32.03 + 1.01**	**1+**	**282.09**
[M+ACN+H]+	M + 41.03 + 1.01	1+	291.09
[M+DMSO+H]+	M + 78.01 + 1.01	1+	328.08

Source: Adapted from Fiehn Laboratory [117][118]. Da: Dalton's; H: Hydrogen; ACN: Acetonitrile; MeOH: Methanol; DMSO: Dimethyl sulfoxide.

It has been reported that adduct ion formation often occurs within the ion source, whereby two species, namely a precursor ion and a neutral molecule (e.g., water, methanol, acetonitrile, etc.), interact to form an adduct ion which contains all the constituent atoms of one species as well as an additional atom or atoms [119]. It has been suggested that neutral molecules are present inside the mass spectrometry system as a result of residual mobile-phase [119]. Literature reports that, overall, precursor ion adduct formation should not complicate MS/MS spectra interpretation or result in questionable identifications and inconsistencies in MRM acquisition quantitation [119].

4.5 Method Development and Validation for the Determination of Sulfasalazine and Sulfapyridine in Maternal- and Umbilical Cord- Plasma

An LC-MS/MS method was developed and validated according to FDA [12][13] and EMA [12][14] guidelines for the simultaneous determination of sulfasalazine and sulfapyridine in maternal- and umbilical cord- plasma. However, this method has been nullified as the sulfapyridine reference standard was determined to be defective. A summary of the method development and validation has been provided below, as well as a what led to the conclusion of a problematic reference standard.

4.5.1 Method Development

This method was developed using the publication by Gu *et al.* [83] as a guideline with laboratory-specific adaptations. Various combinations of mobile phases and columns were assessed to obtain optimal peak shape and retention of the analytes of interest. Furthermore, extraction method development was performed to determine the most suitable protein precipitation reagent for the extraction of sulfasalazine and sulfapyridine from human plasma. The results indicated that a mobile phase combination of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B) was optimal. Furthermore, a protein precipitation extraction performed with ice-cold acetonitrile was determined as the most suitable protein precipitation reagent, as it provided the

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highest peak areas with the smallest precision value between the replicates (n= 3). However, because quantification of sulfapyridine was performed using the methanol adduct, 10% methanol was added to both mobile phase B and the protein precipitation reagent to ensure that the methanol content prior to analysis was consistent throughout all samples, to allow for reproducible adduct formation. Moreover, source optimisation was performed, whereby sulfasalazine and sulfapyridine extracts at a mid-level concentration were exposed to different heating block temperatures (450 °C, 400 °C, 350 °C, and 300 °C) and desolvation line (300 °C, 250 °C, 200 °C, 150 °C) temperatures. A heating block temperature of 450 °C and a desolvation line temperature of 200 °C was determined to be the most suitable for the ionisation of both sulfasalazine and sulfapyridine.

4.5.2 Summary and Discussion of the Final Method and Validation

LC-MS/MS was used to monitor the mass-to-charge (m/z) transition of the protonated precursor ions m/z 398.90 and m/z 282.95 to the product ions m/z 381.05 and m/z 158.00 for sulfasalazine and sulfapyridine, respectively, using sulfasalazine-d4 as an internal standard. A gradient elution method with a mobile phase combination of water + 0.1% formic acid (A) and acetonitrile:methanol (90:10, v/v) + 0.1% formic acid (B) was used. Separation was achieved using a Poroshell 120 EC-C18 analytical column (3.0 x 100 mm, 2.7 µm). Samples were prepared using a protein precipitation extraction whereby 200 µL of precipitating reagent, which consisted of acetonitrile:methanol (90:10, v/v) and sulfasalazine-d4 at a concentration of 400 ng/mL, was added to 50 µL plasma. All samples were vortexed for 30 seconds, sonicated for 1 minute, and placed at -20 °C to equilibrate. Samples were then centrifuged at 16 000 x g for 10 minutes at 23 °C. 100 µL of supernatant was removed and diluted with 100 µL of water. 200 µL of the sample was transferred to a 96-well plate and 10 µL was injected onto the LC-MS/MS for analysis.

Pre-validation experiments demonstrated stock solution stability in DMSO and working solution stability in methanol at -80 °C, -20 °C, and 4 °C for 24 hours, and at room temperature for six hours. Furthermore, matrix effects were shown to have no impact on the reproducibility of the method when plasma originating from six different sources was analysed. Furthermore, the recovery from plasma was reported as 108.0% (%CV= 1.2) and 99.5% (%CV= 3.1) for sulfasalazine and sulfapyridine, respectively. The high recoveries indicate that the sample extraction procedure was effective as minimal analyte was lost throughout the extraction of sulfasalazine and sulfapyridine from plasma. The recoveries obtained are consistent with those obtained by Gu *et al.* [83] whereby the mean recovery across QC H, M, and L was 95.0% for sulfasalazine and 93.5% for sulfapyridine.

Accuracy and precision were assessed over three consecutive, independent runs. During the intra- and inter-day validations, the average accuracy of calibration standards ranged from 91.4-115.4% (%CV= 2.5-5.4) for sulfasalazine and 94.7-109.1% (%CV= 1.9-12.2) for sulfapyridine. The average accuracy of quality controls ranged from 97.3-111.4% (%CV= 2.5-6.2) and 94.6-104.2% (%CV= 4.4-11.8) for sulfasalazine and sulfapyridine, respectively. The calibration curve fits a quadratic regression (weighted by 1/x, x= concentration) for sulfasalazine and sulfapyridine over the range 160-50 000 ng/mL. In Gu *et al.'s* method [83], the LLOQ of sulfasalazine and sulfapyridine was determined to be 10 ng/mL using a simple protein

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precipitation extraction without concentrating the samples. Although our method did not need to reach such low concentrations as Gu *et al.*'s method, obtaining a sufficient signal-to-noise of sulfapyridine at a concentration of 100 ng/mL proved to be challenging, even though a similar extraction methodology and injection volume was used.

The integrity of a five-fold dilution was illustrated and was determined to be within the guidelines whether the samples were diluted pre- or post-extraction. Additionally, this method illustrated re-injection stability and autosampler stability for up to 48 hours when stored in the autosampler at 10 °C. The method developed by Gu *et al.* [83] only demonstrated autosampler stability for up to 24 hours at 4 °C. Freeze-thaw stability for both analytes was demonstrated for three freeze-thaw cycles, which consisted of a 30 minute thawing time at room temperature and a freezing time of 24 hours at -80 °C. These results are consistent with those obtained by Gu *et al.* [83], as freeze-thaw stability was also demonstrated for three freeze-thaw cycles, whereby samples were thawed at room temperature and frozen at -20 °C. The analytes also demonstrated stability in plasma when left on bench for six hours. The quantification of both analytes was not affected by the presence of 2% haemolysis when EDTA was used as the anti-coagulant. However, sulfasalazine and sulfapyridine demonstrated instability in whole blood for 1 hour, thus blood should be spun down to plasma immediately after it has been drawn.

The method was applied to evaluate the pharmacokinetics of sulfasalazine and sulfapyridine in plasma after oral administration of 1.5 g sulfasalazine twice daily, to 10 pre-eclamptic patients between 24-34 weeks gestation.

4.5.3 Determination of a Defective Reference Standard

Upon analysis of patient samples, the concentrations of sulfasalazine ranged from 2663-14 350 ng/mL in umbilical cord plasma and 2002-36 379 ng/mL in maternal plasma. Moreover, for sulfasalazine, no samples were reported as below the limit of quantitation (BLQ) or required a dilution for re-analysis, indicating that the calibration curve range was adequately chosen for this analyte. However, sulfapyridine was not detected in the maternal- and umbilical cord- plasma samples. Because patient samples have been collected and stored over the past four years, it was initially thought that sulfapyridine degraded in patient samples over time. However, long-term stability of sulfapyridine had not been determined at this stage. Alternatively, it was suggested that either the instrument was contaminated at a m/z of 250.00, resulting in the inability to detect the compound at this mass, or the reference standard was defective, causing the incorrect identification of an adduct ion.

To test these hypotheses, a sulfapyridine EP reference standard was ordered from Merck and compared to sulfapyridine obtained from TRC. When the sulfapyridine purchased from Merck was infused into the ionisation source of the mass spectrometer, the protonated precursor ion $[M+H]^+$ was identified as 250.07, and the product ions were 156.00; 92.10; 108.00; and 184.10, which were almost identical to those achieved by Choi *et al*. (250.06 > 108.04; 156.01; 184.08; and 92.04) [102] and Gu *et al*. (250.00 > 156.2; 183.9; 108.1; and 157.2) [83]. The comparison between sulfapyridine obtained from Merck versus sulfapyridine obtained

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from TRC eliminated the possibility that the instrument was operating incorrectly, thus confirming that the sulfapyridine reference standard obtained from TRC was indeed defective.

There have not been any other claims in literature regarding the determination of a defective active pharmaceutical reference standard purchased from any manufacturer. This indicates how rare it was for this situation to have occurred, and also emphasises why it was not originally suspected that the reference standard was defective. However, in hindsight, there were several occurrences that took place which were overlooked and ultimately led to the incorrect identification of a molecular ion adduct.

Firstly, after performing a full mass spectrometer scan, as illustrated in **Figure 4.3** above, the complete absence of a m/z ratio at 250.00 Da should have been a warning sign that the reference standard was potentially defective. Although it is possible for adduct formation to occur and for adducts to be used during the quantitation of the analytes of interest, in this case it was unusual that only the “adduct” was seen with no identifiable intensity at the original protonated mass of 250.00 Da.

Secondly, after infusing sulfapyridine, the protonated m/z was determined as 282.95 Da, whereas the mass of sulfapyridine with a methanol adduct was determined to be 282.09 Da. These two values differ by almost an entire mass unit, indicating that the m/z transition used in this project was not that of a methanol adduct ion but potentially that of a contaminant within the original reference standard.

Lastly, sulfapyridine did not elute at the same time as its deuterated internal standard which should have been an indication that the m/z transition for sulfapyridine was potentially incorrect. The retention times of sulfapyridine and sulfapyridine-d4 were 1.7 and 1.4 minutes, respectively. Stable isotopically labelled internal standards display almost identical retention and ionisation properties as the analyte of interest [95], however in this study the retention times differed by 30 seconds. It was incorrectly assumed that the internal standard was defective and thus was not used during method development and validation for the plasma method. Instead, sulfasalazine-d4 was used as an internal standard for both sulfasalazine and sulfapyridine. Chromatograms of sulfapyridine (282.95 > 158.00; 107.95; 64.90) and sulfapyridine-d4 are illustrated in **Figure 4.4** and **Figure 4.5**, respectively.

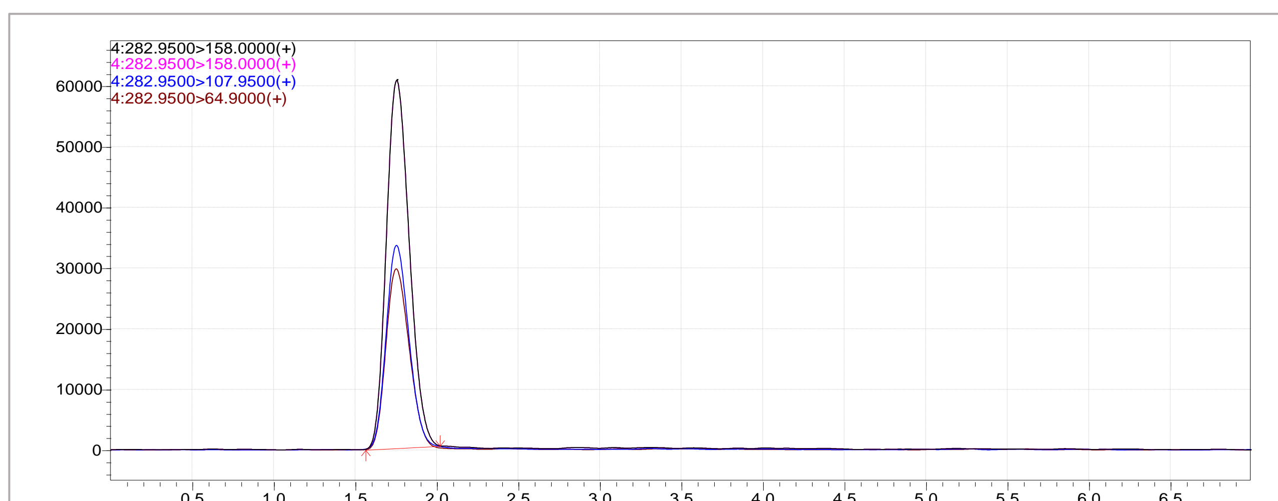

Figure 4.4 – A chromatogram of sulfapyridine (40 000 ng/mL) to illustrate the retention time.

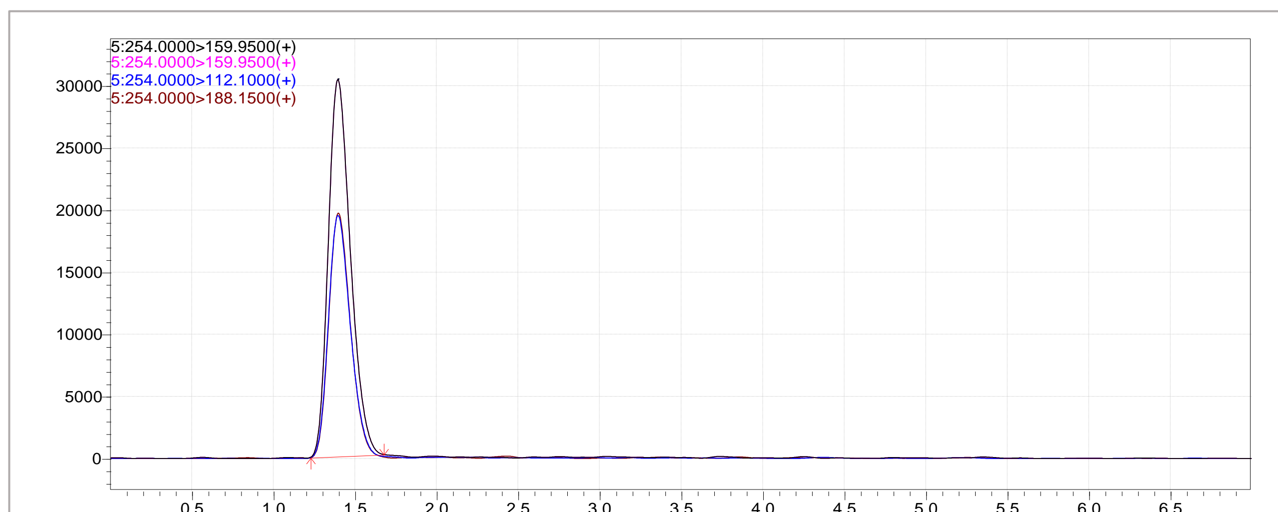

Figure 4.5 – A chromatogram of sulfapyridine-d4 (400 ng/mL) to illustrate the retention time.

4.6 Chapter Conclusion

An LC-MS/MS method was developed for the simultaneous quantitation of sulfasalazine and sulfapyridine in maternal- and umbilical cord- plasma. However, upon the analysis of patient samples, no sulfapyridine was detected, and it was determined that the reference standard used to develop the method did not contain an adduct as initially thought, but rather, was defective. Because the Division of Pharmacology laboratory records show that the reference standards and internal standards obtained from TRC were appropriately stored on the day of their arrival, the cause of obtaining a defective reference standard may be attributed to factors outside of our control such as the quality of its preparation, or perhaps the time spent in transit and in customs where it may not have been stored at appropriate conditions.

Although several oversights occurred which led to the incorrect identification of a molecular ion adduct, it was not initially suspected that the reference standard was defective due to the unlikelihood of such a situation transpiring.

Many hours were spent on developing, troubleshooting, optimizing, and validating the plasma LC-MS/MS method. However, due to time constraints and for the purposes of this MSc, a BSc Honours student re-validated the plasma method using the correct m/z transition for sulfapyridine by following the developed protocols summarised in this chapter. Because the placenta is the site of disease in pre-eclampsia, quantitating sulfasalazine and sulfapyridine in this organ will assist the clinical trial in determining whether the dose given as an intervention for preterm pre-eclampsia was therapeutic. Therefore, it was decided to focus on method development and validation for the simultaneous quantitation of these analytes in placenta.

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CHAPTER 5

Development and Validation of an LC-MS/MS Method for the Determination of Sulfasalazine and Sulfapyridine in Placenta

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5.1 Introduction

The main objective of this chapter was to develop and validate an LC-MS/MS method according to FDA [12][13] and EMA [12][14] guidelines for the quantitation of sulfasalazine and sulfapyridine in human placenta. This method was used to analyse samples obtained from the clinical trial, contributing towards a better understanding of the pharmacokinetics of these analytes when administered to pregnant women as a treatment for preterm pre-eclampsia. Inter- and intra-day validations took place over three days and validation experiments such as matrix effects, recovery, process efficiency, sensitivity and specificity, dilution integrity, and stability of the analytes under various conditions were performed.

5.2 Sample Preparation

5.2.1 Placental Structure and Composition

The placenta may be described as a nutrient-rich, structurally complex organ and is composed of four different tissues, namely the placental disk, umbilical cord, amniotic fluid, and amniotic sac, as illustrated in **Figure 5.1** [120]. For the purposes of this study, tissue obtained from the placental disk will be analysed to determine analyte concentrations. The placental disk consists of trophoblasts, connective tissues, fibroblasts, vascular cells, mesenchymal stem cells, and a highly vascularised extracellular matrix containing collagens I, III, IV, and VI, and non-collagenous glycoproteins and proteoglycans [120]. Owing to the role of the placenta in nutrient transport, it is also rich in electrolytes, water, glucose, proteins, vitamins, lipids, and triglycerides [120].

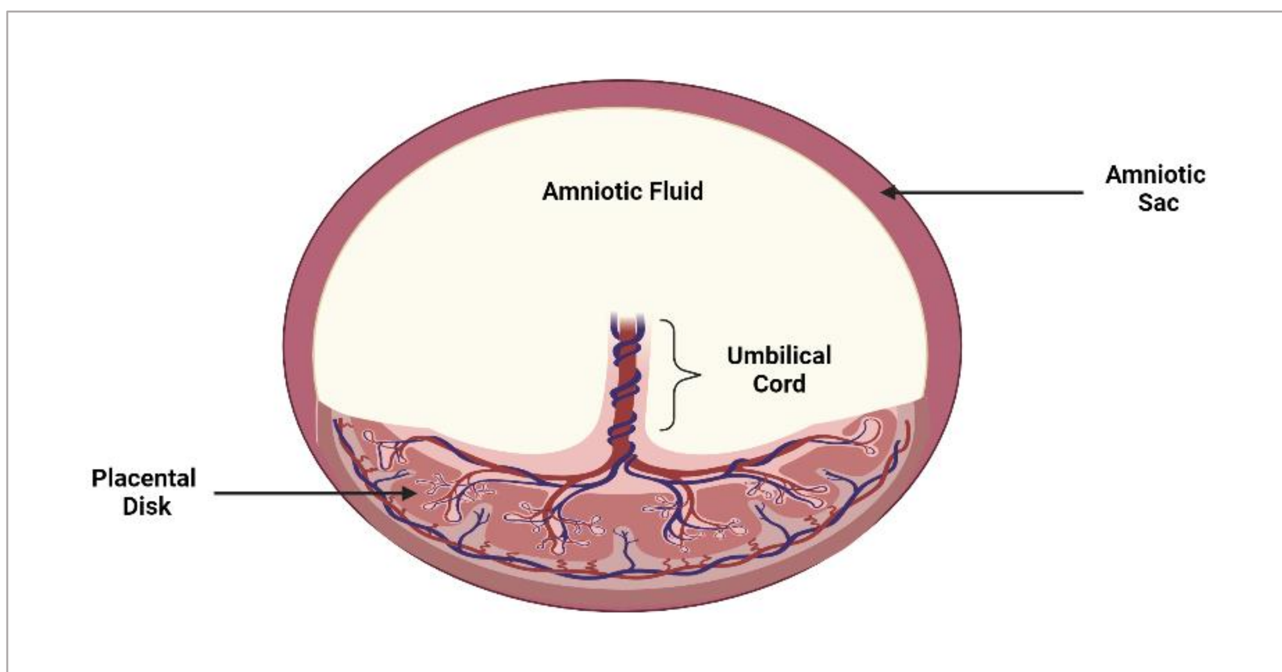

Figure 5.1 - A simplified diagram to illustrate the four types of placental tissues, namely the placental disk, umbilical cord, amniotic sac, and amniotic fluid. Figure adapted from Lim and Koob [120] and created with BioRender.com [40].

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5.2.2 Tissue Sample Preparation Methods

A critical step in the development of an LC-MS/MS method for tissue sample analysis is the preparation of solid tissue into a form from which the analyte(s) of interest can be readily and reproducibly extracted for analysis [121]. The process of transforming a solid tissue sample into a liquid that is compatible with commonly used analyte extraction procedures is referred to as homogenisation [108][121]. There are four commonly used techniques for tissue homogenisation, namely mechanical shearing, acoustic disruption, enzymatic digestion and chemical digestion [121]. The most appropriate homogenisation technique is determined by the physical and histological properties of the tissue samples [121].

Mechanical homogenisation may be achieved via bead beating, whereby tissue samples are exposed to high speed shaking and agitation against small, hard beads inside sealed homogenising tubes [121]. A typical protocol for the mechanical homogenisation of fibrous tissue involves adding a homogenising solvent in a three- to five-fold dilution prior to bead beating to ensure that homogenous tissue particles of uniform size are produced [121]. Acoustic disruption involves the use of sonication or ultrasonication, whereby strong acoustic energy is directed to the tissues [108][121]. The resulting pressure disturbances and intense vibrations contribute towards the agitation and lysing of tissue cells [108][121]. A typical enzymatic digestion protocol requires incubation of a pre-weighed tissue sample in an appropriate enzymatic digestion agent (e.g., collagenase) at 37 °C for 2-12 hours, depending on how fibrous the sample is, to allow for digestion of the extracellular matrix [121]. Chemical methods of digestion involves exposing the tissue sample to strong acids or bases, such as solutions of 1 M hydrochloric acid and 1-10 M sodium hydroxide [121]. However the use of 30% hydrogen peroxide mixed with 2 M ammonium hydroxide or 20% trichloroacetic acid has also been reported [121]. A neutralisation step is required to stop the chemical digestion reaction [121]. After enzymatic or chemical digestions, the samples may be homogenised via bead beating [121].

It has been suggested that dense fibrous connective tissues may be resistant to mechanical shearing, and therefore, performing a chemical or enzymatic digestion prior to mechanical homogenisation may ensure that the fibrous structure is completely disrupted to allow for adequate release of the analyte(s) from tissue [108][121][122]. However, in the context of this study, there were several reasons why an enzymatic or chemical digestion was deemed unsuitable. Firstly, the time-consuming incubation period of an enzymatic digestion significantly adds to sample preparation time and, in the context of patient sample analysis whereby many samples are required to be extracted and analysed in the same batch, enzymatic digestions may be considered impractical and result in a lower through-put. Secondly, chemical methods of digestion raise concern for the stability of the analytes in extreme pH environments. Moreover, the neutralisation step required to stop the digestion results in salt formation which, if not removed, may cause ion suppression during LC-MS/MS analysis [121]. Therefore, it was decided to perform mechanical homogenisation and acoustic disruption of placental tissue without any prior digestion steps.

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5.2.3 Final Method for the Preparation of Placental Tissue Homogenate

After delivery, blank placental disk tissue was dissected into pieces of approximately 1 g which were flash-frozen in liquid nitrogen and stored at -20 °C to maintain stability of the tissue. To prepare placental homogenate, the flash-frozen samples were removed from the freezer, dissected into smaller pieces, and weighed. The placental tissue samples were placed into 2 mL homogenising tubes, five metal homogenising beads were added into each tube, and homogenising solvent (water:methanol (1:1)) was added at a volume (mL) five times that of the weighed mass (g), resulting in a five-fold dilution. The speed of the bead mill homogeniser was set to 8 metres/second for three cycles of 30 seconds each. In between the homogenising steps, the homogenising tubes were sonicated in an ice bath for 1 minute. Sonication was performed to further disrupt and break down the cells of the tissue, and the use of ice served two purposes: (a) mechanical homogenisation results in heat generation, thus ice was used to stabilise the temperature of the analytes; (b) placing homogenate on ice may assist with more rapid dispersion of foam generated during homogenisation, as foam may affect the accuracy with which the homogenate is pipetted [121]. For the preparation of STDs and QCs from a single drug-free matrix, the liquid from each homogenising tube was pooled into one 15 mL tube and stored in the -20 °C freezer. On the day that homogenate was required, it was vortexed thoroughly before use. **Figure 5.2** illustrates homogenised placental tissue. Some heterogeneity of the placental liquid exists, as evident by the pieces of connective tissue present on the sides of the 15 mL centrifuge tube. Due to the presence of large cellular components in the tissue matrix, pipette tips often became obstructed. Thus, to allow for more accurate and precise pipetting, prior to aliquoting or pipetting any matrix the 20-200 µL pipette tips were manually altered by cutting off 5 mm of each tip using sharp scissors. **Figure 5.3** provides an example of a 20-200 µL pipette tip that (A) was unaltered; and (B) has been cut to increase its diameter.

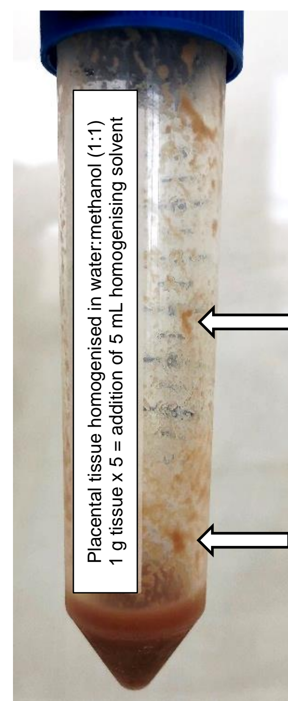

Figure 5.2 – An illustration of mechanically homogenised placenta.

Figure 5.3 - 20-200 µL pipette tips. (A) Unaltered tip; (B) Manually altered tip.

5.2.4 Extraction Method Development

As previously mentioned in **Chapter 3**, no methods have been reported for the extraction of sulfasalazine and sulfapyridine from placental tissue. An LC-MS/MS method has been developed and optimised for the analysis of veterinary drug resides in meat homogenate, and although seemingly unrelated to this study, the major constituents of meat are similar to the components of placenta (i.e., water, proteins, fats, and phospholipids) [123]. In the published method by Young and Tran (2015), meat homogenate was prepared by using a simple protein precipitation and centrifugation step for the removal of proteins, followed by passing the acetonitrile-based extracts through Oasis PRiME hydrophilic-lipophilic balanced (HLB) cartridges [123]. It was found that more than 90% of fats and phospholipids were effectively removed from meat extracts and the recoveries of the analytes were not impacted as a result of the HLB cartridges [123]. Although traditional methods of fat

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removal such as hexane defatting steps or the use of reversed-phase sorbents (e.g., C_{18} silica) may be effective, it has been reported that neither of these methods remove phospholipids [123]. If significant amounts of fats and phospholipids are present after sample clean-up, this may lead to interference in LC-MS analysis and may also result in contamination of the column and instrument [123]. Therefore, sample preparation methods which effectively removed both fats and phospholipid contaminants from the matrix were evaluated.

To determine a suitable method for the extraction of sulfasalazine and sulfapyridine from placental homogenate, four different sample preparation conditions were performed in triplicate, whereby the initial step of all four conditions was a protein precipitation extraction. A volume of 100 µL placental homogenate containing sulfasalazine (10 ng/mL) and sulfapyridine (10 ng/mL) was aliquoted into 1.5 mL polypropylene micro-centrifuge tubes. 400 µL of ice-cold acetonitrile:methanol (90:10, v/v) which contained both sulfasalazine-d4 (50 ng/mL) and sulfapyridine-d4 (50 ng/mL) internal standards was added to each sample. The samples were vortexed for 30 seconds and centrifuged at 16 000 x g for 10 minutes at 23°C. After protein precipitation, the supernatant of the samples was exposed to either of the following four treatment conditions:

Condition 1: The Oasis PRiME HLB 3cc (60 mg) extraction cartridges were mounted on a pre-cleaned vacuum manifold (CEREX System 48-II Positive Pressure Processor) and conditioned with 2 mL acetonitrile. 400 µL of supernatant from each respective sample was eluted through the cartridges and collected in borosilicate glass tubes. 200 µL of acetonitrile:methanol (90:10) was used to elute any remaining analytes and was also collected in the same borosilicate glass tubes. The samples were placed in the heating block at 30 °C and evaporated to dryness under nitrogen gas. Samples were reconstituted in 200 µL of [acetonitrile:methanol (90:10)]:water (1:1).

Condition 2: The same methodology as described in condition 1 was performed for condition 2, however the supernatant was not evaporated to dryness. Rather, 100 µL of the supernatant was removed and diluted with 100 µL of water prior to analysis.

Condition 3: The same methodology as described in condition 1 was performed for condition 3, however the supernatant was not evaporated to dryness. Rather, the supernatant was directly injected for analysis.

Condition 4: 400 µL of the sample supernatant was diluted in 8600 µL of 50 mM ammonium acetate (total volume 9000 µL). By diluting the sample in an aqueous solution, the organic content of the supernatant was reduced to less than 5% to avoid analyte break-through during the loading step. The C18 cartridges were conditioned with 2 mL of methanol and equilibrated with 2 mL of 50 mM ammonium acetate. Because the total volume of the diluted sample was 9000 µL, 3mL of the sample was added to the cartridges three times. The first wash step was performed twice using 2 mL of 5 mM ammonium acetate. The second wash step was also performed twice using 2 mL of 2% methanol. Finally, an elution step was performed twice with 1 mL of methanol and the sample was collected in borosilicate glass tubes. The samples were then evaporated to dryness using nitrogen gas and reconstituted in 200 µL [acetonitrile:methanol (90:10)]:water (1:1).

The average peak areas were analysed and compared, as shown in **Table 5.1-5.2** and **Figure 5.4**.

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Table 5.1 - The average peak areas of sulfasalazine (10 ng/mL) for the various extraction methodologies.

Sulfasalazine	Condition 1	Condition 2	Condition 3	Condition 4
Average Peak Area	9213	1169	3925	10 182
STDEV	245	150	182	2104
%CV	2.7	12.8	4.6	20.7

Table 5.2 - The average peak areas of sulfapyridine (10 ng/mL) for the various extraction methodologies.

Sulfapyridine	Condition 1	Condition 2	Condition 3	Condition 4
Average Peak Area	19 507	6017	13 169	10 914
STDEV	1389	1111	532	2418
%CV	7.1	18.5	4.0	22.2

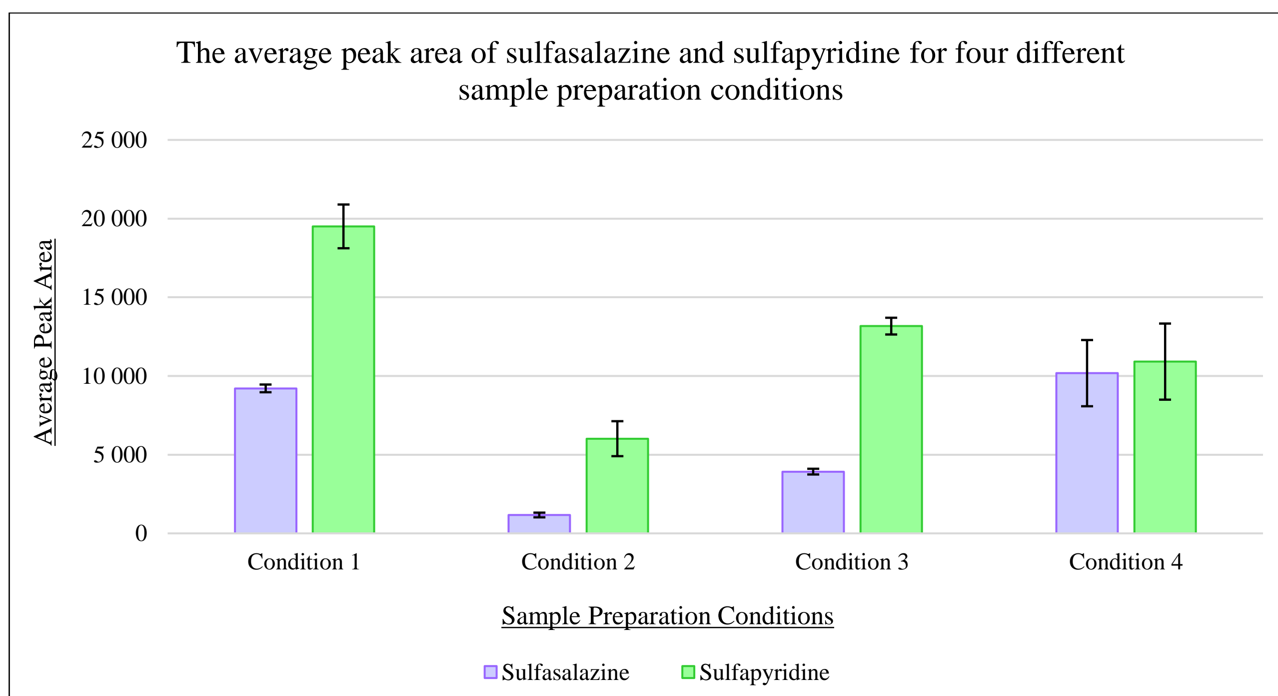

Figure 5.4 - The average peak area of sulfasalazine (10 ng/mL) and sulfapyridine (10 ng/mL) for four different sample preparation conditions.

Condition 1 yielded the second highest peak area with the lowest precision value (%CV= 2.7) for sulfasalazine and the highest peak area for sulfapyridine (%CV= 7.1). Condition 2 yielded the lowest peak area for both sulfasalazine (%CV= 12.8) and sulfapyridine (%CV= 18.5), and the precision value for sulfapyridine was unacceptable as it was larger than 15%. Compared to condition 2, condition 3 had higher peak areas and lower precision values for sulfasalazine (%CV= 4.6) and sulfapyridine (%CV= 4.0). Condition 4 yielded similar peak areas for sulfasalazine and sulfapyridine, however the precision values of both analytes were above the acceptable value of 15%. Therefore, condition 1 and condition 3 were deemed most suitable. The LLOQ of the method was 30 ng/mL, and although sufficient signal-to-noise was obtained for both methods at 10 ng/mL as indicated in the graph, in the case that instrument sensitivity decreased throughout method development and validation, it was decided to err on the side of caution and concentrate the samples to obtain improved signal-to-noise. Therefore, condition 1 was chosen as the extraction method.

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5.2.5 Final Extraction Methodology

Calibration standards, quality controls, blanks, and double blanks were prepared in placental homogenate on the day of extraction. Unknown patient samples were only homogenised on the day of extraction. A volume of 100 µL placental homogenate was aliquoted into 1.5 mL polypropylene micro-centrifuge tubes and spiked with either 20 µL of working solution or 20 µL of blank methanol. Next, 400 µL of ice-cold acetonitrile:methanol (90:10, v/v) containing sulfasalazine-d4 and sulfapyridine-d4 internal standards at a concentration of 250 ng/mL and 200 ng/mL, respectively, was added to all the samples, except for the double blank in which 400 µL of blank precipitation reagent was added (i.e., no internal standard). All samples were vortexed for 30 seconds and centrifuged at 16 000 x g for 10 minutes at 23°C. The HLB cartridges were mounted on a pre-cleaned vacuum manifold and conditioned with 2 mL acetonitrile. A volume of 400 µL supernatant from each respective sample was eluted through the cartridges and collected in borosilicate glass tubes. Furthermore, 200 µL of acetonitrile:methanol (90:10, v/v) was used to elute any remaining analytes and was also collected in the same borosilicate glass tubes. The samples were placed in the heating block at 30 °C and evaporated to dryness under nitrogen gas. Samples were reconstituted in 200 µL of [acetonitrile:methanol (90:10)]:water (1:1). Finally, 200 µL of the sample was transferred to 96-well plates, and 10 µL was injected onto the LC-MS/MS for analysis of sulfasalazine and sulfapyridine. If patient samples were reported as above the upper limit of quantitation (i.e., > 30 000 ng/mL) for sulfasalazine and sulfapyridine, a five-fold post-extraction dilution was validated, whereby 40 µL of supernatant from the extracted sample was diluted with 160 µL of blank placental homogenate that was extracted in the same way as the patient samples.

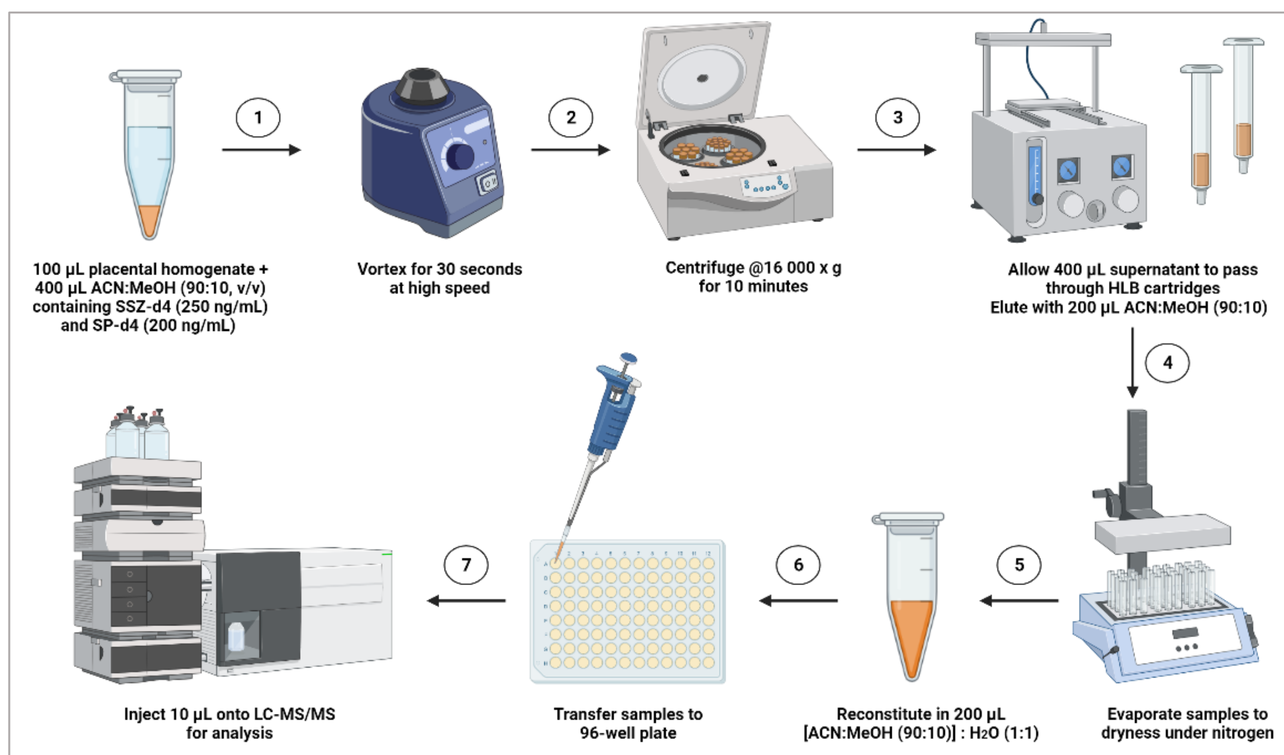

Figure 5.5 - Protocol for the extraction of sulfasalazine and sulfapyridine from placental homogenate. Figure created with BioRender.com [40].

(ACN: acetonitrile; MeOH: methanol; SSZ-d4: sulfasalazine-d4; SP-d4: sulfapyridine-d4; H_2O: water).

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5.3 Chromatography and Mass Spectrometry Method

5.3.1 Chromatographic Conditions

A chromatographic method was developed to separate the analytes of interest from endogenous interferences, and to achieve baseline separation of the compounds, as co-elution may result in ion suppression or enhancement [124]. Various combinations and ratios of mobile phases and types of analytical columns were investigated to obtain optimum separation of the analytes of interest. The final method consisted of a gradient elution which was performed at a flow rate of 0.450 mL/min over a total run time of 7 minutes with a mobile phase combination of water + 0.1% formic acid (A) and acetonitrile:methanol (90:10) + 0.1% formic acid (B). Acetonitrile was used as the needle rinse. The final chromatographic conditions for the gradient elution are presented in **Table 5.3**. Separation of sulfasalazine and sulfapyridine was achieved using reverse-phase chromatography with a Poroshell 120 EC-C18 analytical column (3.0 x 100 mm, 2.7 µm) kept at 30 °C. The sample injection volume was 10 µL and the autosampler was set at 10 °C.

Table 5.3 - Gradient elution for chromatographic conditions.

Time (min)	Mobile Phase A%	Mobile Phase B%	Flow (mL/min)
0	60	40	0.450
3.00	10	90	0.450
3.50	10	90	0.450
4.00	60	40	0.450
7.00	60	40	0.450

Pre-validation extractions of calibration standards and quality controls in duplicate showed good accuracy and precision, excellent peak shapes, and reproducible retention times using the reported chromatographic conditions. Sulfasalazine was retained on the analytical column at 2.3 minutes and sulfapyridine was retained at 1.4 minutes. Chromatograms of sulfasalazine and sulfapyridine at the highest calibration standard are illustrated in **Figure 5.6** and **Figure 5.7**, respectively.

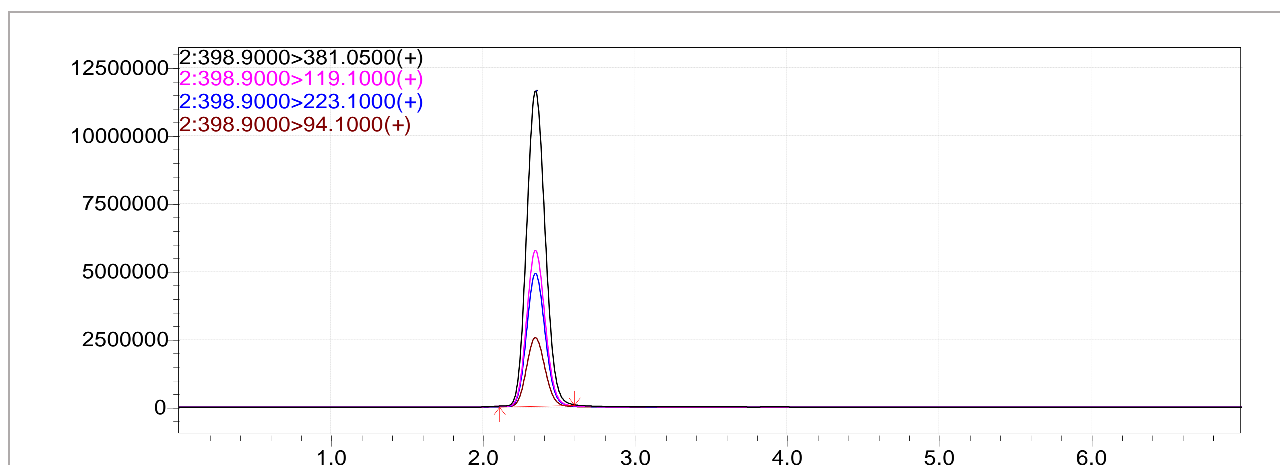

Figure 5.6 – Chromatogram of sulfasalazine at the highest calibration standard (30 000 ng/mL) using the final chromatographic conditions.

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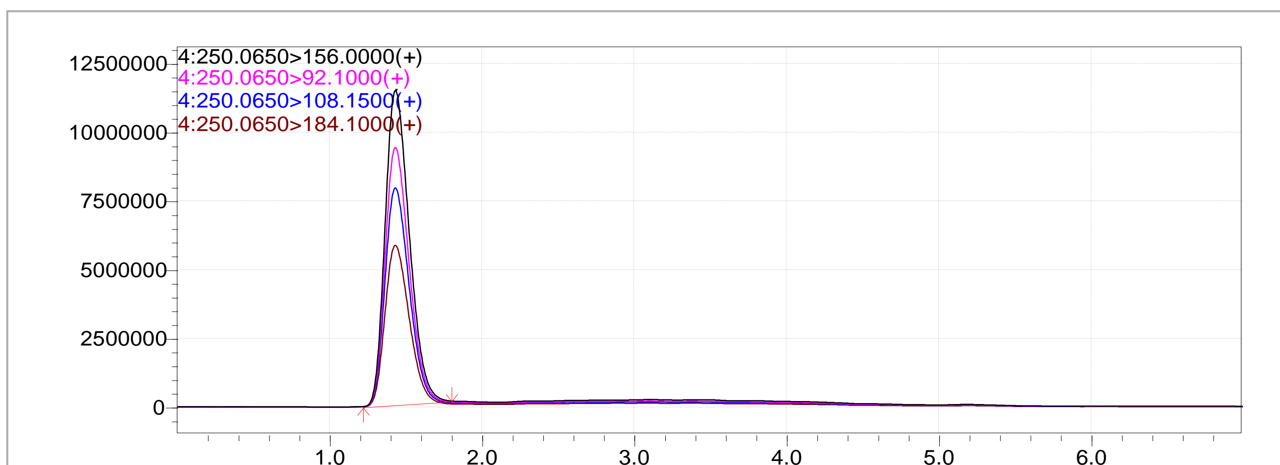

Figure 5.7 – Chromatogram of sulfapyridine at the highest calibration standard (30 000 ng/mL) using the final chromatographic conditions.

5.3.2 Mass Spectrometry Conditions

The instrument was operated in MRM scanning mode and each compound contained several transitions. In **Table 5.4** below, the first product ion of each analyte was identified as the quantifier ion which was used for the quantitation of calibration standards, quality controls, and the unknown patient samples. The second product ion was identified as the qualifier ion and was used to confirm that the peaks seen in unknown samples were indeed the analyte of interest. Both analytes and their internal standards were ionised in positive ESI mode. The nebulising gas flow and drying gas flow was set at 3 mL/min and 15 mL/min, respectively. The desolvation line temperature was set at 200 °C and the heating block temperature was set at 450 °C. The interface voltage was set to 4500 V and the dwell time was set at 300 milliseconds. Argon was used as the collision-induced dissociation gas delivered at 230 kPa.

Table 5.4 – The ESI mode and MRM transitions for the analytes and their respective internal standards.

Compound	ESI Mode	Precursor Ion m/z	Product Ion m/z
Sulfasalazine	Positive	398.90	381.05; 119.10; 223.10; 94.10
Sulfasalazine-d4	Positive	403.00	384.95; 223.15; 119.00; 97.95
Sulfapyridine	Positive	250.07	156.00; 92.10; 108.00; 184.10
Sulfapyridine-d4	Positive	254.00	159.95; 96.00; 112.10; 188.15

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5.4 Bioanalytical Method Validation

5.4.1 Preparation of Reference Standard and Internal Standard Stock Solutions

Stock solutions were prepared at a known concentration by weighing out an appropriate amount of high purity reference standard or internal standard and adding a solvent in which the compound was soluble. The preparation of reference standards and internal standards are illustrated in **Table 5.5** and **Table 5.6**, respectively. Stock solutions of sulfasalazine and sulfapyridine were prepared in dimethyl sulfoxide (DMSO) at a concentration of 2500 µg/mL by adjusting the weighed mass to purity. For the internal standard stock solutions, sulfasalazine-d4 was prepared in DMSO and sulfapyridine-d4 was prepared in methanol (MeOH) to achieve a concentration of 1000 µg/mL for each, and the weighed mass was not adjusted to purity. For each stock solution, aliquots of 100 µL were made and were stored at -80°C until required.

Table 5.5 – Preparation of reference standard stock solutions.

Analyte	Molecular Mass (g/moL)	Solvent	Mass of Analyte (mg)	Purity (%)	Adjusted Mass of Analyte (mg)	Solvent Volume (mL)	Stock Solution Concentration (µg/mL)
Sulfasalazine	398.39	DMSO	2.39	98.0	2.34	0.937	2500
Sulfapyridine	249.29	DMSO	2.41	98.0	2.36	0.945	2500

Reason for adjustment (e.g., purity, salt, hydrate): Purity
Calculation:
1. Sulfasalazine = 2.39 mg x 98.0% = 2.34 mg / 2.50 = 0.937 mL solvent
2. Sulfapyridine = 2.41 mg x 98.0% = 2.36 mg / 2.50 = 0.945 mL solvent

Table 5.6 - Preparation of internal standard stock solutions.

Analyte	Molecular Mass (g/moL)	Solvent	Mass of Analyte (mg)	Purity (%)	Solvent Volume (mL)	Stock Solution Concentration (µg/mL)
Sulfasalazine-d4	402.42	DMSO	2.50	96.0	2.50	1000
Sulfapyridine-d4	253.31	MeOH	1.00	96.0	1.00	1000

5.4.2 Accuracy of Stock Solutions

Stock solutions need to be accurate because they are used to prepare a series of dilutions, referred to as working solutions, which are then used to prepare calibration standards and quality controls. Thus, to determine the accuracy of stock solutions, sulfasalazine (2.5 mg/mL) and sulfapyridine (2.5 mg/mL) were prepared by two different analysts. The stock solutions of each analyte were diluted in triplicate to 10 µg/mL and the peak areas of the reference sample and test sample were determined using HPLC analyses and compared, as shown in **Table 5.7**. The UV absorbance on the HPLC was set at 364 nanometres (nm) for sulfasalazine and 268 nm for sulfapyridine.

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Table 5.7 - Stock solution accuracy of sulfasalazine and sulfapyridine.

Stock Solution Accuracy	Sulfasalazine		Sulfapyridine	
	Reference	Test	Reference	Test
Average Peak Area	359 291	361 167	323 953	316 975
STEDEV	11 003	2179	3520	4216
% CV	3.1	0.6	1.1	1.3
% Difference from reference	0.5		2.1	

The percentage difference in average peak area between the reference stock solution and test stock solution was 0.5% for sulfasalazine and 2.1% for sulfapyridine. Therefore, it can be concluded that stock solutions of sulfasalazine and sulfapyridine were prepared accurately because the difference was less than 5.0% for both analytes.

5.4.3 Preparation of Working Solutions, Calibration Standards and Quality Controls

Working solutions (WS), calibration standards (STDs) and quality controls (QCs) were prepared as shown in **Table 5.8-5.11** below. The final calibration range was chosen as 30-30 000 ng/mL for sulfasalazine and sulfapyridine in human placenta. STDs were extracted in duplicate for all three validations as well as on the day of patient sample analysis. QC high (H), medium (M), low (L), and lowest limit of quantitation (LLOQ) were extracted in six-fold during the three validation batches, however on the day of patient sample analysis, QC H, M, and L were extracted in duplicate. Furthermore, a system suitability sample (SYS) at a concentration of 4800 ng/mL for sulfasalazine and sulfapyridine was included in every batch to serve as a reference sample for the number of injections required to achieve an equilibrated system prior to the injection of a batch.

Because no prior information was available for the expected concentrations of sulfasalazine and sulfapyridine in placenta, several steps were followed to determine a suitable calibration range which was based on the determination of a plasma calibration range first. In a method developed by Gu *et al*. (2011) [83] whereby sulfasalazine, sulfapyridine, and 5-ASA were quantitated in human plasma, the range of sulfasalazine was reported as 10-10 000 ng/mL and the range for sulfapyridine was reported as 10-1000 ng/mL. However, this method had an application to a clinical study whereby volunteers were administered a single oral dose of 250 mg. In the early phase clinical study performed by the University of Melbourne, study participants diagnosed with preterm pre-eclampsia were administered 1.5 mg of sulfasalazine twice daily until delivery. Therefore, not only were the patients in the early phase clinical study dosed with a much higher concentration of sulfasalazine, but they were also administered multiple doses over the period from when they were first recruited to when they gave birth. Thus, the concentration of sulfasalazine and sulfapyridine in plasma was predicted to be higher than the concentrations reported in the plasma during the study conducted by Gu *et al*. (2011) [83].

For this project, the calibration range for the quantitation of sulfasalazine and sulfapyridine in plasma was determined as 160-50 000 ng/mL, and the determination of a suitable placental tissue ranged was based on this

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plasma range. Sulfasalazine is extensively bound to proteins (>99%) whereas sulfapyridine is bound to proteins to a lesser extent (70%). Drugs that exhibit high protein-binding capacity remain in circulation and often have smaller volumes of distribution, however drugs with a lower affinity for protein binding have a higher free plasma fraction and consequently, a larger volume of distribution. Therefore, it was expected that less sulfasalazine will cross the placenta due to its high protein-binding affinity, which means that the plasma concentrations would be higher than the placenta concentrations. Because sulfapyridine is not as protein bound, it was expected that the concentration of this metabolite would be higher in the placenta than in the plasma. However, these were theoretical assumptions. Due to uncertainties about what concentrations to expect in the placenta, a wide calibration range of 30-30 000 ng/mL was utilised to accommodate both the multiple dosing strategy and the volume of distribution of both analytes. Moreover, the choice of a dynamic range ensured that the re-analysis of specimens due to high concentrations was avoided, which reduced overall cost and analysis time. However, a definite oversight that occurred when choosing the concentration range was determining the concentration range in ng/mL instead of in ng/g tissue. When the range of 30-30 000 ng/mL was expressed as a weight per weight (ng/g) concentration, the range was reported as 150-150 000 ng/g tissue. Although this range was largely suited for patient sample analysis, perhaps a range of 50-50 000 ng/g (i.e., 10-10 000 mg/mL) would have been far more suited based on the concentrations reported in **Chapter 6**.

5.4.3.1 Working Solution and Calibration Standard Preparation

The initial working solution (WS1) was prepared by spiking 30 µL of sulfasalazine (1500 µg/mL) and 30 µL sulfapyridine (1500 µg/mL) into 190 µL methanol, as shown in **Table 5.8** and **Table 5.9**. The remainder of the working solutions (WS2-WS10) were prepared by performing a series of dilutions. STDs were prepared by directly spiking 20 µL of each respective working solution into 100 µL placenta to obtain the desired concentration range.

5.4.3.2 Working Solution and Quality Control Preparation

The initial working solution (WSQ1) was prepared by spiking 60 µL of sulfasalazine (1500 µg/mL) and 60 µL sulfapyridine (1500 µg/mL) into 505 µL methanol, as shown in **Table 5.10** and **Table 5.11**. The remainder of the working solutions (WSQ2-WSQ10) were prepared by a performing a series of dilutions. QCs were prepared by spiking 20 µL of each respective working solution into 100 µL placenta to obtain the desired concentration range. QC dilution (DIL) did not form part of the dilution series and was prepared separately. The initial working solution (WSQDIL) was prepared by spiking 60 µL of sulfasalazine (1500 µg/mL) and 60 µL sulfapyridine (1500 µg/mL) into 130 µL methanol. QCDIL was prepared by spiking 20 µL WSQDIL into 100 µL placenta. The purpose of QCDIL was to validate the dilution process should the observed concentration of the analytes in the patient samples be reported as above the upper limit of quantitation (ULOQ) of the assay (i.e., greater than 30 000 ng/mL for sulfasalazine and sulfapyridine). In **Table 5.11**, the QCs that are denoted as "X" were not prepared and extracted, but rather, their working solutions were used to perform a series of additional dilutions to obtain the QC L and LLOQ concentrations. The ULOQ of the assay is equal to STD1 and the LLOQ is equal to STD10. QC H is calculated as 80% of the ULOQ, QC M is calculated as 50% of QC H, and the concentration of QC L must fall within 3x the LLOQ.

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Table 5.8 - Preparation of spiking volume of stock solutions (SS) for STDs.

Analyte	Concentration (µg/mL)	Solvent	Volume (µL) of Stock Solution (SS) Spiked into WS1
Sulfasalazine	1500	DMSO	30.0
Sulfapyridine	1500	DMSO	30.0

Table 5.9 - Preparation of working solutions (WS) and calibration standards (STDs).

Working Solution (WS)	Blank Solvent (µL) (Methanol)	Spiking Solution Source	Spiking Solution Volume (µL)	Sulfasalazine Working Solution Concentration (µg/mL)	Sulfapyridine Working Solution Concentration (µg/mL)	Volume (mL) into 0.100 mL Placenta	Calibration Standard (STD)	Placenta Sulfasalazine Concentration (ng/mL)	Placenta Sulfapyridine Concentration (ng/mL)
WS1	190	SS	SS1	180	180	0.0200	STD 1	30 000	30 000
WS2	100	WS1	100	90.0	90.0	0.0200	STD 2	15 000	15 000
WS3	100	WS2	100	45.0	45.0	0.0200	STD 3	7500	7500
WS4	120	WS3	80.0	18.0	18.0	0.0200	STD 4	3000	3000
WS5	100	WS4	100	9.00	9.00	0.0200	STD 5	1500	1500
WS6	100	WS5	100	4.50	4.50	0.0200	STD 6	750	750
WS7	120	WS6	80.0	1.80	1.80	0.0200	STD 7	300	300
WS8	100	WS7	100	0.900	0.900	0.0200	STD 8	150	150
WS9	120	WS8	80.0	0.360	0.360	0.0200	STD 9	60.0	60.0
WS10	50.0	WS9	50.0	0.180	0.180	0.0200	STD 10	30.0	30.0

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Table 5.10 - Preparation of spiking volume of stock solutions (SS) for QCs.

Analyte	Concentration (µg/mL)	Solvent	Volume (µL) of Stock Solution (SS) Spiked into WS1
Sulfasalazine	1500	DMSO	60.0
Sulfapyridine	1500	DMSO	60.0

Table 5.11 - Preparation of working solutions (WS) and quality controls (QC's).

Working Solution (WSQ)	Blank Solvent (µL) (Methanol)	Spiking Solution Source	Spiking Solution Volume (µL)	Sulfasalazine Working Solution Concentration (µg/mL)	Sulfapyridine Working Solution Concentration (µg/mL)	Volume (mL) into 0.100 mL Placenta	Quality Control (QC)	Placenta Sulfasalazine Concentration (ng/mL)	Placenta Sulfapyridine Concentration (ng/mL)
WSQDIL	130	SS	SS1	360	360	0.0200	QC DIL	60 000	60 000
WSQ1	505	SS	SS1	144	144	0.0200	QC H	24 000	24 000
WSQ2	200	WSQ1	200	72.0	72.0	0.0200	QC M	12 000	12 000
WSQ3	240	WSQ2	160	28.8	28.8	0.0200	SYS	4800	4800
WSQ4	200	WSQ3	200	14.4	14.4	0.0200	X	2400	2400
WSQ5	200	WSQ4	200	7.20	7.20	0.0200	X	1200	1200
WSQ6	200	WSQ5	200	3.60	3.60	0.0200	X	600	600
WSQ7	200	WSQ6	200	1.80	1.80	0.0200	X	300	300
WSQ8	200	WSQ7	200	0.900	0.900	0.0200	X	150	150
WSQ9	200	WSQ8	200	0.450	0.450	0.0200	QC L	75.0	75.0
WSQ10	120	WSQ9	80.0	0.180	0.180	0.0200	QC LLOQ	30.0	30.0

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5.4.4 Stock Solution and Working Solution Stability

To determine stock solution stability, stock solutions of sulfasalazine (1 mg/mL) and sulfapyridine (1 mg/mL) were prepared in DMSO and stored at -80 °C, -20 °C, and 4 °C, respectively for 24 hours, and were left on-bench at room temperature for four hours. All stocks were diluted in triplicate to 10 µg/mL and evaluated against freshly prepared stocks (also diluted to 10 µg/mL) using a spectrophotometer, as illustrated in **Table 5.12-5.13**. Similarly, to determine working solution stability, sulfasalazine and sulfapyridine were prepared at 1 mg/mL in methanol for each of the four storage conditions. The working solutions were stored at -80 °C, -20 °C, and 4 °C, respectively for 24 hours, and were left on-bench at room temperature for four hours. All stocks were diluted in triplicate to 10 µg/mL and evaluated against freshly prepared working stocks (also diluted to 10 µg/mL) using HPLC analyses, as illustrated by **Table 5.14-5.15**. For the stability experiments, the UV absorbance on the spectrophotometer and HPLC was set at 364 nm for sulfasalazine and 268 nm for sulfapyridine.

Table 5.12 - Stock solution stability of sulfasalazine at the relevant test conditions compared to a fresh stock.

Sulfasalazine	Fresh Stock	-80 °C	-20 °C	4 °C	On-bench
Average Absorbance	0.552	0.534	0.535	0.532	0.534
STDEV	0.000635	0.00121	0.00229	0.00121	0.00121
%CV	0.1	0.2	0.4	0.2	0.2
% Difference from fresh stock	----	3.3	3.0	3.6	3.2

Table 5.13 - Stock solution stability of sulfapyridine at the relevant test conditions compared to a fresh stock.

Sulfapyridine	Fresh Stock	-80 °C	-20 °C	4 °C	On-bench
Average Absorbance	0.876	0.863	0.860	0.858	0.882
STDEV	0.00150	0.00644	0.00439	0.00387	0.00312
%CV	0.2	0.7	0.5	0.5	0.4
% Difference from fresh stock	----	1.5	1.9	2.1	-0.7

Both tables above indicate that stock solutions of sulfasalazine and sulfapyridine prepared in DMSO were stable for 24 hours when stored at -80 °C, -20 ° C, and 4 °C for 24 hours, and when stored on-bench at room temperature for four hours. For both compounds, the %CV between the average absorbances at each storage condition was less than 15% and the percentage difference of each condition from the fresh stock was less than 15%, indicating stability of the compounds in the stock solutions at the different storage conditions for the specified time period.

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Table 5.14 - Working solution stability of sulfasalazine at the relevant test conditions versus a fresh stock.

Sulfasalazine	Fresh Stock	-80 °C	-20 °C	4 °C	On-bench
Average Peak Area	351 908	315 530	310 378	306 066	313 423
STDEV	413	2161	715	746	2012
%CV	0.1	0.7	0.2	0.2	0.6
% Difference from fresh stock	----	10.3	11.8	13.0	10.9

Table 5.15 - Working solution stability of sulfapyridine at the relevant test conditions versus a fresh stock.

Sulfapyridine	Fresh Stock	-80 °C	-20 °C	4 °C	On-bench
Average Peak Area	478 987	437 003	430 626	428 791	431 663
STDEV	1898	1516	607	1195	2829
%CV	0.4	0.3	0.1	0.3	0.7
% Difference from fresh stock	----	8.8	10.1	10.5	9.9

Both tables above indicate that working solutions of sulfasalazine and sulfapyridine prepared in methanol were stable for 24 hours when stored at -80 °C, -20 ° C, and 4 °C for 24 hours, and when stored on-bench at room temperature for four hours. For both compounds, the %CV between the average peak areas at each relevant storage condition was less than 15% and the percentage difference of each condition from the fresh stock was less than 15%, indicating stability of the compounds in the working solution at the relevant storage conditions for the specified time period. Because working solutions were freshly prepared on the day of analysis, it was not necessary to determine working solution stability for a period of longer than 24 hours.

5.4.5 Linearity, Accuracy, and Precision of Intra- and Inter- day Validations

To demonstrate acceptable intra- and inter-day accuracy and precision of the method over the desired concentration range, STDs and QCs were prepared on the day of extraction and assayed in three validation batches which took place over three days. Each validation run consisted of STDs in duplicate to generate one calibration curve, and six replicates of the prepared QCs. Using LabSolutions software version 5.109, the ratios of the analyte peak area to internal standard peak area were plotted against the nominal concentrations to generate a calibration curve which fits a quadratic regression (weighted by 1/x, x= concentration) over the range 30-30 000 ng/mL for both sulfasalazine and sulfapyridine, as illustrated by **Figure 5.8** below.

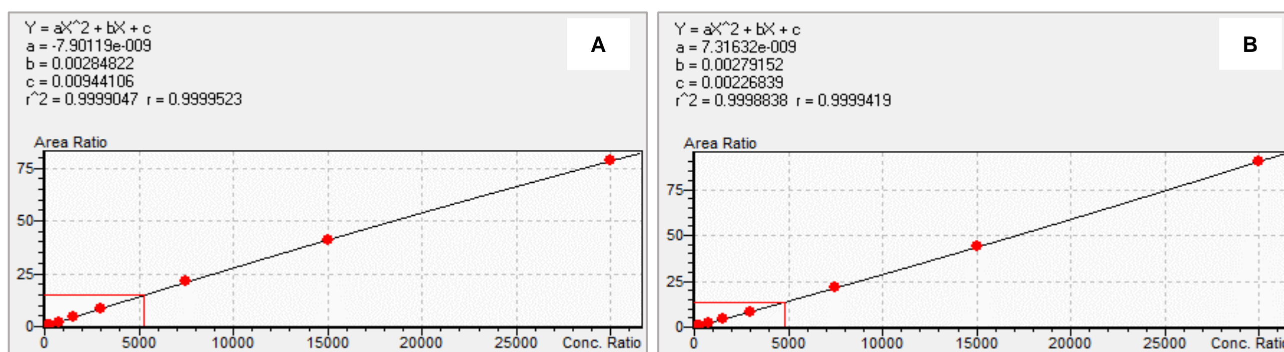

Figure 5.8 – Calibration curve from validation day 1 for (A) sulfasalazine; and (B) sulfapyridine

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The sulfasalazine curves showed a good fit for validation 1, 2, and 3 with the regression co-efficient (r) being 0.9999, 0.9999, and 0.9999, respectively. Similarly, the sulfapyridine curves showed a good fit for validation 1, 2, and 3 with the regression co-efficient (r) being 0.9999, 0.9999, and 0.9999 respectively.

To determine intra-day accuracy and precision, the three individual batches were analysed. To determine inter-day accuracy and precision, the combined results of the three batches were assessed. A summary of the combined accuracy and precision statistics for STDs and QCs obtained from validation 1, 2, and 3 for sulfasalazine and sulfapyridine are presented in **Table 5.16-5.19** below. According to the guidelines, STDs that fail to meet acceptance criteria may be excluded, however QCs that do not meet acceptance criteria must be reported (including statistical outliers) [105].

Table 5.16 – Inter-day accuracy and precision of STDs from validation 1-3 for sulfasalazine.

Batch	Replicates	STD 1 30 000 ng/mL	STD 2 15 000 ng/mL	STD 3 7 500 ng/mL	STD 4 3 000 ng/mL	STD 5 1 500 ng/mL	STD 6 750 ng/mL	STD 7 300 ng/mL	STD 8 150 ng/mL	STD 9 60.0 ng/mL	STD 10 30.0 ng/mL
Validation 1	1	29 798	14 990	7 517	2 988	1 492	748	290	154	60.1	25.0
	2	30 256	14 790	7 749	2 949	1 524	696	303	154	68.4	31.9
Validation 2	1	30 841	14 963	7 759	3 153	1 589	737	312	147	63.0	30.9
	2	29 378	14 645	7 139	3 031	1 521	729	311	155	57.3	24.5
Validation 3	1	29 539	15 025	7 341	3 111	1 521	753	301	147	61.1	29.6
	2	30 639	14 527	7 824	3 017	1 491	696	318	153	61.5	27.8
	n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
	Average	**30 075**	**14 823**	**7 555**	**3 042**	**1 523**	**726**	**306**	**152**	**61.9**	**28.3**
	STDEV	**598**	**204**	**273**	**76.8**	**35.7**	**25.1**	**10.0**	**3.48**	**3.70**	**3.06**
	%CV	**2.0**	**1.4**	**3.6**	**2.5**	**2.3**	**3.5**	**3.3**	**2.3**	**6.0**	**10.8**
	%Accuracy	**100.2**	**98.8**	**100.7**	**101.4**	**101.5**	**96.8**	**101.9**	**101.1**	**103.2**	**94.2**

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Table 5.17 - Inter-day accuracy and precision of QCs from validation 1-3 for sulfasalazine.

Batch	Replicates	QC H 24 000 ng/mL	QC M 12 000 ng/mL	QC L 75.0 ng/mL	QC LLOQ 30.0 ng/mL
Validation 1	1	24 174	11 944	83.8	33.6
	2	24 590	11 927	84.9	32.8
	3	24 609	12 106	87.5	30.8
	4	24 136	12 100	84.3	30.6
	5	22 593	11 846	93.3	32.2
	6	23 558	11 989	83.4	27.9
Validation 2	1	24 543	12 430	85.5	31.3
	2	26 102	12 325	82.7	31.8
	3	24 091	12 965	79.3	29.8
	4	22 180	12 348	83.7	29.3
	5	22 984	12 218	85.3	27.1
	6	24 310	11 007	78.1	30.2
Validation 3	1	26 377	12 852	85.8	28.5
	2	27 098	13 179	82.2	28.2
	3	26 349	12 797	88.2	29.6
	4	25 624	12 925	80.3	34.2
	5	23 126	13 279	90.2	28.4
	6	25 457	13 337	83.1	32.1
	n	**18**	**18**	**18**	**18**
	Average	**24 550**	**12 421**	**84.5**	**30.5**
	STDEV	**1392**	**607**	**3.71**	**2.03**
	%CV	**5.7**	**4.9**	**4.4**	**6.7**
	%Accuracy	**102.3**	**103.5**	**112.7**	**101.6**

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Table 5.18 - Inter-day accuracy and precision of STDs from validation 1-3 for sulfapyridine.

Batch	Replicates	STD 1 30 000 ng/mL	STD 2 15 000 ng/mL	STD 3 7 500 ng/mL	STD 4 3 000 ng/mL	STD 5 1 500 ng/mL	STD 6 750 ng/mL	STD 7 300 ng/mL	STD 8 150 ng/mL	STD 9 60.0 ng/mL	STD 10 30.0 ng/mL
Validation 1	1	28 564	15 154	7 489	2 991	1 475	730	300	151	60.8	27.4
	2	31 286	15 160	7 561	2 831	1 544	689	306	154	62.9	33.1
Validation 2	1	29 158	14 377	7 498	2 877	1 537	730	310	150	57.0	29.8
	2	30 750	15 892	7 358	3 027	1 540	720	317	153	64.5	27.7
Validation 3	1	28 465	15 073	7 455	3 090	1 532	739	304	141	51.1	31.2
	2	31 547	14 943	7 276	3 079	1 519	740	325	161	62.2	28.0
	n	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
	Average	**29962**	**15100**	**7 440**	**2 983**	**1 525**	**725**	**310**	**152**	**59.7**	**29.5**
	STDEV	**1395**	**486**	**104**	**107**	**25.7**	**19.0**	**9.23**	**6.67**	**4.93**	**2.26**
	%CV	**4.7**	**3.2**	**1.4**	**3.6**	**1.7**	**2.6**	**3.0**	**4.4**	**8.3**	**7.7**
	%Accuracy	**99.9**	**100.7**	**99.2**	**99.4**	**101.6**	**96.6**	**103.4**	**101.1**	**99.6**	**98.5**

Table 5.19 - Inter-day accuracy and precision of QCs from validation 1-3 for sulfapyridine.

Batch	Replicates	QC H 24 000 ng/mL	QC M 12 000 ng/mL	QC L 75.0 ng/mL	QC LLOQ 30.0 ng/mL
Validation 1	1	24 152	12 193	80.6	36.4
	2	24 481	12 107	84.3	32.9
	3	24 385	12 305	87.6	30.4
	4	24 642	12 248	86.1	31.2
	5	24 362	12 171	81.9	32.4
	6	24 608	12 059	81.0	30.0
Validation 2	1	24 169	12 549	82.9	28.7
	2	25 172	12 324	84.7	27.6

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	3	22 193	12 287	82.4	29.6
	4	23 989	13 040	80.1	25.6
	5	25 059	13 096	82.1	28.0
	6	25 485	11 937	80.0	27.2
Validation 3	1	25 542	13 080	77.5	29.3
	2	25 818	13 685	79.1	30.2
	3	25 683	13 341	76.9	29.9
	4	25 739	13 378	84.3	25.1
	5	23 984	13 757	80.2	24.9
	6	25 812	13 647	71.9	27.0
	n	**18**	**18**	**18**	**18**
	Average	**24 737**	**12 734**	**81.3**	**29.2**
	STDEV	**919**	**634**	**3.66**	**2.92**
	%CV	**3.7**	**5.0**	**4.5**	**10.0**
	%Accuracy	**103.1**	**106.1**	**108.4**	**97.4**

As illustrated in **Table 5.16-5.19** above, the average accuracy of STDs during the inter-day validations ranged from 94.2-103.2% (%CV= 1.4-10.8) for sulfasalazine and 96.6-103.4% (%CV= 1.4-8.3) for sulfapyridine. The average accuracy of QCs ranged from 101.6-112.7% (%CV= 4.4-6.7) and 97.4-108.4% (%CV= 3.7-10.0) for sulfasalazine and sulfapyridine, respectively. Although the data are not represented in the tables above, for the intra-day validations the accuracies of STDs ranged from 92.3-107.1% (%CV= 0.1-17.1) for sulfasalazine and 94.4-104.8% (%CV= 0.03-13.8) for sulfapyridine. Similarly, the average accuracies of QCs ranged from 99.8-114.9% (%CV= 0.9-8.1) and 92.4-112.3% (%CV= 0.7-8.7) for sulfasalazine and sulfapyridine, respectively.

Therefore, as per the required criteria for both inter- and intra- day validations, 75% of all STDs and 67% of QCs in each batch passed (with 50% at each level) [12][13][14][105][106]. Furthermore, the %CV between the replicates was less than 15% for each STD or QC, and less than 20% for the lowest standard (STD 10) and the LLOQ [12][13][14][105][106]. The data indicate that the method was accurate, precise, and reproducible for the analysis of sulfasalazine and sulfapyridine in placental tissue homogenate over the range 30-30 000 ng/mL.

No previous LC-MS/MS methods have been developed for the quantitation of sulfasalazine and sulfapyridine in placental tissue homogenate, thus calibration ranges could not be compared with other studies.

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5.4.6 Sensitivity

To determine sensitivity of the method, six different sources of placental homogenate from six different sources of human placenta were spiked at the LLOQ (30 ng/mL) for sulfasalazine and sulfapyridine and extracted as per the final extraction protocol. The signal-to-noise (S/N) ratio was calculated by the software (LabSolutions version 5.109). Raw chromatograms of the LLOQ for both sulfasalazine and sulfapyridine are shown in **Figure 5.9** and **Figure 5.10**, respectively. For the method to quantitate both analytes with adequate accuracy and precision, the S/N is required to be equal to or greater than 5 [105].

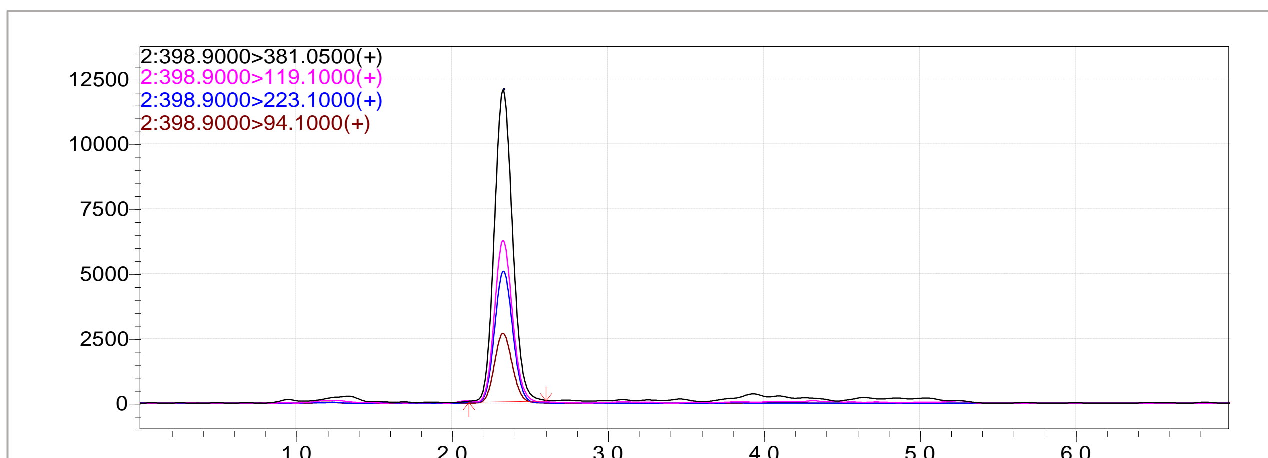

Figure 5.9 - A representative chromatogram of sulfasalazine at the LLOQ (30 ng/mL).

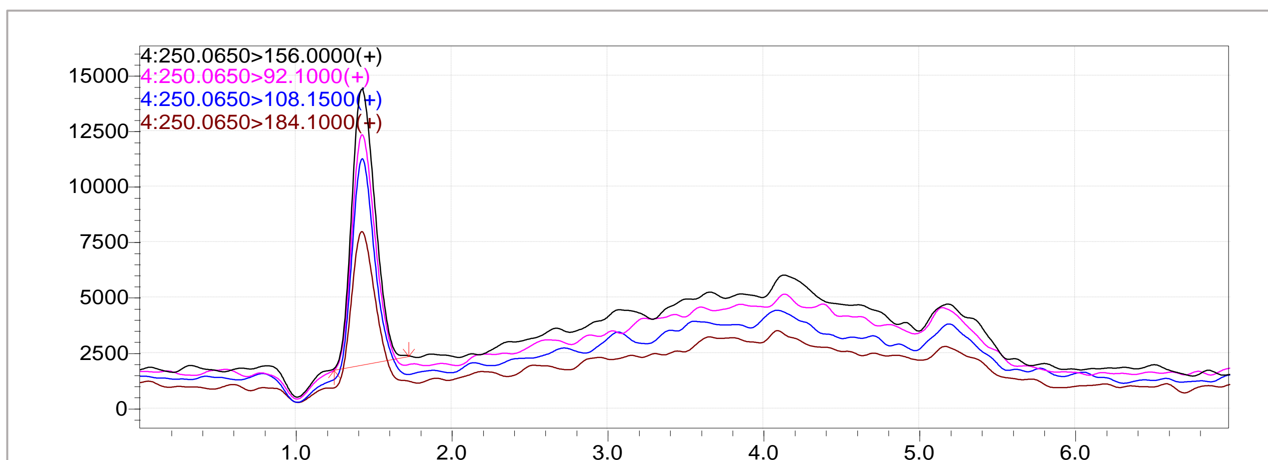

Figure 5.10 - A representative chromatogram of sulfapyridine at the LLOQ (30 ng/mL).

The S/N at the LLOQ level extracted from six different lots of matrices was greater than 5 for sulfasalazine and sulfapyridine, indicating that the mass spectrometry method was sensitive for both analytes, and could accurately and precisely quantitate both analytes at this concentration. Thus, patient samples with a calculated concentration of greater than or equal to 30 ng/mL were reported, however, patient samples with a calculated concentration of below 30 ng/mL were reported as “below the limit of quantitation” (BLQ). It was difficult to compare the sensitivity of this method with other studies, as this is the first LC-MS/MS method developed to quantitate sulfasalazine and sulfapyridine in placental tissue.

5.4.7 Specificity and Carry-Over

To determine specificity, blank placental tissue homogenate obtained from six different sources of placenta was extracted according to the extraction protocol, with the exception that the protein precipitation solvent contains no internal standard (i.e., double blanks). Moreover, to assess potential carry-over effects, a double blank sample was positioned in the injection sequence immediately after the highest calibration standard (STD1). The double blank chromatograms of sulfasalazine, sulfapyridine, and their relevant internal standards are illustrated in **Figure 5.11 and Figure 5.12** below.

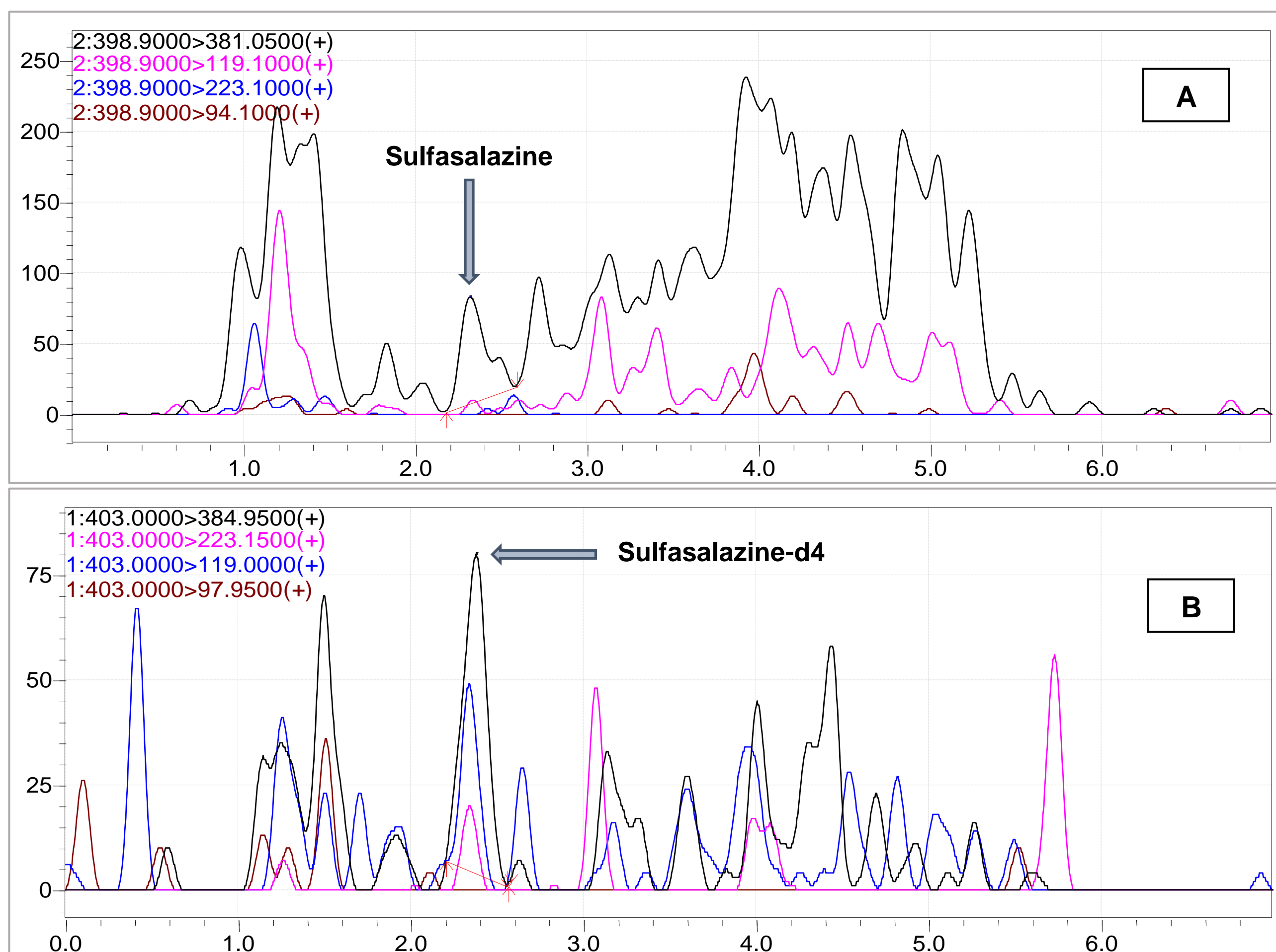

Figure 5.11 – Double blank chromatograms of (A) sulfasalazine; and (B) sulfasalazine-d4.

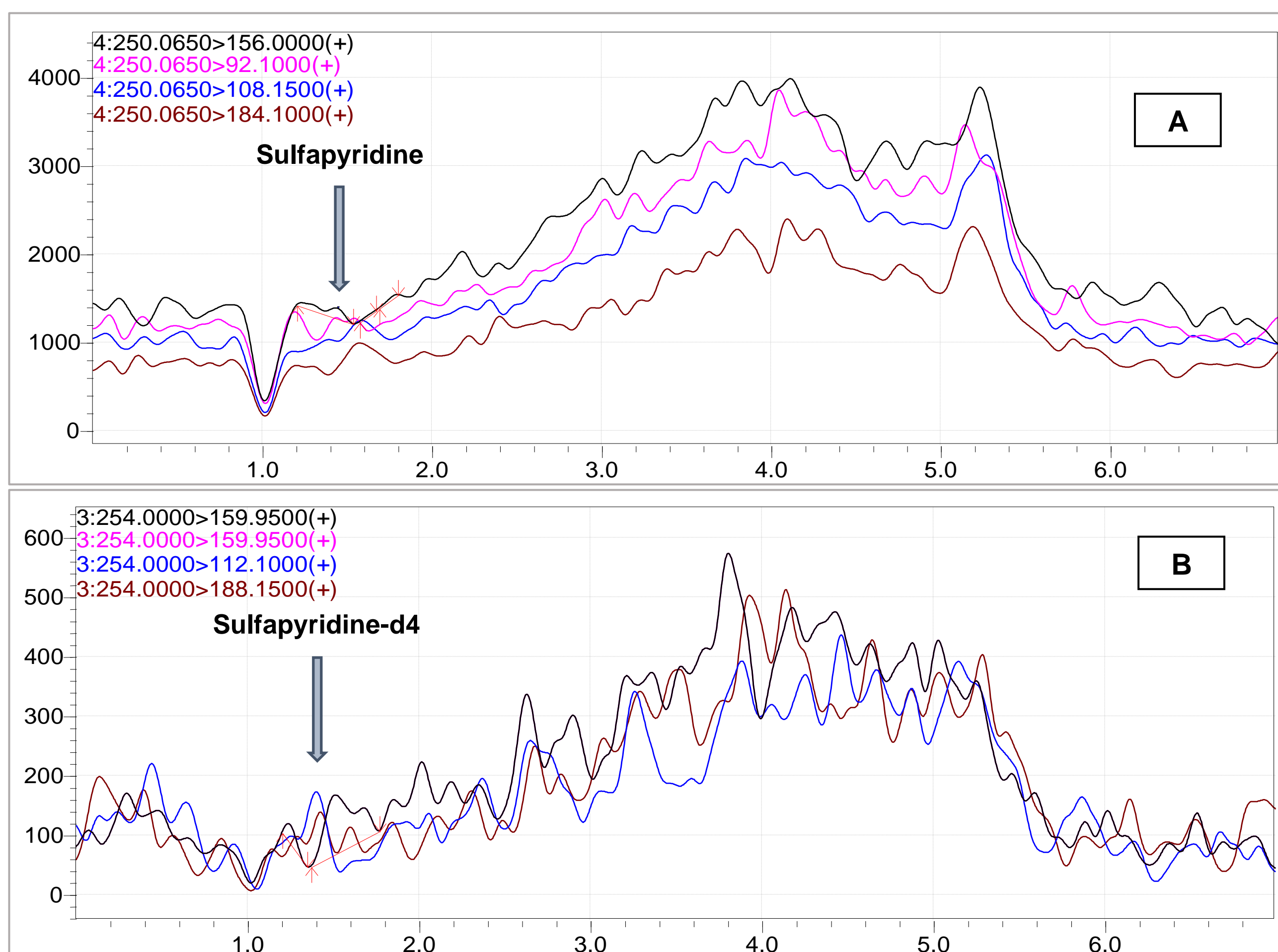

Figure 5.12 – Double blank chromatograms of (A) sulfapyridine; and (B) sulfapyridine-d4.

The chromatograms presented in **Figure 5.11-12** indicate that the method was highly specific and showed no carry-over when injected after the highest standard because there were no identifiable peaks at the retention time of the analytes or their internal standards. The chromatogram for sulfasalazine (**Figure 5.11 A**) showed very low background noise throughout the run time. However, the chromatogram of sulfapyridine (**Figure 5.12 A**) shows evident noise as well as a solvent front, which is not usually visible on chromatograms obtained using LC-MS/MS. The phenomenon was not caused by the matrix itself, because a neat, unextracted sample containing sulfapyridine at a low concentration (10 ng/mL) was injected into the system in triplicate and the same solvent front and noise was visible. Therefore, this occurrence was attributed to a potentially contaminated ionisation source at the specific mass of sulfapyridine and may be resolved with a simple instrument clean. It is important to note that this did not negatively affect the method, as the LLOQ concentrations were detected with adequate accuracy and precision.

5.4.8 Matrix Effects

This experiment was performed using a method described by Matuszewski *et al.* [111] whereby six different sources of placenta were used to account for inter-and intra-patient matrix variabilities present in a clinical setting [113]. Blank placental homogenate from six different sources was extracted as per the final extraction method, except without analyte or internal standard. After extraction, the samples were evaporated to dryness

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and reconstituted in 200 µL of [acetonitrile:methanol (90:10)] : water (1:1) which was spiked at a concentration that represented a theoretical high (9231 ng/mL), medium (4615 ng/mL), or low (29 ng/mL) concentration for each analyte, and a single theoretical internal standard concentration for sulfasalazine-d4 (385 ng/mL) and sulfapyridine-d4 (308 ng/mL). The theoretical concentration takes into account appropriate adjustments for dilution and concentration steps that occurred during the extraction process. The calculations used to determine the theoretical concentrations can be found in **Appendix B**. The ratios of analyte to internal standard peak area for QC H, M, and L versus their respective nominal concentrations were used to generate regressions over the concentration range for each individual matrix, as shown in **Table 5.20** and **Table 5.21**.

Table 5.20 - Regression results from six different matrices to determine matrix effects for sulfasalazine.

Placenta Source	High Conc. 24 000 ng/mL Peak Area Ratio	Medium Conc. 12 000 ng/mL Peak Area Ratio	Low Conc. 75 ng/mL Peak Area Ratio	Area Ratio vs Conc. Regression Slope
Patient 1	52.5	25.8	0.153	0.00219
Patient 2	51.9	25.3	0.155	0.00216
Patient 3	51.8	25.2	0.160	0.00216
Patient 4	50.5	25.2	0.163	0.00210
Patient 5	51.4	25.8	0.159	0.00214
Patient 6	52.0	25.8	0.157	0.00217
Average	**51.7**	**25.5**	**0.158**	**0.00215**
STDEV	**0.701**	**0.328**	**0.00365**	**0.0000294**
% CV	**1.4**	**1.3**	**2.3**	**1.4**

Table 5.21 - Regression results from six different matrices to determine matrix effects for sulfapyridine.

Placenta Source	High Conc. 24 000 ng/mL Peak Area Ratio	Medium Conc. 12 000 ng/mL Peak Area Ratio	Low Conc. 75 ng/mL Peak Area Ratio	Area Ratio vs Conc. Regression Slope
Patient 1	56.2	25.9	0.164	0.00234
Patient 2	57.8	26.9	0.156	0.00241
Patient 3	56.6	26.1	0.152	0.00236
Patient 4	58.5	26.9	0.160	0.00244
Patient 5	57.2	25.8	0.160	0.00238
Patient 6	57.9	26.4	0.143	0.00241
Average	**57.4**	**26.4**	**0.156**	**0.00239**
STDEV	**0.846**	**0.483**	**0.00761**	**0.0000355**
% CV	**1.5**	**1.8**	**4.9**	**1.5**

The mean variability of the individual regression slopes was determined to be 1.4% for sulfasalazine and 1.5% for sulfapyridine, meeting the acceptance criteria of less than 5.0% for mean variability as stipulated in the FDA and EMA guidelines [14][111][112]. Moreover, %CV's generated from the six different matrix sources were

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below 15% for QC H, M, and L. In comparison with biological fluids, tissues consist of a more complex mixture of cellular components with varying amounts of phospholipids, which may be more likely to contribute towards variable matrix effects [108]. Despite this challenge, the method displayed insignificant matrix effects, indicating that background components which may impact the ionisation of analytes were effectively removed during the extraction method, and that the internal standard adequately compensated for any matrix effects.

5.4.9 Recovery

Six different lots of matrices were spiked at QC H, M, and L concentrations (24 000 ng/mL, 12 000 ng/mL, and 75 ng/mL, respectively) for both sulfasalazine and sulfapyridine and extracted as per the final extraction method. These were referred to as the "test" samples. The same six lots of blank matrices were extracted in the same way as the test samples (except without internal standard), and spiked post-extraction with a single concentration of each internal standard and an analyte concentration which represented 100% recovery for QC H, M, and L, respectively . These were referred to as the "reference samples". To obtain a percentage recovery, the analyte peak areas of the test samples were compared to the analyte peak areas observed in the reference samples, as shown in **Table 5.22** and **Table 5.23**.

Table 5.22 - Recovery experiment results from six different matrices for sulfasalazine.

Placenta Source	High Concentration (24 000 ng/mL)		Medium Concentration (12 000 ng/mL)		Low Concentration (75 ng/mL)	
	Test Sample Analyte Peak Area	Reference Sample Analyte Peak Area	Test Sample Analyte Peak Area	Reference Sample Analyte Peak Area	Test Sample Analyte Peak Area	Reference Sample Analyte Peak Area
Patient 1	25 676 001	21 198 038	12 097 076	11 397 449	109 190	71 958
Patient 2	22 220 595	20 049 136	12 282 998	10 834 832	95 751	70 072
Patient 3	21 474 790	20 019 702	11 938 716	10 313 443	99 621	73 399
Patient 4	23 002 800	20 380 533	11 628 719	10 810 694	104 050	73 929
Patient 5	22 407 151	19 915 687	12 861 204	11 488 742	106 187	72 624
Patient 6	22 451 192	19 699 865	12 768 749	11 130 131	96 453	75 064
Average	**22 872 088**	**20 210 494**	**12 262 910**	**10 995 882**	**101 875**	**72 841**
STDEV	**1 459 398**	**531 836**	**479 382**	**435 464**	**5456**	**1728**
% CV	**6.4**	**2.6**	**3.9**	**4.0**	**5.4**	**2.4**
% Recovery	----	**113.2**	----	**111.5**	----	**139.9**
				Average % Recovery		**121.5**
				Average % CV		**13.1**

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Table 5.23 - Recovery experiment results from six different matrices for sulfapyridine.

Placenta Source	High Concentration (24 000 ng/mL)		Medium Concentration (12 000 ng/mL)		Low Concentration (75 ng/mL)	
	Test Sample Analyte Peak Area	Reference Sample Analyte Peak Area	Test Sample Analyte Peak Area	Reference Sample Analyte Peak Area	Test Sample Analyte Peak Area	Reference Sample Analyte Peak Area
Patient 1	26 865 084	24 107 495	15 904 438	13 670 018	179 496	145 569
Patient 2	24 890 281	22 742 851	15 956 146	13 330 265	176 482	130 661
Patient 3	23 763 861	21 705 507	15 101 361	12 321 503	187 463	142 261
Patient 4	24 598 326	22 454 491	14 754 956	13 000 614	174 250	138 748
Patient 5	23 190 019	21 884 528	15 049 779	12 606 482	175 342	135 878
Patient 6	23 524 307	21 238 646	15 364 439	12 172 856	167 194	124 201
Average	**24 471 980**	**22 355 586**	**15 355 187**	**12 850 290**	**176 705**	**136 220**
STDEV	**1 338 718**	**1 012 027**	**485 982**	**587 010**	**6662**	**7820**
% CV	**5.5**	**4.5**	**3.2**	**4.6**	**3.8**	**5.7**
% Recovery	----	**109.5**	----	**119.7**	----	**129.7**
				Average % Recovery		**119.6**
				Average % CV		**8.5**

The average recovery was determined to be 121.5% (%CV = 13.1%) for sulfasalazine and 119.6% (%CV = 8.5%) for sulfapyridine. The recovery was consistent and precise across the different concentrations and matrix sources with a required average precision and a precision at QC H, M, and L of less than 15%. However, a challenge encountered when working with tissue homogenate was that the approach used to determine recovery did not necessarily mimic the true distribution of the analytes in real tissue samples [108]. Thus, the unknown nature of tissue-drug distribution *in vivo* complicated the true assessment of method recovery.

5.4.10 Process Efficiency

Neat, un-extracted samples which represented 100% efficiency (i.e., 100% recovery and zero matrix effects) were prepared in triplicate by spiking the theoretical concentrations of QC H, M, and L into solvent composed of [acetonitrile:methanol (90:10)] : water (1:1), respectively. These were referred to as the "spiked solution" samples. Six different lots of matrices were spiked at QC H, M, and L concentrations (24 000 ng/mL, 12 000 ng/mL, and 75 ng/mL, respectively) for both sulfasalazine and sulfapyridine and extracted as per the final extraction method. These were referred to as the "extracted samples". To obtain a percentage process efficiency, the ratios of the analyte peak area to internal standard peak area of the spiked solution samples were compared to the ratios of the analyte peak area to internal standard peak area of the extracted samples. The data for both analytes are illustrated in **Table 5.24** and **Table 5.25**. As with average percentage recovery, the average percentage process efficiency need not be 100% but should be consistent across the different concentrations and plasma sources.

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Table 5.24 - Process efficiency experiment results for sulfasalazine.

Placenta Source	High Concentration (24 000 ng/mL)		Medium Concentration (12 000 ng/mL)		Low Concentration (75 ng/mL)	
	Extracted Analyte Peak Area Ratio	Spiked Solution Analyte Peak Area Ratio	Extracted Analyte Peak Area Ratio	Spiked Solution Analyte Peak Area Ratio	Extracted Analyte Peak Area Ratio	Spiked Solution Analyte Peak Area Ratio
Patient 1	64.7	51.5	33.4	26.3	0.282	0.169
Patient 2	69.7	52.1	35.4	25.7	0.259	0.171
Patient 3	70.8	53.1	34.0	26.2	0.266	0.158
Patient 4	68.6	----	34.8	----	0.282	----
Patient 5	65.7	----	35.9	----	0.268	----
Patient 6	68.3	----	33.7	----	0.257	----
Average	**68.0**	**52.2**	**34.5**	**26.1**	**0.269**	**0.166**
STDEV	**2.33**	**0.798**	**1.01**	**0.312**	**0.0107**	**0.00683**
% CV	**3.4**	**1.5**	**2.9**	**1.2**	**4.0**	**4.1**
% Process Efficiency	----	**130.1**	----	**132.3**	----	**162.2**
				Average %Process Efficiency		**141.5**
				Average % CV		**12.7**

Table 5.25 - Process efficiency experiment results for sulfapyridine.

Placenta Source	High Concentration (24 000 ng/mL)		Medium Concentration (12 000 ng/mL)		Low Concentration (75 ng/mL)	
	Extracted Analyte Peak Area Ratio	Spiked Solution Analyte Peak Area Ratio	Extracted Analyte Peak Area Ratio	Spiked Solution Analyte Peak Area Ratio	Extracted Analyte Peak Area Ratio	Spiked Solution Analyte Peak Area Ratio
Patient 1	72.6	55.2	33.9	26.5	0.251	0.148
Patient 2	79.2	55.8	38.4	26.8	0.261	0.152
Patient 3	76.2	58.3	34.8	26.4	0.241	0.151
Patient 4	78.5	----	37.0	----	0.249	----
Patient 5	70.8	----	36.5	----	0.248	----
Patient 6	76.9	----	36.8	----	0.249	----
Average	**75.7**	**56.4**	**36.2**	**26.5**	**0.250**	**0.151**
STDEV	**3.34**	**1.63**	**1.62**	**0.244**	**0.00654**	**0.00225**
% CV	**4.4**	**2.9**	**4.5**	**0.9**	**2.6**	**1.5**
% Process Efficiency	----	**134.1**	----	**136.5**	----	**166.1**
				Average %Process Efficiency		**145.6**
				Average % CV		**12.2**

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The mean process efficiency across the QC H, M, and L concentrations was determined to be 141.5% (%CV = 12.7%) for sulfasalazine and 145.6% (%CV = 12.2%) for sulfapyridine. The %CV at QC H, M, and L does not exceed 15%, and the average %CV does not exceed 15%, thus the acceptance criteria were met for both analytes. The mean process efficiency of sulfasalazine was 20.0% higher than its mean recovery. Similarly, the mean process efficiency of sulfapyridine was 26.0% higher than its mean recovery. When analysing the data in **Table 5.24-5.25**, it is evident that the analyte to internal standard peak area ratios for the extracted samples were higher than that of the spiked solutions across the QC H, M, and L concentrations for both analytes. This suggests that potential ion enhancement occurred in the mass spectrometer during analysis of the extracted samples as a result of matrix being present. However, as illustrated by the matrix effects experiment for sulfasalazine and sulfapyridine, these effects were consistent across different lots of matrices and were compensated for by the internal standard, and therefore, did not negatively impact the method. While noting the differences that have occurred between the recovery and process efficiency results may be interesting, this does not determine whether a method has met the acceptance criteria. Working with tissue was far more challenging than working with matrices such as plasma or urine because tissue homogenate contains large quantities of cellular membrane particles that may affect the extraction process and efficiency [108]. However, despite these challenges, the method proved to be adequate and efficient, because the recovery and the presence of matrix components did not impact the response of sulfasalazine and sulfapyridine.

5.4.11 Re-injection Stability

Following the initial injection of the second validation batch (validation day 2), the extracted samples were left in the autosampler at 10 °C for 48 hours after which the batch was re-injected and subjected to the same criteria as for a validation batch. Therefore, it was evaluated whether a batch passed or failed as a whole. The re-injection reproducibility data obtained for the STDs and QCs for sulfasalazine and sulfapyridine from validation day 2 after 48- hours are summarised in **Table 5.26** and **Table 5.27**.

Table 5.26 – Re-injection reproducibility of STDs after 48 hours in the autosampler at 10 °C.

Calibration Standards (STDs)	Sulfasalazine			Sulfapyridine		
	%CV	%Accuracy	n	%CV	%Accuracy	n
STD 1	4.7	100.6	2 of 2	6.6	100.4	2 of 2
STD 2	0.6	97.4	2 of 2	0.1	97.7	2 of 2
STD 3	1.8	100.9	2 of 2	6.5	101.4	2 of 2
STD 4	3.6	102.3	2 of 2	0.2	101.0	2 of 2
STD 5	2.1	105.9	2 of 2	0.6	104.6	2 of 2
STD 6	3.2	96.0	2 of 2	0.8	101.0	2 of 2
STD 7	3.1	103.6	2 of 2	2.2	105.3	2 of 2
STD 8	5.1	106.6	2 of 2	0.4	103.7	2 of 2
STD 9	0.5	98.7	2 of 2	4.4	98.2	2 of 2
STD 10	5.9	88.1	2 of 2	0.5	86.6	2 of 2

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Table 5.27 – Re-injection reproducibility of QCs after 48 hours in the autosampler at 10 °C.

Quality Controls (QCs)	Sulfasalazine			Sulfapyridine		
	%CV	%Accuracy	n	%CV	%Accuracy	n
QC H	3.9	101.1	6 of 6	4.7	99.4	6 of 6
QC M	4.0	102.9	6 of 6	4.1	105.4	6 of 6
QC L	3.9	114.3	6 of 6	3.0	110.3	6 of 6
QC LLOQ	10.7	94.4	6 of 6	11.9	92.6	6 of 6

For sulfasalazine, the accuracy of STDs ranged from 88.1 - 106.6% and QCs ranged from 94.4 - 114.3%. For sulfapyridine, the accuracy of STDs and QCs ranged from 86.6-105.3% and 92.6-110.3%, respectively. For both analytes, the %CV for STDs and QCs was equal to or less than 11.9%. Therefore, the data indicate that the method remained accurate and precise for both sulfasalazine and sulfapyridine following a re-injection of the batch 48 hours after the initial injection. If it is not possible to inject the batch on the day of extraction or should instrument disruptions or malfunctions occur [114], the extracted samples may be re-injected and accurately analysed for up to 48 hours when stored in the autosampler at 10 °C.

5.4.12 Autosampler (On-instrument) Stability

The same set of results used to assess re-injection stability were used to determine autosampler stability at 10 °C following a 48-hour re-injection of the second validation batch. The analyte to internal standard peak area ratios of QC H and L for both analytes were compared to the results obtained from the initial validation day 2 injection. The percentage difference between the mean peak area ratios of the initial injection and the 48-hour re-injection for sulfasalazine and sulfapyridine are shown in **Table 5.28** and **Table 5.29**, respectively.

Table 5.28 - Summary of 48- hour autosampler stability of sulfasalazine in extracted samples.

Summary Statistics	High Concentration		Low Concentration	
	Initial Injection	Re-injection after 48 hours	Initial Injection	Re-injection after 48 hours
Average Peak Area Ratio	61.0	62.8	0.249	0.265
STDEV	3.21	2.32	0.00837	0.0106
%CV	5.3	3.7	3.4	4.0
% Difference	----	2.9	----	6.1

Table 5.29 - Summary of 48- hour autosampler stability of sulfapyridine in extracted samples.

Summary Statistics	High Concentration		Low Concentration	
	Initial Injection	Re-injection after 48 hours	Initial Injection	Re-injection after 48 hours
Average Peak Area Ratio	77.0	70.1	0.274	0.235
STDEV	4.04	3.81	0.00528	0.00617
%CV	5.2	5.4	1.9	2.6
% Difference	----	-9.0	----	-14.2

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For sulfasalazine, the percentage difference in the average peak area ratio between the initial injection and the 48-hour re-injection was 2.9% for the high concentration, and 6.1% for the low concentration. For sulfapyridine, the percentage difference in the average peak area ratio for the high concentration was 9.0%, and for the low concentration the percentage difference was 14.2%. Because the re-injected peak area ratios of both analytes do not differ by more than 15% of their initial peak area ratio, the results indicated post-extraction stability of the analytes for up to 48 hours at 10 °C. Therefore, if instrument malfunction occurs causing a batch to stop running, partial or entire batch re-injections may be performed for sulfasalazine and sulfapyridine, starting from the last accepted STD that was injected prior to instrument disruption [107][114].

5.4.13 Fresh vs Frozen Stability

To determine whether the process of freezing in the matrix has an impact on the analytes, STDs in validation batch 3 were freshly prepared on the day of extraction and compared to QCs that were frozen for at least 24 hours prior to extraction. Thus, the data obtained has also been used to demonstrate stability of the analytes in matrix at the specified storage condition of -80°C for 24 hours. Freshly prepared STDs were extracted in duplicate and QC H, M, L, and LLOQ which were frozen at -80°C for at least 24 hours were extracted in six-fold. The data obtained were subjected to the requirements as per a validation batch. Additionally, as per FDA and EMA guidelines, the freeze-thaw, on-bench, and matrix stability assessments were included in this validation batch whereby STDs were freshly prepared on the day of extraction [12]. The accuracy and precision statistics for STDs and QCs are illustrated in **Table 5.30** for sulfasalazine and **Table 5.31** for sulfapyridine.

Table 5.30 - Accuracy and precision summary statistics from validation day 3 for sulfasalazine.

Calibration Standards (STDs)	Nominal Concentration (ng/mL)	Mean Observed Concentration (ng/mL)	STDEV	%CV	%Accuracy	n
STD 1	30 000	30 089	778	2.6	100.3	2 of 2
STD 2	15 000	14 776	352	2.4	98.5	2 of 2
STD 3	7 500	7 582	342	4.5	101.1	2 of 2
STD 4	3 000	3 064	66.7	2.2	102.1	2 of 2
STD 5	1 500	1 506	21.0	1.4	100.4	2 of 2
STD 6	750	724	40.2	5.6	96.6	2 of 2
STD 7	300	309	12.2	3.9	103.1	2 of 2
STD 8	150	150	4.59	3.1	100.1	2 of 2
STD 9	60.0	61.3	0.266	0.4	102.1	2 of 2
STD 10	30.0	28.7	1.29	4.5	95.6	2 of 2
Quality Controls (QCs)	**Nominal Concentration (ng/mL)**	**Mean Observed Concentration (ng/mL)**	**STDEV**	**%CV**	**%Accuracy**	**n**
QC H	24 000	25 672	1380	5.4	107.0	6 of 6
QC M	12 000	13 061	232	1.8	108.8	6 of 6
QC L	75.0	82.9	3.76	4.4	113.3	6 of 6
QC LLOQ	30.0	30.2	2.44	8.1	100.6	6 of 6

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Table 5.31 - Accuracy and precision summary statistics from validation day 3 for sulfapyridine.

Calibration Standards (STDs)	Nominal Concentration (ng/mL)	Mean Observed Concentration (ng/mL)	STDEV	%CV	%Accuracy	n
STD 1	30 000	30 006	2179	7.3	100.0	2 of 2
STD 2	15 000	15 008	92.3	0.6	100.1	2 of 2
STD 3	7 500	7 366	127	1.7	98.2	2 of 2
STD 4	3 000	3 085	8.13	0.3	102.8	2 of 2
STD 5	1 500	1 525	9.21	0.6	101.7	2 of 2
STD 6	750	739	0.930	0.1	98.6	2 of 2
STD 7	300	314	14.4	4.6	104.8	2 of 2
STD 8	150	151	14.6	9.6	100.7	2 of 2
STD 9	60.0	56.6	7.79	13.8	94.4	2 of 2
STD 10	30.0	29.6	2.20	7.4	98.7	2 of 2
Quality Controls (QCs)	**Nominal Concentration (ng/mL)**	**Mean Observed Concentration (ng/mL)**	**STDEV**	**%CV**	**%Accuracy**	**n**
QC H	24 000	25 429	716	2.8	106.0	6 of 6
QC M	12 000	13 481	259	1.9	112.3	6 of 6
QC L	75.0	78.3	4.11	5.2	104.5	6 of 6
QC LLOQ	30.0	27.7	2.41	8.7	92.4	6 of 6

From the tables above, it is evident that all STDs and QCs for sulfasalazine and sulfapyridine met the required criteria for a validation batch. For sulfasalazine, the accuracy of STDs ranged from 95.6 - 103.1% and the QCs ranged from 100.6 - 113.3%. For sulfapyridine, the accuracy of STDs and QCs ranged from 94.4 - 104.8% and 92.4 - 112.3%, respectively. Furthermore, the %CV was less than 15% for all STDs and QCs. Therefore, the method remained accurate and precise for both analytes following the comparison of STDs which were freshly prepared on the day of extraction to QCs frozen for 24 hours prior to extraction. Therefore, freezing in the matrix at -80 °C for 24 hours did not have an impact on the ability of the method to quantitate the analytes. The data also demonstrated stability of both analytes in the matrix at -80 °C for a storage period of 24 hours.

5.4.14 Freeze-Thaw Stability

Freeze-thaw stability was tested for by mimicking the sample handling conditions as closely as possible. To ascertain freeze-thaw stability, frozen QCs at high (24 000 ng/mL) and low concentrations (75 ng/mL) for both sulfasalazine and sulfapyridine were subjected to three consecutive freeze-thaw cycles. Each cycle consisted of a 30-minute thawing time at room temperature followed by a 24-hour freezing time at -80 °C. Furthermore, samples were frozen for at least 24 hours before starting this experiment. The QC H and L samples were then extracted in six-fold, analysed against a freshly prepared validation calibration curve within validation batch 3, and assessed for accuracy against the nominal QC concentrations. The data are illustrated in **Table 5.32**.

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Table 5.32 - Stability of sulfasalazine and sulfapyridine in matrix after three freeze-thaw cycles.

Statistical Analysis for Freeze-Thaw Stability	Sulfasalazine		Sulfapyridine	
	High Concentration 24 000 ng/mL	Low Concentration 75 ng/mL	High Concentration 24 000 ng/mL	Low Concentration 75 ng/mL
Average	24 419	83.6	24 496	78.2
n	6	6	6	6
STDEV	309	8.07	504	6.89
%CV	1.3	9.7	2.1	8.8
% Difference from Nominal Concentration	1.7	11.5	2.1	4.3

The results presented in the table above indicate that the percentage differences from the nominal concentrations for QC H and L for sulfasalazine and sulfapyridine were within 15%. Moreover, the %CVs do not exceed 15%. Thus, both analytes were stable in placental tissue homogenate for three freeze-thaw cycles when stored at -80 °C and thawed at room temperature for 30 minutes.

5.4.15 Benchtop Stability

Bench-top stability was tested for by mimicking the expected amount of time that samples will be on-bench at room temperature prior to and during an extraction. To determine benchtop stability, frozen QCs at high (24 000 ng/mL) and low concentrations (75 ng/mL) for both sulfasalazine and sulfapyridine were removed from the -80 °C freezer and left on-bench for approximately six hours. QC H and L were then extracted in six-fold, tested against a freshly prepared calibration curve within validation batch 3, and assessed for accuracy against the nominal QC concentrations. The data are illustrated in **Table 5.33**.

Table 5.33 – Stability of sulfasalazine and sulfapyridine in matrix for six hours on-bench.

Statistical Analysis for Benchtop Stability	Sulfasalazine		Sulfapyridine	
	High Concentration 24 000 ng/mL	Low Concentration 75 ng/mL	High Concentration 24 000 ng/mL	Low Concentration 75 ng/mL
Average	24 634	72.3	23 298	70.0
n	6	6	6	6
STDEV	711	5.13	610	6.28
%CV	2.9	7.1	2.6	9.0
% Difference from Nominal Concentration	2.6	-3.6	-2.9	-6.6

For both sulfasalazine and sulfapyridine, the percentage differences from the nominal concentrations for QC H and L were within 15%. Furthermore, all %CVs were less than 15%, thus falling within the accepted criteria. Therefore, the data indicate that both analytes were stable in placental tissue homogenate for up to six hours when left on-bench at room temperature.

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5.4.16 Matrix Stability

High and low QC's were stored at -80 °C for 10 days. The QC's were removed from storage and extracted in six-fold, tested against a freshly prepared calibration curve within validation batch 3, and assessed for accuracy against the nominal QC concentrations. This experiment provided storage stability information for each analyte in matrix when stored at -80 °C for 10 days. The data are illustrated in **Table 5.34**.

Table 5.34 – Stability of sulfasalazine and sulfapyridine in matrix after storage at -80 °C for 10 days.

Statistical Analysis for Matrix Stability	Sulfasalazine		Sulfapyridine	
	High Concentration 24 000 ng/mL	Low Concentration 75 ng/mL	High Concentration 24 000 ng/mL	Low Concentration 75 ng/mL
Average	25 729	80.9	26 466	84.8
n	6	6	6	6
STDEV	1616	4.08	1535	4.03
%CV	6.3	5.0	5.8	4.8
% Difference from Nominal Concentration	7.2	7.9	10.3	13.0

The percentage differences of QC H and L from the nominal concentrations and the %CVs were all less than 15% for both sulfasalazine and sulfapyridine, thus falling within the accepted criteria. Therefore, the data indicate that both analytes were stable in placental tissue homogenate when stored at -80 °C for 10 days.

5.4.17 Dilution Integrity

To determine the integrity of a dilution post-extraction, undiluted QCDIL samples were extracted in six-fold as per the final extraction protocol. Post-extraction, the QCDIL samples were diluted 1:5 with blank samples extracted in the same way (containing internal standard). The data are shown in **Table 5.35**.

Table 5.35 - Dilution integrity after a five-fold post-extraction dilution for sulfasalazine and sulfapyridine.

Statistical Analysis for Dilution Integrity	Sulfasalazine	Sulfapyridine
	Dilution Post-extraction 60 000 ng/mL	Dilution Post-extraction 60 000 ng/mL
Average	65 469	68 926
STDEV	2553	4110
% CV	3.9	6.0
% Accuracy	109.1	114.9

The average percentage accuracy was 109.1% for sulfasalazine and 114.9% for sulfapyridine. Thus, patient samples reported as above the ULOQ (>30 000 ng/mL) may undergo a five-fold post-extraction dilution. A post-extraction dilution was useful for this assay because if samples were above the ULOQ, they did not need to be re-extracted, but rather, could be diluted post-extraction for reliable re-analysis. Because sulfasalazine and sulfapyridine have been determined to be stable in the autosampler at 10 °C for up to 48 hours, the entire batch may be re-run with the diluted samples, which meant that no re-extractions were necessary.

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5.5 Chapter Summary

This chapter describes the first LC-MS/MS method that has been developed and validated for the extraction, separation, determination, and quantitation of sulfasalazine and sulfapyridine in human placenta.

A suitable sample clean-up method was developed whereby the analytes of interest were extracted from placental tissue homogenate using protein precipitation followed by elution through HLB cartridges. During bioanalytical method validation, the method was rigorously tested according to FDA [12][13] and EMA [12][14] guidelines, and was shown to be robust, accurate, and precise over the concentration range of 30-30 000 ng/mL (150-150 000 ng/g) for both sulfasalazine and sulfapyridine. The accuracies of STDs ranged from 92.3-107.1% (%CV= 0.1-17.1) for sulfasalazine and 94.4-104.8% (%CV= 0.03-13.8) for sulfapyridine. Similarly, the average accuracies of QCs ranged from 99.8-114.9% (%CV= 0.9-8.1) and 92.4-112.3% (%CV= 0.7-8.7) for sulfasalazine and sulfapyridine, respectively.

The mean variability of the individual regression slopes was determined to be 1.4% for sulfasalazine and 1.5% for sulfapyridine, indicating that endogenous matrix components were found to have no adverse effects on the reproducibility of the method when placenta originating from six different sources was analysed. Furthermore, the average recovery was reproducible for both analytes and was determined to be 121.5% (%CV = 13.1%) for sulfasalazine and 119.6% (%CV = 8.5%) for sulfapyridine. The mean process efficiency for sulfasalazine and sulfapyridine across the QC H, M, and L concentrations was determined to be 141.5% (%CV = 12.7%) and 145.6% (%CV = 12.2%), respectively.

For both analytes, stock solutions prepared in DMSO and working solutions prepared in methanol were shown to be stable at -80 °C, -20 °C, and 4 °C for 24 hours, and on-bench at room temperature for four hours. Because both analytes could be accurately and precisely quantitated at the LLOQ, the method was deemed sensitive. Moreover, the method was highly specific and showed no carry-over when injected after the highest standard because there were no identifiable peaks at the retention time of the analytes or their internal standards. The integrity of a five-fold post-extraction dilution was illustrated such that samples reported as above the ULOQ (>30 000 ng/mL) for sulfasalazine and sulfapyridine could be re-analysed reliably when diluted to a concentration within the calibration range.

Sample extracts were reported to be stable in the autosampler for up to 48 hours at 10 °C, and re-injection reproducibility experiments indicated that a batch may be re-injected for up to 48 hours after the initial injection. It was shown that the process of freezing in the matrix for 24 hours at -80 °C did not have an impact on the analytes because the accuracy and precision of the method was not affected. Both analytes were shown to be stable in placental tissue homogenate for three freeze-thaw cycles when stored at -80 °C and thawed at room temperature for 30 minutes. Analytes were also reported to be stable in placental tissue homogenate for up to six hours at room temperature. Moreover, both analytes were stable in placental tissue homogenate when stored at -80 °C for 10 days.

The clinical application of this method has been described in **Chapter 6**.

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CHAPTER 6

Patient Sample Analysis of Sulfasalazine and Sulfapyridine in Placenta

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6.1 Introduction

The main objective of this chapter was to determine the concentrations of sulfasalazine and sulfapyridine in placental tissue samples obtained from the clinical trial. The patient samples were extracted as per the final method protocol and analysed using the validated LC-MS/MS method to quantitate the analyte concentrations within the range 30-30 000 ng/mL. Patient samples were reported as below the limit of quantitation (BLQ) if they yielded an observed concentration of below 30 ng/mL. To determine the measured concentration of the analytes expressed as a weight per weight (ng/g), a calculation adjustment of the observed concentrations (ng/mL) was required [108][121][125].

6.2 Placental Tissue Patient Samples

6.2.1 Addition of Homogenising Solvent

As mentioned in **Chapter 5**, a standard protocol for the mechanical shearing of tissue samples involves the addition of a homogenising solvent at a three- to- five-fold dilution [121]. For this project, for every 1 g of tissue weighed out, a volume of homogenising solvent (mL) was added at five times the mass (g), resulting in a five-fold dilution.

6.2.2 Drug Levels Measured in Patient Samples

After the patients gave birth at the Mercy Hospital for Women (Australia), the placentas were sent to the clinical trial site at the University of Melbourne to be dissected into smaller pieces and flash-frozen in liquid nitrogen for the preservation of the tissue and analytes of interest. Patient samples were stored at -80 °C at the clinical trial site, during transportation to the analytical site (Division of Clinical Pharmacology, Tygerberg Medical Campus), and at the analytical site until analysis. At the analytical site, each patient sample was further dissected, weighed (the weight was recorded) and added into homogenising tubes on the day of analysis. Ten patient samples were supposed to be analysed however one sample went missing at the clinical site before it was sent to the laboratory for analysis.

It was important to note that the flash-frozen placental samples thawed very quickly, which meant that due to the highly perfused nature of the placenta, the blood in and around the tissue samples thawed quickly as well. Not only did the placental tissue samples contain the analytes of interest, but it is highly likely that the residual blood may also have contained the analytes, potentially affecting the reproducibility and accuracy of the results. Caution was taken to prevent contamination of the placental tissue during dissection of each sample. Specifically, all equipment (i.e., the scale, cutting board, scalpel, etc.) was properly sanitised between the weighing of each sample, a new pair of gloves was used each time, samples were dissected and weighed as quickly as possible, and all samples were kept on ice prior to and after dissection.

The patient samples were homogenised and extracted as per the final protocol reported in **Chapter 5**. The placental tissue extracts were analysed using the developed and validated LC-MS/MS method to determine the observed sulfasalazine and sulfapyridine concentrations (ng/mL). To determine the measured concentration of the analytes expressed as a weigh per weight (ng/g tissue), the observed concentrations (ng/mL) obtained after

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LC-MS/MS analysis were normalised by tissue weight (g) per volume (mL) of homogenising solvent added. More specifically, the observed concentration (ng/mL) was multiplied by the amount of homogenising solvent (mL) that was added to the tube and this value was then divided by the mass of dissected placenta (g). **Table 6.1** and **Table 6.2** represents the measured concentrations of sulfasalazine and sulfapyridine per gram of tissue (ng/g tissue).

Table 6.1 - The measured concentration of sulfasalazine per gram of tissue (ng/g tissue).

ID	Mass of Placenta (g)	Volume of Homogenising Solvent (mL)	Observed Sulfasalazine Concentration (ng/mL)	Measured Sulfasalazine Concentration (ng/g tissue)
APL_001	0.16671	0.83355	344.8	1724
APL_002	0.12356	0.61780	293.8	1469
APL_003	0.14723	0.73615	BLQ	BLQ
APL_004	0.15066	0.75330	98.2	491
APL_005	0.12671	0.63355	253.4	1267
APL_006	0.15332	0.76660	284.2	1421
APL_007	0.14540	0.72700	322.7	1614
APL_008	0.13564	0.67820	BLQ	BLQ
APL_009	0.16243	0.81215	840.1	4201

Table 6.2 - The measured concentration of sulfapyridine per gram of tissue (ng/g tissue).

ID	Mass of Placenta (g)	Volume of Homogenising Solvent (mL)	Observed Sulfapyridine Concentration (ng/mL)	Measured Sulfapyridine Concentration (ng/g tissue)
APL_001	0.16671	0.83355	2294.4	11 472
APL_002	0.12356	0.61780	172.3	861
APL_003	0.14723	0.73615	BLQ	BLQ
APL_004	0.15066	0.75330	127.3	637
APL_005	0.12671	0.63355	2615.6	13 078
APL_006	0.15332	0.76660	1420.9	7104
APL_007	0.14540	0.72700	2206.9	11035
APL_008	0.13564	0.67820	BLQ	BLQ
APL_009	0.16243	0.81215	5351.2	26 756

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As is evident by the data represented in **Table 6.1-6.2**, the patient sample concentrations ranged from 491-4201 ng/g tissue for sulfasalazine, and 637-26 756 ng/g tissue for sulfapyridine, with two patient samples (APL_003 and APL_008) reported as below the limit of quantitation of the assay for both analytes. The data are summarised in **Figure 6.1** below. Because extraction recovery is not 100%, the measured analyte concentrations represent only the concentrations extracted from placental tissue homogenate and does not necessarily represent the total concentrations within the tissue.

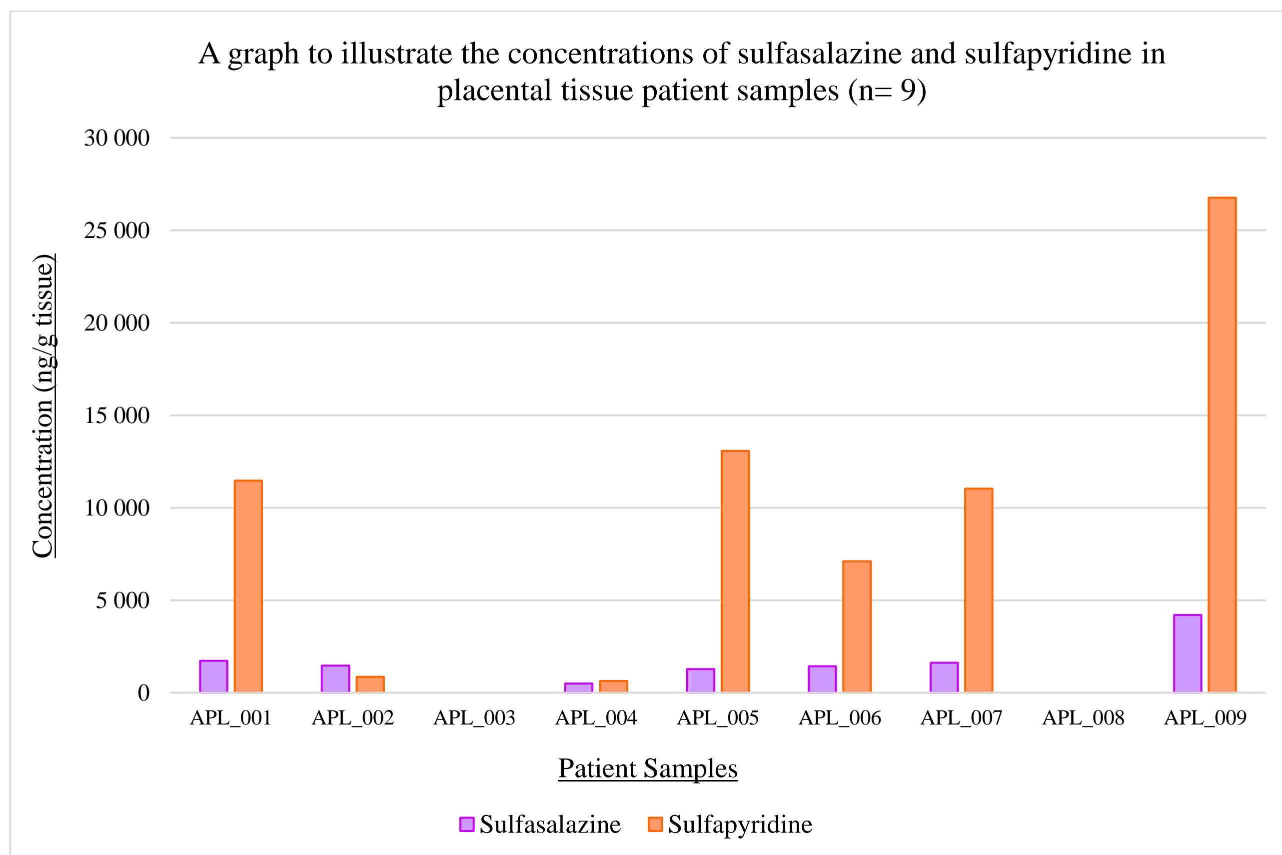

Figure 6.1 – The concentration of sulfasalazine and sulfapyridine in ng/g tissue for the clinical trial patient samples (n= 9).

Based on the results obtained after patient sample analysis, perhaps a more suitable concentration range for the determination of sulfasalazine and sulfapyridine in human placenta based on the dosing strategy of this trial would have been 10-10 000 ng/mL (50-50 000 ng/g) instead of 30-30 000 ng/mL (150-150 000 ng/g). Because two patient samples yielded concentrations of below the limit of quantitation of the assay, the LLOQ of 30 ng/mL (150 ng/g) could have been decreased. Moreover, the highest concentration of sulfasalazine and sulfapyridine was reported as 4201 ng/g and 26 756 ng/g, respectively, such that an ULOQ of 150 000 ng/g (i.e., 30 000 ng/mL) was far too high. However, prior to analysing patient samples, it was uncertain what type of analyte concentrations were to be expected within the placenta, hence for the most part, the validated range chosen was suited for the purposes of this study.

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6.3 Discussion

6.3.1 Patient Sample Analysis

There are several changes that occur during pregnancy that may affect the distribution and protein binding of a drug [126][127]. Firstly, the distribution of a drug to tissues is favoured during pregnancy as a result of several factors: (a) cardiovascular changes (e.g., an increase in cardiac output and stroke volume) occur, such that an increase in plasma volume and total body water can be expected, resulting in an increased volume of distribution of hydrophilic drugs and decreased plasma concentrations; (b) the foetus and amniotic fluid act as additional compartments, contributing towards an increase in drug accumulation and volume of distribution; and (c) the concentration of proteins such as albumin and α1-acid glycoprotein are decreased during pregnancy, thereby reducing plasma protein binding of drugs and increasing the concentration of free drug available to distribute to tissues [126]. Furthermore, as a result of elevations in maternal body fat, the volume of distribution of lipophilic drugs may increase, however insufficient evidence is available to evaluate the effect of adipose tissue on the disposition of drugs during pregnancy [126]. Lastly, glomerular filtration rate (GFR) is increased during pregnancy [126]. Because renal drug excretion is dependent on GFR, renal clearance will parallel changes in GFR during pregnancy if the drug is excreted solely by glomerular filtration [126].

Most studies that evaluate the pharmacokinetic parameters of drugs have been predominantly performed in men, occasionally in women, and rarely in pregnant women due to maternal or foetal safety concerns [126]. Owing to the complexity associated with drug distribution and response during pregnancy, the extrapolation of drug dosage and expected responses from non-pregnant populations is not appropriate and may cause harm for women who are pregnant [126]. Thus, caution should be taken when analysing pharmacokinetic studies of sulfasalazine and sulfapyridine performed in non-pregnant cohorts as the expected drug concentrations in pregnancy will be different due to altered pharmacokinetic parameters. Understanding the pharmacokinetic parameters specific to the drug(s) of interest and being aware of variations specific to gestation will allow for treatment and dosing strategies to be improved during pregnancy, enhancing treatment efficacy and limiting maternal and foetal risks [126].

The placental tissue concentrations of sulfasalazine were determined to be lower than the concentrations of sulfapyridine for all the patient samples, except for APL_002. It has been reported that sulfasalazine is extensively bound to proteins (>99%), whereas sulfapyridine is bound to proteins to a lesser extent (70%) [84]. If a drug has a lower tendency to bind to proteins, a higher concentration of free drug is available to distribute to tissues. Thus, due to the lower protein binding affinity of sulfapyridine, it may be concluded that the drug has a higher volume of distribution, potentially explaining why the metabolite concentrations were higher than the concentrations of sulfasalazine in the placenta. Moreover, as mentioned above, protein concentrations are reduced during pregnancy, which may mean that sulfapyridine's volume of distribution is even higher in comparison with a normal population.

The placental tissue concentrations of sulfasalazine and sulfapyridine varied greatly between the patients and may be attributed to differences in age and inter-individual differences in the rates of drug absorption,

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distribution, metabolism, and excretion [128]. Moreover, genetic and environmental factors may contribute to the differences in analyte concentrations [128]. It is also important to note that the patients were treated with the same amount of investigational drug but for different lengths of time, i.e., from the time they were recruited (anywhere between 24-36 weeks) until the time they gave birth. However, because no data are available regarding the expected concentrations of drugs in placenta after administration of sulfasalazine, it was challenging to interpret the results.

6.3.2 Other LC-MS/MS Methods for the Quantitation of Drugs in Placenta

As previously mentioned, no previous literature has discussed the development and validation of an LC-MS/MS method for the quantitation of sulfasalazine and sulfapyridine in placenta. However, there have been several other reported LC-MS/MS methods for the quantitation of other drugs, which mainly include the detection and quantification of licit and illicit drugs, in human placenta. Although it is worthwhile to note that the nature of these drugs will be different to sulfasalazine and sulfapyridine, these methods will be discussed for comparative purposes.

In a method published by de Castro *et al.* (2009) [129], an LC-MS/MS method was developed and validated for the simultaneous quantitation of methadone, cocaine, 6-acetylmorphine, and its metabolites in human placenta. This method was applied to the analysis of placental tissue samples in opioid-dependent pregnant women following controlled administration of methadone [129]. The method reported a linear range of 10-2000 ng/g for methadone and 2.5-500 ng/g for the other analytes. Tissue sample preparation included the homogenisation of approximately 1 g of placenta in 5 mL 0.1% perchloric acid for 1-2 minutes in a blender. The homogenate was centrifuged, and the supernatant was prepared for LC-MS/MS analysis using solid-phase extraction (SPE). The method reported process efficiency values of between 24.2-201.0% (%CV <15%), and recoveries of the analytes were determined to be 84.4-113.3% (%CV <15%). Matrix effects were reported as less than 27%. A potential oversight of this study was performing centrifugation of the placental homogenate prior to extraction because it has previously been reported that centrifugation may cause heterogeneity of tissue homogenate, resulting in inaccuracy and poor reproducibility [108]. Moreover, it is possible that the analytes of interest may become trapped in the tissue pellet that forms as a result of centrifugation prior to extraction. However, 0.1% perchloric acid was used as a homogenising solvent to assist in the retention of the analytes on the SPE cartridges via a cation exchange mechanism. Thus, if extracted uncentrifuged homogenate were used, the sample may first need to undergo protein precipitation prior to SPE such that large tissue particles do not block the cartridges.

In a method developed by Paniagua-Gonzalez *et al.* (2018) [130], an LC-MS/MS method was developed and validated for the quantitation of nicotine, cotinine, and hydroxycotinine in placenta and umbilical cord. The method was applied to the analysis of 14 placental and umbilical cord samples obtained post-delivery from patients who smoked tobacco throughout pregnancy. A linear range of 5.0-1000 ng/g was validated for all analytes. Placental and umbilical cord tissues were dissected into approximately 1 g samples which were homogenised in 5 mL purified Mili-Q water at maximum speed. Similar to the study above, samples were

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centrifuged and subjected to SPE. Although the method claims to be validated according to EMA guidelines, the %CVs of the matrix effects and process efficiency experiments exceeded 15%.

Firstly, for both of these studies discussed, unacceptable values were obtained for the matrix effects and process efficiency experiments, due to the complexity of using placental tissue as a matrix. These findings emphasise the need for tissue bioanalysis guidelines to be determined from regulatory authorities such as the FDA and EMA with a wider acceptance criterion. Secondly, both studies centrifuge the placental tissue homogenate prior to extraction which may not be an advisable approach. Initially, for this MSc project the placental tissue homogenate was also centrifuged prior to extraction to remove larger tissue components, making the matrix easier to work with. An experiment was then performed to compare the recovery of analytes that were extracted from uncentrifuged homogenate versus analytes that were extracted from centrifuged homogenate. However, it was found that a large proportion of sulfasalazine and sulfapyridine was trapped within the tissue pellet that formed as a result of centrifuging the homogenate because the recovery of these analytes from centrifuged homogenate was far lower compared to the recovery obtained from uncentrifuged homogenate (data not shown in this thesis). Moreover, due to the tissue-bound nature of sulfapyridine, this analyte was trapped within the tissue pellet at a higher proportion than sulfasalazine.

Because the nature of protein binding and tissue binding may be different for licit and illicit drugs in comparison with sulfasalazine and sulfapyridine, it is possible that these drugs might not become trapped in the tissue pellet formed after centrifuging the homogenate prior to an extraction, however it would have been a worthwhile and important experiment for these studies to perform.

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CHAPTER 7

Conclusions, Limitations, and Future Work

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7.1 Conclusion

Pre-eclampsia is a complication unique to pregnancy and is often described as a hypertensive disorder originating in the placenta. Currently, the only treatment for this disease is delivery of the foetus and placenta, however this may be associated with serious risks of prematurity. Thus, the development or repurposing of a treatment which stabilises the disease and allows for the safe prolongation of pregnancy would significantly contribute towards the field of Obstetrics and Gynaecology. Recent literature describes several *in vitro* experiments conducted to investigate the potential of sulfasalazine as a treatment for preterm pre-eclampsia. Therefore, an early phase clinical trial took place at the Mercy Hospital for Women, Australia, to assess the pharmacokinetics and use of sulfasalazine as a treatment for preterm pre-eclampsia.

Although this project set out to develop LC-MS/MS methods for the quantitation of sulfasalazine and sulfapyridine in maternal- and umbilical cord- plasma, as well as in placenta, working with a defective reference standard negatively impacted the timeline, which resulted in the inability to re-validate the plasma method. Several analytical methods have been published for the individual or simultaneous quantitation of sulfasalazine in human plasma, however, no methods have been described for the determination of these compounds in human placenta. Therefore, this project presents the first validated LC-MS/MS methodology for the simultaneous quantitation of sulfasalazine and sulfapyridine in placental tissue which was substantially applied to an early phase clinical trial.

The mass spectrometer was operated in MRM mode to monitor the m/z transition of the protonated precursor ions m/z 398.90 and m/z 250.07 to the product ions m/z 381.05 and m/z 156.00 for sulfasalazine and sulfapyridine, respectively. Placental specimens were homogenised in water:methanol (1:1) prior to extraction to transform the solid tissue samples into a liquid that was compatible with the extraction procedure of the analytes. Different extraction methodologies were evaluated, and the best results were found when protein precipitation using acetonitrile:methanol (90:10) was coupled with elution through HLB cartridges. Deuterated internal standards (sulfasalazine-d4 and sulfapyridine-d4) were incorporated to compensate for any extraction or instrument variability. A reverse-phase gradient method was utilised for analyte separation, resulting in a retention time of 2.3 minutes for sulfasalazine and 1.4 minutes for sulfapyridine. The calibration curve fits a quadratic regression (weighted by 1/x, x= concentration) for sulfasalazine and sulfapyridine over the range 30-30 000 ng/mL (150-150 000 ng/g).

During validation, the assay was shown to produce accurate and precise concentrations over three consecutive, independent runs. Percentage accuracies of STDs ranged from 92.3-107.1% (%CV= 0.1-17.1) for sulfasalazine and 94.4-104.8% (%CV= 0.03-13.8) for sulfapyridine. Similarly, the average accuracies of QCs ranged from 99.8-114.9% (%CV= 0.9-8.1) and 92.4-112.3% (%CV= 0.7-8.7) for sulfasalazine and sulfapyridine, respectively. The method was specific, as no quantifiable peaks were observed in blank placenta specimens from endogenous compounds in placenta. When placenta originating from six different sources was analysed, it was found that endogenous matrix components did not affect the reproducibility of the method. Furthermore, the recovery was shown to be reproducible for both analytes and was determined to be 121.5% (%CV = 13.1%)

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for sulfasalazine and 119.6% (%CV = 8.5%) for sulfapyridine. Extraction efficiency was reported as 141.5% for sulfasalazine and 145.6% for sulfapyridine, and the corresponding %CVs were below 15% for both analytes. The analysis of sulfasalazine and sulfapyridine in placental tissue played a vital role in understanding the safety, efficacy, and distribution of the compounds. However, working with tissue homogenate was a labour intensive and tedious process that was associated with several of its own challenges.

The analytical method was developed and validated according to FDA and EMA guidelines and facilitated the analysis of sulfasalazine and sulfapyridine over a wide concentration range in human placenta collected from women post-birth as part of an early phase clinical trial. The placental tissue concentrations ranged from 491-4201 ng/g tissue for sulfasalazine, and 637-26 756 ng/g tissue for sulfapyridine, with two patient samples reported as below the limit of quantitation of the assay for both analytes.

7.2 Contributions

The method described in this thesis has been used to analyse samples as part of the primary objective of an early phase clinical trial intended to evaluate the pharmacokinetics of sulfasalazine and sulfapyridine after the administration of 1.5 g of sulfasalazine twice daily to 10 pre-eclamptic pregnant women. The validation data described in **Chapter 5** were presented as an oral presentation at the 66th Annual Academic Day at Stellenbosch University 2022.

7.3 Limitations

As with all scientific research, there are always limitations to a study which should be identified and acknowledged.

A major limitation of this study that was beyond our control was unknowingly purchasing a defective reference standard and incorrectly identifying it as a molecular ion adduct, as this impacted the timeline and outputs of this project.

Another limitation of this study was the disconnected timeline between the execution of the clinical trial and the analysis of patient samples. Specifically for this project, patient samples were collected from 2018 to 2020 and have been stored at -80 ° C since. Ideally, LC-MS/MS method development should have started prior to 2021, such that when all sample collection from the clinical trial was complete, the pharmacokinetic study and patient sample analysis could start immediately to minimise long-term storage of study samples, especially if there is a concern for long-term storage stability in the respective matrices. Thus, experiments were not performed to evaluate the stability of analytes at conditions to which the samples were expected to be exposed prior to receipt at the analytical site (e.g., at the clinical site, during shipment, and at all other secondary sites [105]) because the patient samples were delivered to the laboratory before any method existed. Moreover, because the clinical study involved obtaining plasma from patients, studies such as whole-blood stability should be performed to determine how soon after blood collection the samples must be spun down. However, these experiments were performed long after the samples were already obtained from patients.

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Other limitations to this study were associated with tissue bioanalysis. The absence of guidance for LC-MS/MS analysis of tissue samples from regulatory authorities such as the FDA and EMA [108] posed a challenge for the validation of the analytical method. Given the variety and complexity of solid tissue matrices, wider acceptance criteria may be required during LC-MS/MS bioanalyses [108]. Spiked tissue homogenate samples did not necessarily have the same tissue-drug binding or intra- and inter-cellular distribution as the study sample tissues [108]. Therefore, it was difficult to prepare STDs and QCs in a manner that accurately represented the study samples. Additionally, the unknown nature of tissue-drug distribution *in vivo* complicated the assessment of method recovery and analyte stability [108]. Moreover, owing to the highly perfused nature of the placenta, residual blood may have contained sulfasalazine and sulfapyridine at different concentrations to that of the tissue samples, which possibly affected the reproducibility and accuracy of the results [108].

7.4 Future Work

It is possible to overcome several of the study's limitations, provided there is sufficient time, access to funding, and access to specialised equipment. Several recommendations have been outlined for research that may be conducted in the future to improve the developed and validated LC-MS/MS method and to enhance the reproducibility of the assay.

Firstly, certain drugs are commonly administered to pregnant women for pain relief during labour or prior to and during a C-section. A very interesting and worthwhile addition to this project would be to examine the effects of anaesthesia and/or analgesics on the quantitation of sulfasalazine and sulfapyridine in various matrices. However, anaesthetics and several analgesics (e.g., opioids) are controlled substances, hence obtaining access to such reference standards may be exceptionally difficult, time-consuming, and expensive.

To further enhance the accuracy, precision, and reproducibility of tissue sample LC-MS/MS bioanalysis, the use of a more effective homogenisation technique which may reduce tissue sample heterogeneity should be examined. Specifically, the use of cryogenic grinding should be assessed. However, this technique requires a cryo mill which is not available in the analytical laboratory in which this project was conducted. Prior to cryogenic grinding, samples are submerged in liquid nitrogen until they are completely frozen [121]. They will then be placed in a cryo mill whereby the system delivers a calibrated and repeatable mechanical force to cryofracture the flash-frozen samples [121]. Subsequently, the tissue gets broken down into a powder or smaller particles which can be resuspended in a suitable solvent and accurately extracted and analysed [121]. Cryogenic grinding increases the surface area of the tissue and ensures that the extracellular matrix is broken up properly, thus improving extraction efficiency [121]. This type of technique will eliminate virtually all heterogeneity associated with poor homogenisation and will decrease the possibility of analytes being trapped in large pieces of tissue [121].

Because the usual approach to determine analyte recovery does not truly mimic the distribution of the analyte in real tissue samples, different approaches of assessing recovery should be incorporated. These approaches may include spiking the analyte onto or directly injecting the analyte into the blank tissue followed by homogenisation and extraction [121].

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Appendices

Appendix A: Ethics Approval Letter

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Approval Notice

New Application

09/04/2021

Project ID: 21742

HREC Reference No: X21/03/006

Project Title: LC-MS/MS Methods for the Quantification of Sulfasalazine and its Main Metabolites in Various Matrices

Dear Miss Vanessa Louw

The **response to stipulations** received on 08/04/2021 18:43 was reviewed by members of **Health Research Ethics Committee** via **expedited** review procedures on 09/04/2021 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Date: 06 April 2021

Protocol Expiry Date: 05 April 2022

Please remember to use your Project ID 21742 and Ethics Reference Number X21/03/006 on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Translation of the informed consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Please note that for studies involving the use of questionnaires, the final copy should be uploaded on Infonetica.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/21742

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Brightness Nxumalo
HREC 2 Coordinator

Stellenbosch University https://scholar.sun.ac.za

Appendix B: Calculations of Theoretical Concentrations

QC H= 24 000 ng/mL

100 µL of 24 000 ng/mL= 24 000 ng/mL x 0.100 mL= 2400 ng

2400 ng in 100 µL placental homogenate

+ 400 µL protein precipitation solvent

+ 20 µL working solution/blank

= 2400 ng in 520 µL total

2400 ng/0.520 mL= 4615.38 ng/mL

400 µL of 4615.38 ng/mL loaded onto cartridge

= 4615.38 ng/mL x 0.400 mL= 1846.15 ng in 400 µL

Elute with 200 µL solvent but dilution step is disregarded because samples are evaporated to dryness

Left with 1846.15 ng which is reconstituted in 200 µL solvent

= 1846.15 ng/0.200 mL= 9230.77 ng/mL

QC M= 12 000 ng/mL

100 µL of 12 000 ng/mL= 12 000 ng/mL x 0.100 mL= 1200 ng

1200 ng in 100 µL placental homogenate

+ 400 µL protein precipitation solvent

+ 20 µL working solution/blank

= 1200 ng in 520 µL total

1200 ng/0.520 mL= 2307.69 ng/mL

400 µL of 2307.69 ng/mL loaded onto cartridge

= 2307.69 ng/mL x 0.400 mL= 923.08 ng in 400 µL

Elute with 200 µL solvent but dilution step is disregarded because samples are evaporated to dryness

Left with 923.08 ng which is reconstituted in 200 µL solvent

= 923.08 ng/0.200 mL= 4615.38 ng/mL

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QC L= 75.0 ng/mL

100 µL of 75 ng/mL= 75 ng/mL x 0.100 mL= 7.50 ng

7.50 ng in 100 µL placental homogenate

+ 400 µL protein precipitation solvent

+ 20 µL working solution/blank

= 7.50 ng in 520 µL total

7.50 ng/0.520 mL= 14.42 ng/mL

400 µL of 14.42 ng/mL loaded onto cartridge

= 14.42 ng/mL x 0.400 mL= 5.77 ng in 400 µL

Elute with 200 µL solvent but dilution step is disregarded because samples are evaporated to dryness

Left with 5.77 ng which is reconstituted in 200 µL solvent

= 5.77 ng/0.200 mL= 28.85 ng/mL

Sulfasalazine-d4= 250 ng/mL

400 µL of 250 ng/mL= 250 ng/mL x 0.400 mL= 100 ng

100 ng in 400 µL protein precipitation reagent

+ 100 µL placental homogenate

+ 20 µL working solution/blank

100 ng in 520 µL total= 100 ng/0.520 mL= 192.31 ng/mL

400 µL loaded onto cartridge

192.31 ng/mL x 0.400 mL= 76.92 ng in 400 µL

Elute with 200 µL solvent but dilution step is disregarded because samples are evaporated to dryness

Left with 76.92 ng which is reconstituted in 200 µL solvent

= 76.92 ng/0.200 mL= 384.615 ng/mL

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Sulfapyridine-d4= 200 ng/mL

400 µL of 200 ng/mL= 200 ng/mL x 0.400 mL= 80.0 ng

80.0 ng in 400 µL protein precipitation reagent

+ 100 µL placental homogenate

+ 20 µL working solution/blank

80.0 ng in 520 µL total= 80.0 ng/0.520 mL= 153.85 ng/mL

400 µL loaded onto cartridge

153.85 ng/mL x 0.400 mL= 61.54 ng in 400 µL

Elute with 200 µL solvent but dilution step is disregarded because samples are evaporated to dryness

Left with 61.54 ng which is reconstituted in 200 µL solvent

= 61.54 ng/0.200 mL= 307.69 ng/mL