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ASSESSING INTER-METHOD AGREEMENT OF DRUG-BASED PHENOTYPING METRICS BETWEEN DRIED BLOOD SPOT AND PLASMA SAMPLING

by

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DECLARATION OF ORIGINALITY

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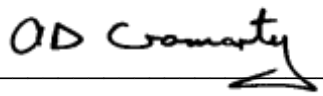
SIGNATURE OF SUPERVISOR: 

SUMMARY

Introduction: Pharmacokinetic variability in response to pharmacotherapy contribute to adverse drug reactions, drug-drug interaction and therapeutic failure seen in clinical practice. Poor therapeutic response to medication has been attributed to inter-individual and interethnic variability in cytochrome P450 (CYP450)-dependent metabolism and altered drug absorption via expressed transport channels such as P-glycoprotein (P-gp). An individualised approach in therapeutic management would be beneficial in a South-African population considering the country's large genetic diversity. A single time point, non-invasive capillary sampling, combined with a low dose probe drug cocktail, to simultaneously quantify *in vivo* drug and metabolite concentrations, would enhance the feasibility and cost-effectiveness of routine phenotyping in clinical practice and guide personalised prescribing to individual patients. A recent development in dried blood spot sampling is the Mitra™ device, using Volumetric Absorptive Micro Sampling (VAMS™) technology to collect an accurate volume (10-30 µL) of whole blood onto a hydrophilic polymeric tip as an alternative to plasma sampling. Small volume blood sampling however presents bioanalytical challenges in terms of the reproducibility and sensitivity of the quantitative method and the agreement between quantitative measurement from a dried blood spot (DBS) and that from plasma sampling. The physicochemical diversity of the structurally related aromatic probe drugs, used together in a drug cocktail, further require optimised analytical procedures for simultaneous quantification. Phenotyping cocktails are compounded from commercially available dosage forms and introduce challenges with regards to dosage homogeneity, chemical interference or degradation and possible incompatibilities of drugs when used in combination.

Aim and objectives: The purpose of this study was to compound the validated "Geneva phenotyping drug cocktail", from available API sources and develop a validated, targeted, analytical LC-MS/MS method to quantify the seven probe drugs and six respective metabolites in dried blood spots when using the Mitra™ volumetric absorptive micro-sampling device for blood collection. The aim was to assess inter-method agreement of the measured probe drug and metabolite concentrations between the low sample volume, from a dried blood spot, and conventional plasma sampling.

Methods: An Agilent binary series LC system coupled to a Sciex 4000 QTRAP triple quadrupole tandem mass spectrometer was used for method optimisation and validation. Targeted LC-MS/MS methods, in both negative and positive ESI mode, were validated

according to ICH guidelines for matrix effects, recovery, linearity, limits of quantitation and detection, carry-over, inter and intraday precision and accuracy and analyte stability. The selectivity of the structurally related ionisable analytes was compared between a Kinetex C18 and Kinetex Biphenyl column and the influence of changes in the analytical conditions (involving mobile phase pH and solvent mixture composition as well as the solvent type) studied. An initial assessment of statistical *in vitro* agreement between plasma and DBS sampling were carried out. USP assays were performed to determine the weight and content uniformity of the compounded phenotyping cocktail containing six of the seven probe drugs. Content uniformity was evaluated with an Acquity UPLC system coupled to a Synapt G2 QTOF mass spectrometer.

Results and discussion: A biphenyl stationary phase in combination with methanol as the organic eluent, provided improved resolution and analyte selectivity of the structurally related aromatic compounds. Results from the robustness experiment further confirmed the importance of controlling analytical conditions to ensure reproducibility and reliability of the quantitative method. Separation selectivity and higher throughput were prioritised over optimised ionisation efficiency, although the sensitivity of the analytical method for individual analytes were still within the expected *in vivo* concentration ranges to infer metabolic and transport phenotypes. This study successfully validated the use of DBS, collected with the volumetrically controlled absorptive microsampling device Mitra™, to measure expected probe drug and metabolite concentrations using the "Geneva phenotyping cocktail". The validated method met all the required standards accepted in bioanalytical chemistry for specificity, sensitivity, linearity, accuracy, precision, carry-over and stability. From the initial *in vitro* assessment of agreement, it was concluded that blood cell distribution kinetics are regulated by the blood-to-plasma concentration ratio and time dependent equilibrium between different blood compartments, the physicochemical properties of the analytes, temperature during extraction, analyte concentration and stability. A conclusive confounding factor was the extent to which the extraction procedure liberated bound drug from either plasma proteins or erythrocytes. It was further concluded that the compounded low dose phenotyping cocktail capsules could be used successfully to assess inter-method agreement of drug-based metabolic ratios and drug transport between plasma and DBS collected with the Mitra™ device.

Conclusion: To our knowledge, this is the first DBS validation study using the Mitra™ device for the purpose of simultaneous phenotyping of the *in vivo* P-gp transport and CYP450 metabolic activity of the CYP1A2, -2B6, -2C9, -2C19, -2D6 and -3A4 enzymes and activity.

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The great Mr Nelson Mandela said: *"It always seems impossible until it's done."* It would not have been possible to complete this work without the support, guidance, assistance and encouragement of a large number of people and institutions.

To my husband Wilhelm and children, Friedrich, Juan and Elrie: You give my life true meaning. Without your moral support and encouragement, I probably would not have survived the past few years. Thank you for enduring the monster I became and your unconditional love.

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Mostly, I am grateful to God for giving me the ability, opportunity and perseverance to complete this PhD study. I pray that it would serve a greater purpose.

ETHICS

The author of this thesis has obtained the applicable research ethics approval, reference number 209/2016, from the University of Pretoria's Research and Ethics Committee for the research presented in this work. The author further declares that the ethical standards, mandated in terms of the University of Pretoria's code of ethics for researchers and the policy guidelines for responsible research, have been honoured.

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ABBREVIATIONS

ABCB1	Adenosine triphosphate-binding cassette transporter type B1
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, excretion
ADR	Adverse Drug Reaction
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon nuclear translocator
ARV	Antiretroviral medication
AUC	Area under the plasma concentration-time curve
CAR	Constitutive androstane receptor
CE	Collision energy
CV	Coefficient of determination (relative standard deviation from the mean)
CYP1A2	Cytochrome P450 enzyme 1A2
CYP2B6	Cytochrome P450 enzyme 2B6
CYP2C19	Cytochrome P450 enzyme 2C19
CYP2C9	Cytochrome P450 enzyme 2C9
CYP2D6	Cytochrome P450 enzyme 2D6
CYP3A4	Cytochrome P450 enzyme 3A4
CYP450	Cytochrome P450 enzymes
CXP	Collision cell exit potential
DBS	Dried Blood Spots
DDI	Drug-drug interactions
DNA	Deoxyribonucleic acid
DP	Declustering potential
EM	Extensive metaboliser
H_2O	Water
HIV	Human immunodeficiency virus
IS	Internal standard
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MDR1	Multidrug resistance gene 1
MeOH	Methanol

MR	Metabolic ratio
m/z	Mass to charge ratio
NRF	National Research Foundation of South Africa
IM	Intermediate metaboliser
PD	Pharmacodynamics
PK	Pharmacokinetics
P-gp	Permeability glycoprotein
PM	Poor metaboliser
mRNA	Messenger ribonucleic acid
PXR	Pregnane X receptor
SD	Standard deviation
TDM	Therapeutic drug monitoring
THF	Tetrahydrofuran
TMD	Transmembrane domains
UM	Ultra rapid metaboliser
UPLC-qTOF-MS/MS	Ultra-performance liquid chromatography tandem quadrupole time of flight mass spectrometer
USP	United States Pharmacopoeia
VAMS	Volumetric absorptive micro-sampling
WHO	World Health Organization
XIC	Extracted ion chromatogram

CHAPTER 1: LITERATURE REVIEW

Individual pharmacokinetic variability to pharmacotherapy remains a problem in clinical practice and a more successful pharmaceutical intervention is warranted, tailoring drug treatment to ensure safe and effective treatment to individual patients. Most drugs are developed in Europe and America and are subsequently marketed worldwide without recognizing the full extent of its efficacy or safety in different population groups.[1] This chapter will introduce pharmacokinetic variability, the intrinsic and extrinsic factors that influence phenotypic response to pharmacotherapy and the advantage of using phenotyping procedures to give a biological snapshot of *in vivo* metabolic and transport activity that could be used in personalised medicine to ensure the right dose is given to the right patient at the right time. Phenotyping could be advantageous when coupled to capillary sampling using dried blood spots (DBS). A detailed overview of the cytochrome P450 (CYP450) enzyme and adenosine triphosphate-binding cassette (ABC) transporter gene expression and functionality will be given, highlighting the clinical consequence of their inter-ethnic and inter-individual variability on the pharmacokinetics and therapeutic outcomes of specific drug substrates. Current phenotyping procedures, focusing on low-dose cocktail approaches, sparse patient pharmacokinetic sampling and advances in the use of dried blood spot sampling will be outlined after a detailed review of published literature. Finally, the importance of optimising analytical quantitation procedures using liquid chromatography tandem mass spectrometry (LC-MS/MS) to detect low concentrations of analytes, with different physicochemical properties in sparse samples, will be discussed.

1.1 INTRODUCTION

Pharmacokinetics (PK) is defined as the study and interrelationship between the absorption, distribution, metabolism and elimination (ADME) of drugs *in vivo*.[2] Inter- and intra-individual PK variability in response to drug therapy is a major causality of subtherapeutic or supratherapeutic exposure to *in vivo* drug concentrations, which in turn leads to adverse drug reactions (ADR), drug-drug interactions (DDI) and therapeutic failure, often resulting in hospitalization and death. [3][4]

Adverse drug reactions are estimated to be a leading cause of mortality in the USA with astronomical economic implications.[5] There are limited data available about the burden of ADR, DDI and therapeutic failure in South Africa. A cross-sectional survey at four South-African hospitals found that 8.4% of admissions were related to ADR and 45% of these were preventable.[6]

Sixty to eighty percent of commercially available drugs today are metabolised by the CYP450 enzymes with great inter-individual and interethnic variability affecting therapeutic outcomes.[7] Oral clearance of drugs through expressed permeability-glycoprotein (P-gp) transport channels (encoded by the adenosine triphosphate-binding cassette (ABC) transporter gene (ABCB1)) are also subject to pharmacokinetic variability and recent studies have shown that many drugs metabolised by the CYP450 enzymes are also ABC transporter protein substrates, indicating that both phase I metabolism and transmembrane transport form a protective barrier against foreign substances entering the body [8]. Pharmacokinetic variability related to CYP450 and P-gp phenotypes will be discussed in Section 1.2.

Several intrinsic and extrinsic factors influence the activity of these drug metabolising enzymes and transmembrane transport proteins, with a high degree of population differences in disease prevalence or outcomes (Figure 1.1). When considering a more individualised approach to pharmacotherapy, it is clear that genotyping (pharmacogenomics) alone cannot infer altered metabolic or transport phenotypes considering the complex interaction between genotype and extrinsic factors influencing metabolic or transport activity.[7] Genotype-phenotype mismatches due to co-administration of medications or comorbidities, altering clinical metaboliser phenotype, have been reported in a number of studies.[7, 9-11] This phenomenon is called phenoconversion and describe a situation where phenotypic response contradict measured genotype.[12] Genotyping with commercially available gene chip technology, AmpliChip, to predict phenotype was found not to be feasible within a demographically representative sample of the South African population.[13] Hiemke *et al.* noted that phenotyping may provide an advantageous alternative where functional significance of genetic polymorphisms are unclear[14], providing a real-time snapshot of individual metabolism or transport activity that take all influencing factors into account.

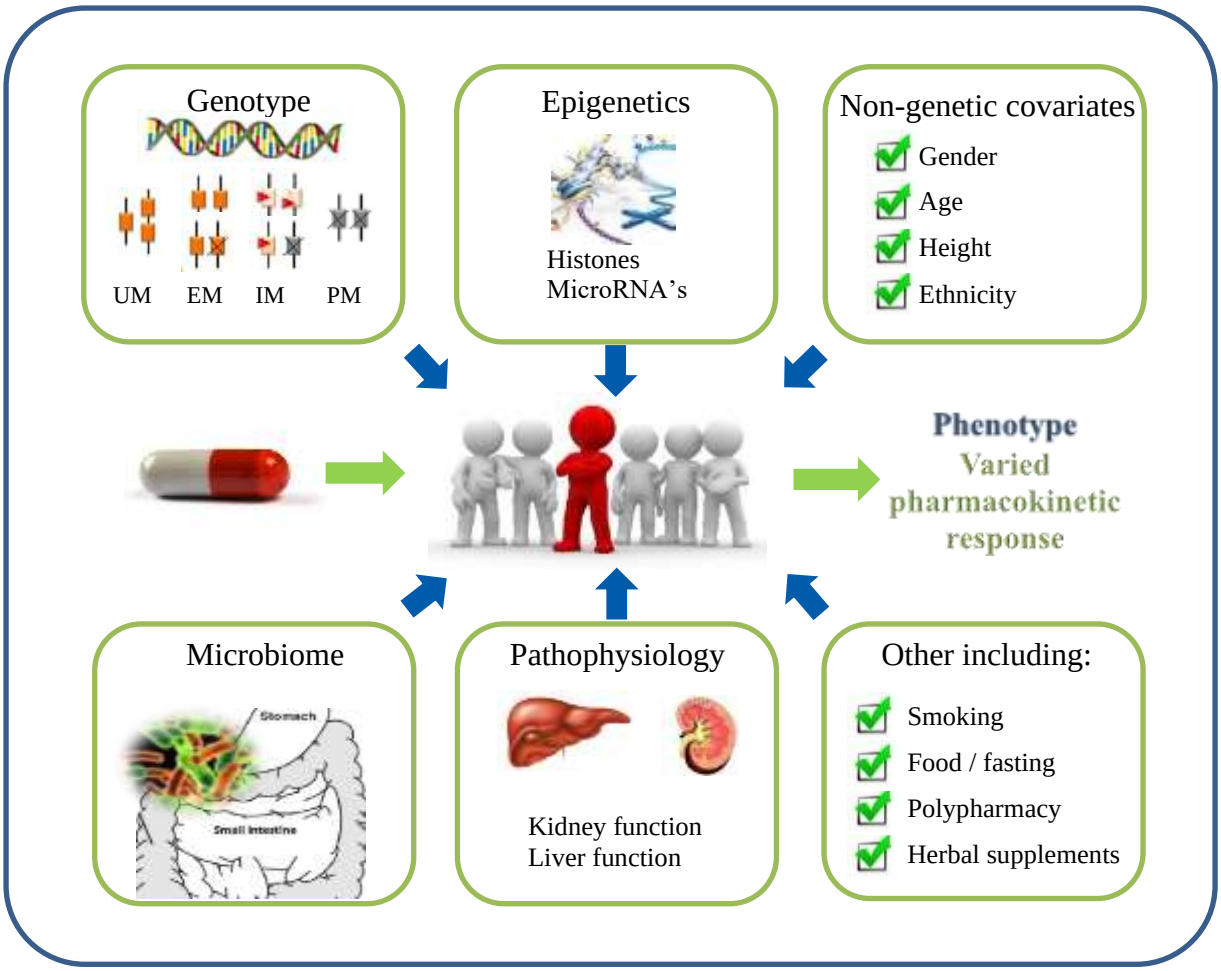


Figure 1.1: Schematic presentation of intrinsic and extrinsic factors influencing pharmacokinetic variability. These factors include, but are not limited to: Inter-ethnic and inter-individual genotypic variability[15-17]; Epigenetic factors regulating expression of drug metabolizing enzymes and transport proteins[18]; Non-genetic covariate factors such as age, gender, race and height[19, 20]; Inter-individual variability in gut microbiome influencing metabolism and bioavailability[21]; Pathophysiological conditions such as diminished kidney and liver function[22]; Other factors such as polypharmacy resulting in pharmacokinetic drug-drug interaction[23-25], Environmental factors, such as smoking, alcohol intake and medication causing CYP450 enzyme induction or inhibition resulting in an altered phenotype[7, 26] Short-term fasting[27], certain foods and herbal remedies may also influence phenotypic expression of specific CYP450 enzymes. [28-30] (Figure created by the author.)

A recent review by Samer *et al.* presented guidelines and both genotyping and phenotyping considerations for CYP2D6, CYP2C19 and CYP2C9 polymorphisms that influence clinical outcomes.[7] Taylor *et al.* further recommended that population studies should be designed to take all variables influencing health into account in order to enhance treatment of disease within all populations.[31] In translating genotype information for CYP2D6 into an activity score predicting phenotype, Gaedigk *et al.* also confirmed that co-factors altering drug metabolising

activity should always be considered in conjunction with genotype predictions.[32] It is important to note that the extend and duration of inhibition are related to dosages and treatment duration and it has been suggested that routine phenotyping should be done in studies focusing on genotype alone and their association with clinical outcomes assessed. The authors argue that any clinical outcome to pharmacotherapy is determined by the drug metabolising phenotype rather than the genotype. Researchers at the Vanderbilt University's School of Medicine in Nashville, USA, proposed a new framework for prediction of adverse drug reactions in preclinical trials as well as post-marketing surveillance of drugs in a large-scale study comparing five machine-learning algorithms. They found that phenotypic data, when available, where most predictive for estimation of adverse drug reactions.[33] A further advantage of phenotyping is its ability to distinguish between a poor metaboliser phenotype versus a possible drug overdose or cases of poor patient compliance versus an ultra-rapid metabolising status.[7] The latter is imperative since patient non-compliance often relates to therapeutic failure.[34]

Phenotyping with single probe drugs or "cocktail" drugs could provide valuable information on the differences in enzyme and transport protein function *in vivo*.[35] A number of phenotyping cocktails have been developed over the years that involve plasma and urine sampling, although lately most phenotyping assays use plasma sampling due to the exclusion of renal functionality and differences in urine pH as well as dilution factors influencing urine sampling. Early phenotyping with a "cocktail" approach was limited due to the use of therapeutic doses that could elicit side-effects, multiple or complicated sampling procedures and use of probe drugs no longer available as registered drugs in most countries. The development and validation of a number of low-dose phenotyping cocktails coupled with highly sensitive bioanalytical assays and simplified sampling are currently being investigated to enhance clinical applicability of phenotyping cocktails. A complete overview of phenotyping CYP450 and P-gp activity towards individualised drug therapy and available phenotyping cocktails, with current challenges of its implementation in clinical practice, will be discussed in Section 1.3.1 and 1.3.2.

Capillary blood sampling could enhance acceptability of phenotyping within a clinical setting in developing countries, as it is non-invasive, requiring a small volume of blood with a simple finger-prick. Improved storage and transport conditions, at ambient temperatures and better analyte stability compared to frozen plasma samples[36-38] are further advantageous of this sampling method.

Dried blood spot sampling (DBS) will therefore be preferable in a clinical setting lacking cold storage and expensive equipment for handling blood and/or plasma samples. DBS could also be applied in clinical practice for therapeutic drug monitoring (TDM) to assess patient adherence on a routine basis and a number of quantitative analytical methods have been validated in this regard.[39-44] A big limitation of DBS is haematocrit bias, influencing quantitative results. In a recent review by Wilhelm *et al.*, the latest development in DBS sampling devices, aiming to overcome or minimise the impact of haematocrit on quantitation, are discussed.[45] Among them a novel micro sampling device, launched by Phenomenex Inc. (Torrance, CA, USA) in 2014 called Mitra™ , using Volumetric Absorptive Micro sampling (VAMS™) technology that collects an accurate volume of blood (10-30 µL) directly from the sampling site into a polymeric tip followed by whole volume extraction, aiming to overcome issues with sample homogeneity and haematocrit.[46] Since its inception numerous articles have been published with a wide range of analytes extracted including DNA, proteins and peptides[47], vitamins[48], metals[49], hormones[50], illicit drugs[51, 52], immunosuppressants[53], antibiotics[54, 55] and other therapeutic drugs.[53, 56-58] This sampling device is not without challenges and research shows that some analytes still show a haematocrit-dependant recovery, others are not stable in DBS and correlation of analyte concentration in DBS and plasma is not straightforward. However, when extraction is optimised, DBS sampling with the Mitra™ device has been successfully applied in TDM. More research needs to be done on individual compounds and methods validated for use in clinical practice.[59] Use of sampling alternatives in phenotyping cocktails will be discussed in Section 1.3.3, focusing on appropriate statistical analysis to assess agreement with the gold standard of plasma sampling. Important pharmacokinetic considerations associated with validation of DBS in phenotyping cocktails will also be elucidated. Finally, the rationale for using the Geneva phenotyping cocktail in a South African population will be stipulated in Section 1.3.4.

From a bioanalytical perspective, implementation of DBS in routine phenotyping, requires sensitive and robust methods to quantify analytes within a limited sample volume. Multi-dose cocktails that consist of analytes with different physicochemical properties pose another challenge when it comes to the simultaneous extraction and analysis within a single bioanalytical method. Liquid-chromatography tandem mass spectrometry (LC-MS/MS) has emerged as the method of choice in DBS analysis due to increases selectivity and sensitivity when coupled to optimised extraction methods. Approaches to optimise selectivity and

resolution in LC-MS/MS analysis of complex biological samples will be discussed in Section 1.4.

1.2 PHARMACOKINETIC VARIABILITY RELATED TO CYP450 ENZYMES AND PERMEABILITY-GLYCOPROTEIN TRANSPORTER

Pharmacokinetic variability is caused by a complex interplay between many different factors (see Figure 1.1) influencing the available drug concentration in the body due to varying rates of absorption, distribution, metabolism and excretion. In this section the focus is on pharmaco-metabolomics, that studies pharmaceutical drug metabolism and on drug transport activity. The CYP450 oxygenase enzymes play an integral role in the biotransformation and detoxification of pharmaceutical drug formulations whereas the P-glycoprotein transporter influence drug bioavailability via gastrointestinal enterocytes, or from altered hepatic and renal clearance as well as via transport across vulnerable tissues like the blood brain barrier.

1.2.1 Cytochrome P450 Oxygenase Enzymes

Most pharmaceutical drugs are lipid soluble ensuring that they readily cross the lipid plasma cell membranes and blood brain barrier to reach their target receptors to elicit a pharmacodynamic response. These pharmaceuticals are subsequently detoxified and eliminated from the body, mostly via the renal system and therefore need to be converted to a more polar hydrophilic form. This biotransformation and detoxification occur mainly in the liver via Phase I catalytic oxidation/ reduction and or hydrolysis reactions and phase II conjugation reactions. During phase I biotransformation a polar hydroxyl group is introduced or unmasked on the pharmaceutical molecule by cytochrome P450 (CYP450) enzymes rendering the molecule more hydrophilic for subsequent elimination.[60] These CYP450 phase one reactions, reviewed by Isin and Guengerich[61] include simple carbon hydroxylation, heteroatom oxygenation, dealkylation, epoxidation and functional group migration, as well as more complex chlorine oxygenation, aromatic dehalogenation, Diels-Alder dimer formation, ring coupling, cleavage or formation and oxidative aryl migration to name a few.

1.2.1.1 CYP450 expression, nomenclature and functionality

The human genome consists of 57 active CYP450 genes[62] classified into 18 families and 44 subfamilies according to similarity in their genetic sequences.[63] These genes regulate the expression of CYP450 enzymes in the endoplasmic reticulum (ER) of hepatocytes (Figure 1.2),

responsible for systemic metabolism, and extrahepatically in respiratory (nasal mucosa, trachea, lung) and gastrointestinal (oesophagus, stomach, small intestine and colon) tissue [64] contributing to first pass and clinically important localised metabolism. The title "cytochrome" is derived from "cyto", (meaning cell, since the enzyme complex is bound to the ER within the cell) and "chrome" (from the containing haem pigment within the enzyme complex). When exposed to carbon dioxide this pigment absorbs light at a wavelength of 450 nm, hence P450. The Arabic number that follows the abbreviated CYP indicates the specific family, the capital letter thereafter the subfamily with the final number indicating the specific enzyme, for example CYP3A4. Expression of CYP450 genes are highly polymorphic and different alleles that are discovered are further indicated by an * followed by a number or numbered letter. Functionally different alleles that are continuously identified are continuously updated on the PharmVar (Pharmacogene Variation) data repository housed at Children's Mercy Hospital in Kansas City, USA from September 2017, being transferred from the original server at the Karolinska Institute.[65] The CYP450 oxidoreductase complex (Figure 1.2) mediates the metabolism of exogenous drug substrates as well as detoxification of other exogenous substances such as nutritional supplementation and environmental pollutants. In addition to xenobiotics, endogenous substances are also metabolised by the CYP450 enzymes and Shahabi *et al.*[66] reported their importance in the metabolism of arachidonic acid and subsequent anti-inflammatory protective effects of the four regioisomer epoxy eicosatrienoic acid metabolites formed by CYP epoxygenases CYP2C8, 2C9, 2C19 and 2J2 on the cardiovascular system.

CYP450 enzymes are further responsible for the synthesis of lipophilic endogenous analytes, such as steroid hormones, cholesterol and fatty acids, by oxidative reactions including N- and S-oxidation, hydroxylation, deamination and N- and O-dealkylation[2] Of the 18 active CYP450 families in the human genome, the first three are mainly responsible for the biotransformation of drug substances with CYP1A2, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6 and CYP3A4/5 catalysing 70-80% of xenobiotic drug metabolism.[16]

A common feature of the 15 CYP enzymes in Class 1-3, responsible for the metabolism of most small drug molecules, are their extensive and overlying substrate specificities.[67] Individual drug response and the incidence of adverse drug reactions or drug-drug interactions are influenced by the polymorphic nature and subsequent expression and activity of CYP450 enzymes.[68]

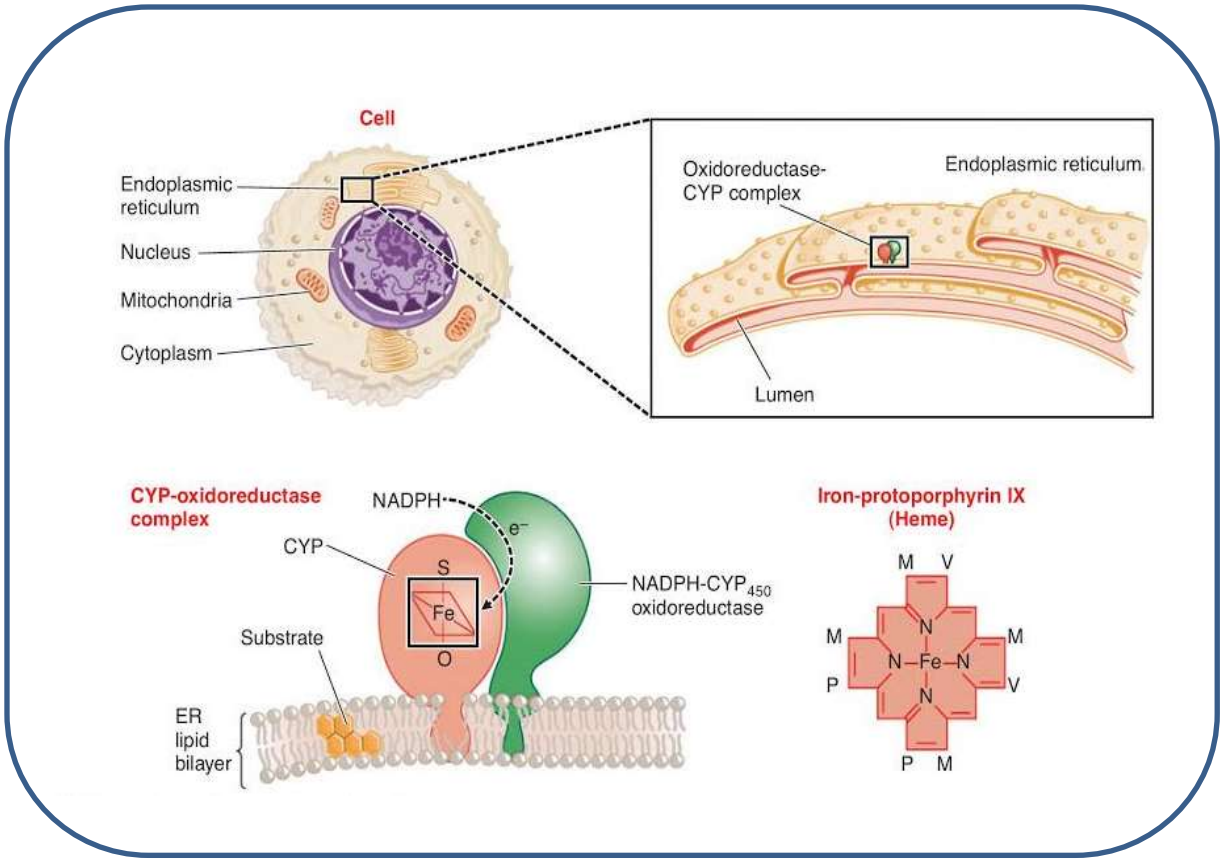


Figure 1.2: Location of the cytochrome P450 oxidoreductase complex, in the endoplasmic reticulum lipid bilayer, illustrating the haem binding site involved in the catalytic oxidation. Nicotinamide adenine dinucleotide phosphate (NADPH) CYP450 oxidoreductase acts as a co-factor in this reaction by binding to the CYP450 enzyme to form the CYP-oxidoreductase complex. The oxygen (O_2) carried by the iron-protoporphyrin IX (haem) molecule and H^+ from NADPH are responsible for the oxidation of substrates with water as a by-product of the reaction. (Reproduced with permission from Goodman and Gillman's The Pharmacological Basis of Therapeutics, 12th edition© [2].)

Zanger and Schwab reviewed 248 known CYP450 drug metabolic pathways and factors influencing variability, from which they compiled a graphic representation (reproduced in Figure 1.3) of ten major CYP450 enzymes indicating the fraction of the drugs metabolised by each enzyme and highlighting the important influencing factors in bold, with their directionality, on the functionality of each enzyme (either induction or inhibition). For clinicians to infer pharmacokinetic variability in the patient population, a basic knowledge of all contributing intrinsic and extrinsic factors on the activity and expression of each individual enzyme related to the individual drug response is a prerequisite. Although monogenetic polymorphisms in CYP2D6 gene expression could explain most of the individual response to pharmacotherapy (due to its non-inducible nature[69]), for most major CYP450 enzymes,

genotype contribution could be as low as only 20% [70] and range between 20-80%, with their clinical functionality depending on a composite of genetic, epigenetic and non-genetic factors, disease state, environmental and nutritional factors. Samer *et al.* underlined the equal importance of drug interactions, especially with polypharmacy, on individual phenotype[7] and an indepth understanding of CYP450 enzyme induction (with increased metabolism and clearance of affected drug substrates) and CYP450 inhibition (imitating genetic defects with decreased metabolism and clearance) is crucial when predicting clinical outcomes toward individualised pharmacotherapy.[71]

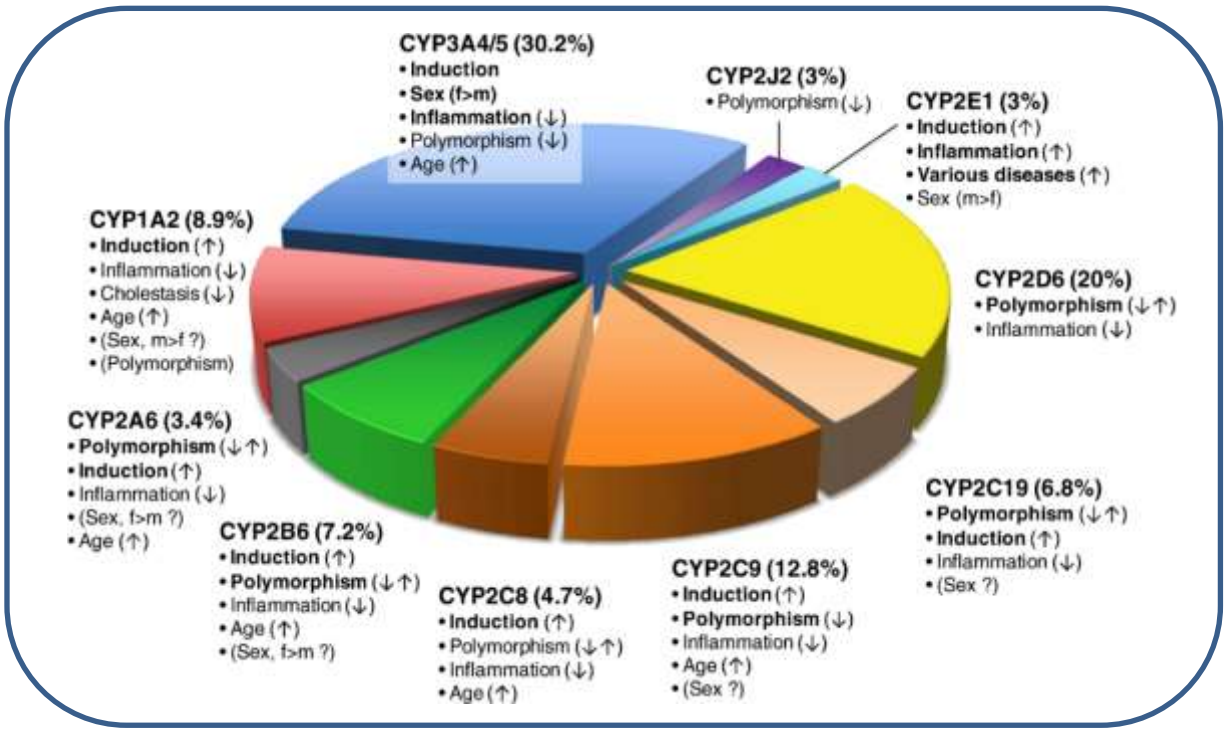


Figure 1.3: Zanger and Schwab's© representation of the intrinsic and extrinsic factors influencing major CYP450 enzyme expression and functionality (with permission).

Mechanisms by which CYP450 enzymes are inhibited or induced have been exemplified during the past 20 years of research. In instances where gene duplication or enzyme induction occurs, more of the enzyme will be expressed, resulting in increased metabolism and reduced drug levels of the active drug. In cases where allele deletion, null allele mutations, inactivating mutations or enzyme inhibition occur, less enzyme activity will be observed, resulting in decreased metabolism and increased drug levels and drug accumulation[72] and vice versa for prodrugs. Herein patient genotype is more indicative of induction than inhibition of enzyme activity.[71] Expression of CYP450 enzymes are further regulated by transcription factors and

nuclear receptors. Studies have documented increased cellular CYP450 protein levels upon activation of the nuclear receptors pregnane X receptor (PXR)[73-75] and constitutive androstane receptor (CAR)[76] (via xenobiotic triggered cytoplasm-nucleus translocation) and of the ligand-activated transcription factor aryl-hydrocarbon receptor (AhR).[77]

Enzyme induction relates to the increase in both the amount and activity of CYP450 enzymes in response to drug exposure. There is a delay in the quantitative increase of an enzyme, which could take anything from hours to days, as a result of the time-consuming process of increased transcription and translation or enzyme stabilisation in response to the induction stimuli. The rate of enzyme induction is determined by both the half-life of the inducing drug and the enzyme degradation half-life.[78] The binding of an inducer to a specific receptor protein results in a transformed transcriptional DNA-binding complex with affinity for specific DNA response elements within the regulatory region of a specific CYP gene, increasing production of the CYP mRNA leading to increased *de novo* synthesis of the enzyme.[79] Induction of CYP1A enzymes by cigarette smoke is a good example, where polycyclic aromatic hydrocarbons in cigarette smoke binds to AhR, translocates to the cell nucleus in a complex with aryl hydrocarbon nuclear translocator (ARNT) protein, binds to the drug response elements in the regulatory region of the CYP1A gene, thereby upregulating its production.[80] The induction of CYP enzymes is usually reversable, dose-dependent and site-specific.

Mechanism-based enzyme inhibition on the contrary is subject to the affinity of a drug substrate for the active site of the CYP450 protein. This inhibitory action is a common cause of potentially harmful drug-drug interactions and can be either reversible or irreversible depending on the strength of the chemical bond forming between the ligand and the enzyme receptor.[81] Irreversible enzyme inhibition develop when a covalent bond is formed between the CYP450 enzyme and a specific drug substrate (rapid onset) or its metabolite (delayed onset) due to stable complex formation.

Restoring baseline enzymatic function will depend on enzyme synthesis and removal of the inhibitory drug and, as a result, the effect is long-lasting. Reversible enzyme inhibition is more common and can be divided in to competitive, non-competitive or un-competitive inhibition. The main difference between irreversible and reversible inhibition is that the inhibitory drug need not be a substrate of the enzyme in reversible inhibitory reactions. Competition between an inhibitor and substrate drug for the same active enzymatic binding site is a function of the available enzyme, the concentration of the competing drugs and their relative enzyme

selectivity. Competitive inhibition usually has a rapid onset of action due to weak chemical bond formation with no subsequent enzyme destruction. Non-competitive inhibition occurs where the inhibitor binds to a different site than the substrate and in un-competitive inhibition the inhibitory drug binds to the substrate-enzyme complex, limiting the rate of metabolism of the substrate.[16, 81, 82]

The potency of the CYP450 inhibition or induction on the available CYP450 substrate concentration *in vivo* further depend on the drug's therapeutic window and the extent to which a drug substrate is metabolised during first-past metabolism. Variation in their phenotypic expression will result in enzymes with increased, normal, or decreased activity.[83] Individuals are then classified as poor (PM), extensive (EM), intermediate (IM) or ultra-rapid (UM) metabolisers for specific enzymes. Table 1.1 summarises the potential pharmacokinetic outcomes and effect on drug metabolism for poor to intermediate versus ultra-rapid metaboliser phenotypes. Prodrugs require biotransformation to be activated with the opposite effect on metabolism to instances where an active drug is administered, causing poor efficacy and therapeutic failure in poor to intermediate metabolisers and a rapid onset of action in ultra-rapid metabolisers.

Table 1.1: Influence of patient phenotype on drug metabolism and potential clinical outcomes.[70]

Drug type	Phenotype	Effect on metabolism	Potential outcome
Prodrug	Poor to intermediate	Slow	Poor efficacy, risk of therapeutic failure. Accumulation of the prodrug could lead to drug induced side-effects
	Ultra-rapid	Fast	Good drug efficacy and rapid effect
Active drug	Poor to Intermediate	Slow	Good drug efficacy, but may need lower doses due to accumulation of drug and possible side-effects
	Ultra-rapid	Fast	Poor efficacy and possible therapeutic failure that require higher doses

Geographical ancestry and ethnicity influence CYP allele frequencies resulting in worldwide variability in genotypic expression and measured phenotypes with significant differences in

treatment response, risk profile and disease prevalence[84] with the largest diversity in the distribution of clinically relevant CYP alleles found in Africa[85] of which a high level genetic and with-in population diversity was found in Southern African Khoisan and Black populations.[86, 87] This has been illustrated with commonly used drugs to treat heart disease, which are known to be less effective in individuals of African descent relative to individuals of European descent.[88] Burroughs *et al.*[89] reported the importance of taking into account differences in drug metabolism, clinical effectiveness and side-effect profiles amongst different racial and ethnic groups. These studies amplify the need for the development of optimised drug dosing strategies for existing drugs and during drug development. Our current knowledge on inter-individual and inter-ethnic differences in the South African population are however based upon a limited number of studies, often pooling data for all African populations inadequately contributing to diverse genetic profiles of the population.[90] Cost-effective routine phenotyping would be advantageous providing real-time information on *in vivo* phenotypes in a clinical setting to assist clinicians on individualised drug therapy.

1.2.1.2 Clinical consequences of inter-individual variability on Cytochrome P450 activity

A more individualised approach in disease management by means of pharmacogenetics testing and phenotyping procedures with subsequent pharmacokinetic/ pharmacodynamics modelling could enhance therapeutic response to pharmacological therapy. Some examples are given below to indicate the clinical consequences of inter-individual variability due to phase one metabolism. By no means is it intended to be an exhaustive or complete review of all factors influencing response to pharmacotherapy of these drug classes. Samer *et al.* published a detailed review on the clinical impact of known CYP450 polymorphisms on drug therapy, including a summary of the consensus dosage recommendations and guidelines based on pharmacogenetics testing of CYP450 expression.[7] A main concern is the lack of published data available on the influence of genotype on Sub-Saharan African populations. A study conducted by Dodgen *et al.*[91] found novel CYP2C19 alleles indigenous to the South African population that contributed to a poor correlation between predicted and measured phenotypes, highlighting the importance of considering the pharmacogenetics and unique confounders present in this population. A similar finding with CYP2C9 alleles confirms the discord between predictive and measured phenotypes, where only a small number of alleles could be successfully attributed to decreased or absent enzyme activity.[92]

1.2.1.2.1 Antihypertensive medication

Effective use of antihypertensive medication and target reduction in arterial blood pressure can greatly reduce medical costs attributed to cerebrovascular events and myocardial infarctions.[93] However, the poor clinical outcomes have been attributed to inter-individual and interethnic variability in cytochrome P450 (CYP)-dependent metabolism of several first-line antihypertensive drugs, including calcium channel blockers, β-blockers and angiotensin receptor blockers that are metabolised by cytochrome P450 enzymes CYP2D6, CYP2C19 and CYP3A4.[94, 95] A clinically relevant example is the CYP2C19*2 loss of function polymorphism that increases cardiovascular adverse events by 30% in patients on treatment with clopidogrel, an antiplatelet prodrug requiring bioactivation.[96]

1.2.1.2.2 Psychiatric medication

Despite the large number of effective psychotropic drugs available[97], therapeutic outcomes for many psychiatric patients are still not optimal due to pharmacokinetic variability[98] resulting in a large inter-individual difference in steady state plasma concentration levels. The major metabolic pathway for elimination is phase-1 metabolism via mainly cytochrome P450 enzymes CYP1A2, CYP2B6, CYP2D6, CYP2C9, CYP2C19 and CYP3A4/5.[14] Therapeutic drug monitoring guidelines have been published and updated in 2011 to improve psychiatric pharmacotherapy and includes information on normal ranges of metabolite to parent drug ratios.[14] An example of a CYP2D6 inhibitor that significantly influence other CYP2D6 substrate drugs with co-administration, is fluoxetine, where the metabolic ratio (MR) for probe drug dextromethorphan increased by 17.1-fold after 28 days of therapy.[9]

1.2.1.2.3 Antiretroviral medication

Human immunodeficiency virus (HIV) prevalence is the highest in South Africa[99] and a number of reviews have underlined the importance of personalised pharmacotherapy to optimise dose response and to prevent unwanted side-effects.[100-102] Both the non-nucleoside reverse transcriptase inhibitors and protease inhibitors are metabolised by the CYP450 enzymes of which CYP2B6 and CYP3A4/5 have been shown to play major roles in the pharmacokinetic variability of nevirapine.[103] Most notable is the high frequency of the CYP2B6*6 allele in Sub-Saharan African populations which could explain the high prevalence of drug induced adverse events reported with efavirenz and nevirapine.[104]

1.2.1.2.4 Antiepileptic medication

Epilepsy is a common non-communicable neurological disease with a high prevalence of active convulsive epilepsy in rural Sub-Saharan Africa[105] and treatment of paediatric and geriatric patients are mostly affected by inter-individual variation. Anti-epileptic drugs, phenobarbital (for children younger than 6 months), phenytoin (if well controlled), lamotrigine, sodium valproate and carbamazepine are listed as essential drug treatment for epilepsy in South-African primary healthcare facilities.[106] Most are subject to inter-individual pharmacokinetic variability and associated with numerous DDI's and ADR with recommended routine therapeutic drug monitoring to establish *in vivo* drug concentrations to optimise drug dosages.[107] Firstline anti-epileptic drugs, carbamazepine, valproate, phenobarbital and phenytoin are all metabolised by the CYP450 enzymes and are also potent inducers of CYP1A, CYP2B, CYP2C and CYP3A enzymes affecting oral bioavailability of substrates for these enzymes.[78, 94] Induction occur via activation of PXR and CAR as described above in 1.2.1.1. Dosing strategies to optimise individual treatment have been published recently.[107, 108]

1.2.1.2.5 Antibiotic rifampicin used in anti-tuberculosis drug regimes

Rifampicin is a well-known inducer of CYP2C8, 2C9, 2C19, 2B6 and 3A4/5.[81, 109] Induction of CYP450 enzymes by rifampicin is mediated by the ligand activated nuclear receptor PXR that regulates the transcription of CYP450 mRNA and finally the translation into CYP450 enzymes with significant effects on the metabolism of drug substrates for these enzymes.[81] Rifampicin has been widely used in validation of phenotyping cocktail studies as enzyme inducer and in drug development and drug interaction studies.

1.2.2 P-glycoprotein membrane transporter

Membrane transporters affects the pharmacokinetics of a large number of xenobiotics through unidirectional transmembrane transport across intestinal and biliary epithelia, renal proximal tubules and endothelia of the blood brain barrier and placenta. The ATP-dependant P-glycoprotein efflux transporter is one of the most ubiquitous transporters in humans and forms an evolutionary protective barrier against a vast number of toxic exogenous substances by actively pumping these substrates across cell membranes of elimination and barrier organs, enhancing their excretion or decreasing systemic and intracellular drug concentrations.[110] P-gp substrates are structurally diverse and often unrelated compounds with relatively hydrophilic or weakly amphoteric properties. Substrates usually contains a N-atom and an aromatic

molecular structure. Common substrates include anti-cancer drugs, tyrosine kinase inhibitors, anti-epileptics, steroids, cardiac glycosides, statins and calcium channel blockers. P-gp also transports endogenous substrates across cell membranes including cytokines, phospholipids and hormones.[111] Many substrates for the P-glycoprotein efflux transporter are also substrates for the CYP450 enzymes contributing to significant drug-drug interactions and drug efficacy or toxicity.

1.2.2.1 P-gp structure, expression and functionality

P-glycoprotein is a large (170 kDa) membrane-bound protein encoded by the adenosine triphosphate-binding cassette type B1 (ABCB1) gene on chromosome 7 and is part of the ABC transporter superfamily (ABC-x). The superfamily of ABC transport proteins consist of seven subfamilies ABCA to ABCG with a total of 49 members identified thus far.[112] The ABCB1 gene was first known as the multidrug resistance gene (MDR1) when it was discovered as the cause of multidrug resistant tumours more than 40 years ago.[111] Structurally, the P-gp protein consist of two identical halves containing 6 lipophilic transmembrane domains (TMD) each and two nucleotide-binding domains (NBD), one on each half, driving conformational change in the TMD via ATP hydrolysis at the NBD to transport substrates against the chemical gradient.[113]

When a drug substrate binds to the TMD binding site from the cytoplasm or the lipid bilayer of the cell membrane the two NBD dimerises and switches the TMD from an inward to outward conformation, releasing the drug extracellularly, thereby actively pumping the drug out of the cell.[114, 115] The protein contains multiple binding sites capable of binding with multiple substrates simultaneously.[113] Expression of the P-gp transporter is mediated by PXR, which could explain overlapping substrate specificity with CYP3A4 and the functional interaction between the two proteins.[116, 117] In addition, these proteins are expressed in the same tissues and organs to act interdependently in the evolutionary protection again exogenous toxins that includes xenobiotics. Intestinal CYP3A4 metabolises drug substrates entering the enterocyte via passive diffusion whilst P-gp act to actively pump substrates out of the cell, thereby controlling the amount of substrate availability for the CYP3A4 enzyme to metabolise.[117] It is therefore difficult to attribute the causes of drug-drug interactions to a specific phenotype in this instance. Genetic polymorphisms of the adenosine triphosphate (ATP) binding cassette transporter gene (ABCB1) influence P-gp transport protein expression and ultimately drug transmembrane transport.[118] ABCB1 SNP variants identified are published on the NCBI's

bdSNP database[119] and although 8643 SNP's have been identified and characterised, for most, their functional consequence on bioavailability remain to be elucidated. One SNP with extensive inter-ethic variability is 3435C>T with the 3435TT polymorphism resulting in lower intestinal P-gp expression and elevated plasma concentrations of digoxin on average compared to homozygous C allele carriers[120] of which frequency of the latter genotype was found to be significantly higher in African populations compared to African American or Caucasian populations.[121] Inter-tissue variability in the expression of P-gp further contribute to the intra-individual variability. In addition to genotype, exogenous compounds influencing P-gp activity include drugs that act as modulators (inducers or inhibitors) as well as certain poisons (pesticides) and natural products (plant flavonoids and St. John's Wort).

The complete mechanisms of action by which P-gp inhibitors or inducers alter the functionality of this transporter is not fully understood. Drug substrates may simultaneously be inhibitors or inducers of P-gp, e.g. cyclosporin, a P-gp substrate, also act as a competitive inhibitor at the binding site. Other substrates inhibit the transporter by strongly binding to the drug-binding pocket without being transported. Steroids on the other hand interfere with ATP hydrolysis through interaction with NBDs.[111] Inhibition of P-gp mediated efflux leads to an increase in the bioavailability of P-gp substrates. This is advantageous when a P-gp inhibitor is used together with anticancer drugs in cancer tissue to increase intercellular concentration of the anticancer drug, reducing multidrug resistance and improving drug efficacy. Similarly, P-gp inhibitors could be used to increase drug delivery in AIDS, epilepsy and other neurological diseases treated by drugs that are also P-gp substrates. Inducers contrarily act at a transcriptional level through PXR activation[113], increasing expression of P-gp and hence the efflux of substrates, leading to decreased systemic bioavailability and possible therapeutic failure. Whether an interaction between a P-gp substrate and inhibitor or inducer will result in a clinically relevant DDI will depend on the drug doses, the therapeutic window of the affected substrate, the potency of the inhibitory or inducing drug, the contribution of other enzymes or transporters to the PK of the affected substrate and the route of administration.[122]

1.2.2.2 Clinical consequences of inter-individual variability in P-glycoprotein transport

Some examples are given below to indicate the clinical consequences of genetic inter-individual variability and drug-drug interactions on the drug disposition of P-gp substrates. The latter should be considered in addition to age, gender, hepatic and renal functionality, since a complex

interplay exist between all factors contributing to clinically significant drug interactions and toxicity. The examples below are not intended to be an exhaustive or complete review of all factors influencing response to pharmacotherapy of these P-gp substrates, but instead highlights the importance of monitoring the real-time *in vivo* P-gp-phenotype to infer possible drug interactions and patient outcomes.

1.2.2.2.1 Antihypertensive medication

A prevalent example of a drug interaction involving P-gp is the higher bioavailability of digoxin when co-administered with the P-gp inhibitor verapamil. Early *in vitro* models showed a 2.5-fold increase in the intracellular concentration of this cardiac glycoside in the presence of verapamil.[123] This increase in digoxin concentration in cardiomyocytes has been confirmed by *in vivo* studies.[124] Digoxin is has a narrow therapeutic index and is a poorly soluble drug, which further emphasises the importance of studying drug interactions in the presence of P-gp inhibitors for this drug, as clinically significant side effects or death could result from co-administration. Alternatively, in the presence of a P-gp inducer, rifampicin, intestinal digoxin concentrations increased and bioavailability decreased significantly.[125] Evidence also suggest that genetic polymorphisms in the ABCB1 gene affect oral clearance of calcium channel blockers, verapamil[126] and amlodipine[127] through P-gp transport channels, influencing bioavailability. Wessler *et al.* recently published a detailed review on cardiovascular drugs mediated by the P-gp transporter and the clinical significance of interactions at this transporter, including antihypertensive, antiarrhythmic and anticoagulant agents and statins.[128]

1.2.2.2.2 Antidepressants and antipsychotics

In addition to the influence of phase 1 metabolism on the therapeutic outcomes of psychotropic drugs, it has been hypothesized that variability may also be as a result of genetic polymorphisms of the ABCB1 genotype and resulting expressed ABC transport channels located in the blood brain barrier.[129]

During investigation into the effects of polymorphisms on the drug disposition of antipsychotics, risperidone has been most studied, with results remaining inconclusive.[115] Intracerebral concentration of risperidone and its active metabolite, 9-hydroxyrisperidone is however significantly affected by co-administration with P-gp inducers or inhibitors regardless of the genotype.[130-132]

The efficacy of antidepressant treatment relies on drug bioavailability across the blood brain barrier (BBB). In a study, done by Uhr *et al.*[133], comparing *abcb1ab* knockout mice to wild-type, it has been shown that P-gp regulates the intracerebral concentrations of citalopram, venlafaxine and its metabolite, O-desmethyl venlafaxine, with significant increase in intracerebral concentration in the mutant mice. This was followed by a pharmacogenetic study involving 443 inpatients, randomised into two groups; patients receiving P-gp substrates (amitriptyline, paroxetine, citalopram or venlafaxine) and a second group receiving mirtazapine, a non P-gp substrate. Patients were genotyped for 95 SNPs in the ABCB1 gene and the genotype compared to phenotypic remission of antidepressant symptoms after 4,5 and 6 weeks on treatment with P-gp substrate drugs compared to the control group. The authors concluded that clinical outcomes in patients treated with P-gp substrate antidepressants could be predicted by SNPs in the ABCB1 gene.[133] Not all studies done on genotype phenotype relation have been able to show correlation however[115], indicating the importance of considering all factors contributing to clinical outcomes, including overlapping substrate specificities with CYP450.

1.2.2.2.3 Antiretroviral medication

The high prevalence of HIV infection in Sub-Saharan Africa amplifies the need for optimised therapy to ensure adequate therapeutic exposure to antiretroviral treatment. In addition to CYP450 predisposition influencing metabolism[134], ABC-x drug transporters contribute to multidrug resistant phenotypes of a number of antiretroviral drugs that are substrates, inducers or inhibitors of most notably P-gp. The protease inhibitors and several of the nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors are transported by P-gp.[135] Specific polymorphisms of the ABCB1, unique to South-African populations, were found to influence drug plasma levels of the antiretroviral drugs nevirapine[136] and efavirenz.[137] The 3435C>T polymorphism resulted in a lower expression of the efflux protein with subsequent decreased hepatotoxic risk. Combination therapy of antiretroviral medication (ARVs) with no expected drug interaction, i.e. Atripla® (efavirenz, emtricitabine and tenofovir disoproxil fumarate) have been the cornerstone in the treatment of HIV to overcome drug resistance. In addition to genetic polymorphisms, drug interactions at the P-gp level also affect therapeutic drug delivery through induction of P-gp expression via PXR activation or P-gp inhibition.[134]

1.2.2.2.4 Antiepileptic medication

Similar to antidepressants and antipsychotics, intracerebral concentration of anti-epileptic drugs depends on their bioavailability across the BBB. Overexpression of active transporters in the endothelial cells of the BBB could decrease entry of anti-epileptic medication into the CNS resulting in treatment failure.[138] The role of ABCB1 polymorphisms in therapeutic failure with anti-epileptic medication remains inconclusive, possibly due to involvement of other genes. Furthermore, most studies have been conducted in Caucasian and Asian cohorts and is not representative of all ethnic groups. Carbamazepine, phenytoin and phenobarbital are all substrates and inducers of P-gp and may affect the bioavailability of concomitant medication.

1.3 PHENOTYPING CYTOCHROME P450 AND P-GP TRANSPORT WITH A COCKTAIL APPROACH TOWARDS INDIVIDUALISED DRUG THERAPY

Measuring specific drug concentrations of substrates for either metabolising enzymes or drug transporter proteins, provides us with a fingerprint of metabolic or transport activity *in vivo* which is then correlated to real-time phenotype. Unlike functional genotype, that depend on epigenetic regulation or posttranslational modifications, this approach measures biochemical activity directly correlated with functional phenotype. It considers all intrinsic and extrinsic factors influencing variability in a dynamic way, because it will change depending on pathophysiology, age, lifestyle and co-medication changing over time for an individual and should therefore be done routinely in order to assist clinicians in drug selection and dosing toward personalised pharmacotherapy.[139] A limitation with genotyping alone is that the fold-change in enzyme activity related to a specific allele is dependent on the substrate.[92] Enzyme activity of a known variant allele may also display diverse results and may be subject to change due to epigenetic or other influencing factors.[140]

A number of phenotyping cocktails aimed at assessing *in vivo* CYP450 metabolic activity and in some instances P-gp activity have been developed (Section 1.3.1), but their implementation in clinical practice have been limited by a wide variety of challenges (Section 1.3.2). Non-invasive sampling using DBS in resource limited countries, like South-Africa, coupled with limited population PK sampling may increase the feasibility of phenotyping provided that pharmacokinetic and bioanalytical challenges are addressed during the validation and appropriate statistical analysis is employed to assess agreement of the quantitative measurement to the gold standard of plasma sampling (Section 1.3.3). The chosen phenotyping cocktail for

implementation in a South African cohort should further meet the required criteria set out in the proposed study (Section 1.3.4).

1.3.1 Review of phenotyping cocktails developed over the last two decades

Published articles on phenotyping cocktails over the past 20 years were reviewed by a literature search on the University of Pretoria's library databases (36 databases for Health Sciences, including AccessMedicine, BMJ, ClinicalKey, MEDLINE (Ovid), PubMed, Scopus and TOXLINE). The search was limited to *in vivo* cocktails conducted in human populations consisting of 5 or more probe drugs to phenotype phase I metabolising enzymes and/or the P-gp transporter with a cocktail approach. Only articles available in the English language were included. A summary of the multiple probe phenotyping cocktails is given in Table 1.2, reviewing the sampling matrix, the enzyme and/or transporter investigated in the cocktail with corresponding phenotyping drug and dosage, the phenotyping metric (i.e. concentration-time profiles with drug area under the curve (AUC) , probe drug to metabolite concentration ratio in plasma/ urine or absolute urinary recovery) used to assess metabolic or transport activity and bioanalytical methods used for quantitation. Additionally, all proposed cocktails were scrutinised for statistical methods employed to assess agreement between different sampling methods (if applicable), whether interaction studies between probe drugs were carried out and for sample size and population demographics (healthy vs diseased, age, genotype etc.) summarised in Table 1.3.

Present phenotyping cocktails, containing multiple probe drugs, are used for simultaneous assessment of drug metabolism during drug development in drug-drug interaction and toxicology studies and regulated by the EMA and the FDA.[141] Due to the safety concerns of possible drug-drug interactions with new chemical entities (NCE), it has to be clinically evaluated during early drug development. Earlier cocktails used plasma and urine sampling to phenotype mostly phase I metabolism[142-149] and in some cocktails also phase II metabolism[150-152]. Three of the recent multiple drug cocktails included a P-gp probe, either digoxin[153] to assess renal P-g activity or fexofenadine[37, 154] assessing intestinal P-gp transport. Alternative non-invasive sampling strategies, using DBS and/or saliva, were explored in two cocktails, namely the Geneva[37] and Basel[38] cocktails.

Table 1.2: Summary of *in vivo* phenotyping cocktails with 5 or more probes used in human populations during the past 20 years.

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
1999 ‘‘GW cocktail’’	Plasma and Urine	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP2E1 CYP3A4	caffeine 100 mg diclofenac 10 mg mephenytoin 25 mg debrisoquine 10 mg chloroxazone 250 mg midazolam 5 mg	Concentration -time profiles for caffeine, chloroxazone, midazolam and metabolites. Absolute urinary recovery over 12-hours for *S*-mephenytoin and diclofenac.	Online-SPE LC-MS/MS	(142)
2001 Zhu *et al.*	Plasma and Urine	CYP1A2 CYP2C19 CYP2D6 CYP2E1 CYP3A4	caffeine 100 mg mephenytoin 100 mg metoprolol 100 mg chloroxazone 200 mg midazolam 7.5 mg	[par]/ [caf] 6 hours plasma [mep]/[OH-mep] 8h collective urine [met]/[OH-met] 8h collective urine [OH-chlor]/[chlor] 4h plasma [OH-mdz]/[mdz] 1-hour plasma	β-glucuronidation + liquid extraction LLE HPLC-UV	(143)
2003 Karolinska cocktail	Plasma and Urine	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	caffeine 100 mg losartan 25 mg omeprazole 20 mg debrisoquine 10 mg quinine 250 mg	[par]/ [caf] 3.5, 4 hours plasma [los]/ [E 3174] 8h collective urine [OH-opz]/[opz] 3, 3.5 hours plasma [deb]/[OH-deb] 8h collective urine [OH-qui] 16h plasma	PPT of plasma with ACN, LLE HPLC-UV HPLC-FL detection	(144)
2003 Cooperstown 5+1 cocktail	Plasma and Urine	CYP1A2, NAT2, XO CYP2C19 CYP2D6 CYP3A4	caffeine 2 mg/kg caffeine 2 mg/kg caffeine 2 mg/kg omeprazole 40 mg dextromethorphan 30 mg midazolam 0.025 mg/kg (plus, vit K) S-warfarin 10 mg	[1X + 1U + AFMU]/[17U]12h collective urine [AFMU]/ [1X + 1U] 12h collective urine [1U]/ [1X + 1U] 12h collective urine [OH-opz]/[opz] plasma [dtp]/[dex] 12h collective urine [OH-mdz]/[mdz] plasma AUC 0-∞ *S*-warfarin	LLE, SPE HPLC-UV HPLC-FL detection	(150)

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
2004 Quebec cocktail Sharma *et al.*	Urine	CYP1A2, NAT2, XO CYP2C9 CYP2D6 CYP2E1 CYP3A4	caffeine 100 mg caffeine 100 mg caffeine 100 mg tolbutamide 250 mg metoprolol 25 mg chloroxazone 250 mg dapsone 100 mg	[1X + 1U + AFMU]/[17U] 8h collective urine [AFMU]/[AFMU+1X+1U] 8h collective urine [1U]/[1X+1U] 8h collective urine [COOH-tol+OH-tol]/[tol] 8h collective urine [Met]/[OH-met] 8h collective urine [OH-chlor]/[chlor] 8h collective urine [dap-HA]/[dap+dap-HA] 8h collective urine	β-glucuronidase /arylsulphatase + LLE HPLC-UV LC-MS/MS	(151)
2004 Loughborough -Blakey *et al.*	Plasma and Urine	CYP1A2 CYP2C9 CYP2D6 CYP2E1 CYP3A4	caffeine 100 mg tolbutamide 250 mg debrisoquine 5 mg chloroxazone 250 mg midazolam 0.025 mg/kg	[par]/ [caf] 6.5 hours plasma [COOH-tol+OH-tol]/[tol] 6-12h urine [deb]/[OH-deb] 0-6h urine [OH-chlor]/[[chlor] 2h 32min plasma AUC last plasma MDZ	Dilute and shoot/ β-glucuronidase +/ SPE/ ACN PPT LC-MS	(145)
2004 Jerdi *et al.* (Geneva University Hospital)	Plasma	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	caffeine 100 mg flurbiprofen 50 mg omeprazole 40 mg dextromethorphan 25 mg midazolam 7.5 mg	*PK parameters and clinical study where to be published elsewhere. No reference found in English language.*	LLE / PPT HPLC-UV and HPLC-FL detection	(155)

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
2004 Yin *et al.*	Plasma and Urine	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	caffeine 100 mg tolbutamide 500 mg omeprazole 40 mg debrisoquine 10 mg midazolam 3.75 mg	[par]/ [caf] $_{2/3\ hours\ plasma}$ [COOH-tol+OH-tol]/[tol] $_{6\text{-}12h\ urine}$ [OH-opz]/[opz] $_{2/3\ hours\ plasma}$ [OH-deb]/[deb] $_{0\text{-}6h\ urine}$ [OH-mdz]/[mdz] $_{2/3\ hours}$	SPE LC-MS	(146)
2005 Tomalik-Scharte *et al.* (Note: 30 mg of dextromethorphan-HBr also given, results not reported)	Plasma and Urine	CYP1A2 CYP2C9 CYP2C19 CYP3A4 $_{Hepatic}$ CYP3A4 $_{Intestinal}$	caffeine 150 mg tolbutamide 125 mg mephenytoin 50 mg midazolam 2 mg iv midazolam 1 mg po	[par]/ [caf] $_{6\ hours\ plasma}$ [COOH-tol+OH-tol]/[tol] $_{6\text{-}12h\ urine}$ AND $AUC_{0\text{-}\infty,}$ $C_{max\ oral}$, $t_{max\ oral}$, $t_{1/2,\lambda z}$, CL/F, [tol] $_{24\ hours\ plasma}$ 4'-Hydroxymephenytoin $_{0\text{-}8h\ urine}$ $AUC_{0\text{-}\infty\ i.v.}$, $CL_{i.v.\ mid}$, $F_{hepatic}$ F_{oral}, $F_{intestinal}$, $AUC_{0\text{-}\infty\ oral}$, $C_{max\ oral}$, $t_{max\ oral}$, $t_{1/2,\lambda z}$	β-glucuronidase deconjugation/ SPE / plasma PPT HPLC-UV LC-MS/MS	(147)
2006 Pittsburg +1	Plasma and Urine	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP2E1 NAT2	caffeine 100 mg flurbiprofen 50 mg mephenytoin 100 mg debrisoquine 10 mg chloroxazone 250 mg dapsone 100 mg	[par]/ [caf] $_{8\ hours\ plasma}$ [OH-flb]/[OH-flb+flb] $_{0\text{-}8h\ urine}$ 4'-Hydroxymephenytoin $_{0\text{-}8h\ urine}$ [OH-deb]/[OH-deb+deb] $_{0\text{-}8h\ urine}$ [OH-chlor]/[chlor] $_{4h\ plasma}$ [MA-dap]/[dap] $_{8\ hours\ plasma}$	No sample prep mentioned HPLC	(152)

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
2006 Darmstadt - Krösser *et al.*	Plasma and Urine	CYP1A2	caffeine 100 mg	AUC $_{0–24h\ par}$/ AUC $_{0–24h\ caf}$	SPE	(149)
		CYP2C9	diclofenac 50 mg	AUC $_{0–24h\ OH\text{-}dic}$/ AUC $_{0–24h\ dic}$		
		CYP2C19	mephenytoin 100 mg	4'-Hydroxymephenytoin $_{0\text{-}8h\ urine}$	HPLC-FL	
		CYP2D6	metoprolol 100 mg	AUC $_{0–72h\ OH\text{-}met}$/ AUC $_{0–72h\ met}$	LC-MS/MS	
		CYP3A4	midazolam 7.5 mg	$AUC_{0\text{-}24\ mdz}$		
2007 Inje cocktail	Plasma and Urine	CYP1A2	caffeine 93 mg	[par]/ [caf] $_{4\ hours\ plasma}$	LLE	(148)
		CYP2C9	losartan 30 mg	[los]/ [E 3174] $_{8h\ collective\ urine}$		
		CYP2C19	omeprazole 20 mg	[OH-opz]/[opz] $_{4\ hours\ plasma}$	LC-MS/MS	
		CYP2D6	dextromethorphan 30 mg	log[dtp]/[dex] $_{8h\ collective\ urine}$	HPLC-FL detection	
		CYP3A4	midazolam 2 mg	[mdz] $_{4\ hours\ plasma}$		
2008 Petsalo *et al.*	Urine	CYP1A2	melatonin 3 mg	[mel] AND [OH-mel] $_{8h\ collective\ urine}$	β-glucuronidase hydrolysis	(156)
		CYP2A6	nicotine 2 mg	[nic] AND [cot] $_{8h\ collective\ urine}$		
		CYP2B6	bupropion 150 mg	[bup] AND [OH-bup] $_{8h\ collective\ urine}$		
		CYP2C8	repaglinide 1 mg	[rep] AND [OH-rep] $_{8h\ collective\ urine}$	UPLC-MS/MS	
		CYP2C9	losartan 50 mg	[los] AND [E 3174] $_{8h\ collective\ urine}$	LC-MS/MS	
		CYP2C19	omeprazole 20 mg	[opz] AND [OH-opz] $_{8h\ collective\ urine}$		
		CYP2D6	dextromethorphan 12.5 mg	[dex] AND [dtp] $_{8h\ collective\ urine}$		
		CYP2E1	chloroxazone 62.5 mg	[chlor] AND [OH-chlor] $_{8h\ collective\ urine}$		
		CYP3A4	midazolam 3.75 mg	[mdz] AND [OH-mdz] $_{8h\ collective\ urine}$		
		CYP3A4	omeprazole 20 mg	[opz] AND [opz-sulphone] $_{8h\ collective\ urine}$		

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
2009 Ghassabian *et al.*	Plasma	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	caffeine 100 mg losartan 25 mg omeprazole 20 mg dextromethorphan 30 mg midazolam 2 mg	[par]/ [caf] $_{4\ hours}$ AUC $_{0–6h\ E\text{-}3174}$/ AUC $_{0–6h\ los}$ [OH-opz]/[opz] $_{4\ or\ 6\ hours}$ AUC $_{0–6h\ dtp}$/ AUC $_{0–6h\ dex}$ AUC $_{0–6h\ OH\text{-}mdz}$/ AUC $_{0–6h\ mdz}$	SPE and LLE after initial PPT with ACN HPLC-MS/MS	(157)
2009 Sanofi-Aventis cocktail - Turpault *et al.*	Plasma	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	caffeine 100 mg *S*-warfarin 10 mg omeprazole 20 mg metoprolol 100 mg midazolam 0.03 mg/kg IV	$AUC_{0\text{-}\infty\ caffeine}$ $AUC_{0\text{-}\infty\ S\text{-}warfarin}$ $AUC_{0\text{-}\infty\ omeprazole}$ $AUC_{0\text{-}\infty\ metoprolol}$ $AUC_{0\text{-}\infty\ midazolam}$	SPE and LLE LC-MS/MS separate analysis	(158)
2010 CIME cocktail **NOTE: initial cocktail included amodiaquine as CYP2C8 probe. Repaglinide was added in 2016*	Plasma	CYP1A2 CYP2C8 CYP2C9 CYP2C19 CYP2D6 CYP3A4 OATP UGT Renal P-gp	caffeine 73 mg repaglinide 0.25 mg * tolbutamide 10 mg omeprazole 10 mg dextromethorphan 18 mg midazolam 4 mg Rosuvastatin 5 mg Acetaminophen 60 mg Memantine 5 mg Digoxin 0.25 mg	C_{max}, AUC_{∞}, $t_{1/2}$, CL/F were calculated for all substrates AND in addition $AUC_{\infty substrate}/AUC_{\infty metabolite}$ for CYP450 substrates and metabolites	SPE UPLC-MS/MS	(153, 159)

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
2012 Inje – low dose Oh *et al*	Plasma	CYP1A2	caffeine 10 mg	$AUC_{0–12h\ caf}$, $AUC_{0–12h\ par}$	LLE	(160)
		CYP2C9	losartan 2 mg	$AUC_{0–12h\ los}$, $AUC_{0–12h\ EXP3174}$		
		CYP2C19	omeprazole 200 µg	$[OH\text{-}opz]_{1.5\ hours}$, $[opz]_{1.5\ hours}$	LC-MS/MS	
		CYP2D6	dextromethorphan 2 mg	$AUC_{0–12h\ dex}$, $AUC_{0–12h\ dtp}$		
		CYP3A4	midazolam 100 µg	$C_{max\ OH\text{-}mdz\ at\ 6h}$, $AUC_{0\text{-}12h\ OH\text{-}mdz}$		
2012 Wohlfarth *et al.*	Plasma	CYP1A2	caffeine 100 mg	$[par]/[caf]_{4\ hours}$	SPE	(161)
		CYP2C9	tolbutamide 125 mg	$[tol]_{24\ hours\ plasma}$		
		CYP2C19	omeprazole 20 mg	$[OH\text{-}opz]/[opz]_{4\ hours}$	LC-MS/MS	
		CYP2D6	dextromethorphan 30 mg	$[dex]/[dtp]_{4\ hours}$		
		CYP3A4	midazolam 2 mg	$[mdz]_{4\ hours}$		
2014 Geneva cocktail	Plasma and DBS	CYP1A2	caffeine 50 mg	$[par]/[caf]_{2\ hours}$	DBS – MeOH	(37, 162)
		CYP2B6	bupropion 20 mg	$[OH\text{-}bup]/[bup]_{3\ hours}$	Plasma – ACN PPT	
		CYP2C9	flurbiprofen 10 mg	$[OH\text{-}flb]/[flb]_{3\ hours}$		
		CYP2C19	omeprazole 10 mg	$AUC_{2,3,6h\ opz}/AUC_{2,3,6h\ OH\text{-}opz}$	LC-MS/MS	
		CYP2D6	dextromethorphan 10 mg	$[dtp]/[dex]_{3\ hours}$		
		CYP3A4	midazolam 1 mg	$[OH\text{-}mdz]/[mdz]_{2\ hours}$		
		P-gp	fexofenadine 25 mg	Limited sampling $AUC_{2,3,6h}$		
2014 Basel cocktail	Plasma, saliva and DBS	CYP1A2	caffeine 100 mg	$[par]/[caf]_{8\ hours\ plasma}$; $[par]/[caf]_{8\ hours\ DBS}$;	PPT	(38)
		CYP2B6	efavirenz 50 mg	$[par]/[caf]_{8\ hours\ saliva}$	LC-MS/MS	
		CYP2C9	losartan 12.5 mg	$[efv]/[OH\text{-}efv]_{8\ hours\ plasma}$		
		CYP2C19	omeprazole 10 mg	$[los]/[E\ 3174]_{8\ hours\ plasma}$		
				$[opz]/[OH\text{-}opz]_{2h\ plasma}$;		
				$[opz]/[OH\text{-}opz]_{2h\ DBS}$;		
		CYP2D6	metoprolol 12.5 mg	$[opz]/[OH\text{-}opz]_{2h\ saliva}$		
		CYP3A4	midazolam 2 mg	$[met]/[OH\text{-}met]_{8\ hours\ plasma}$		
				$[mdz]/[OH\text{-}mdz]_{2\ hours\ plasma}$		

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
2016 Lammers *et al.*	Plasma	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	caffeine 100 mg warfarin 5 mg omeprazole 20 mg metoprolol 100 mg midazolam 0.03 mg/kg IV	$AUC_{0-\infty}$ caffeine $AUC_{0-\infty}$ *S*-warfarin $AUC_{0-\infty}$ omeprazole $AUC_{0-\infty}$ metoprolol $AUC_{0-\infty}$ midazolam	PPT with 42:8 ACN: MeOH LC-MS/MS nonchiral and chiral methods	(163)
2017 Puris *et al.* *NOTE: repaglinide excluded as metabolite 3'-hydroxyrepaglinide not detected from samples and interference of another compound with similar m/z*	Urine and Serum	CYP1A2 CYP2A6 CYP2B6 CYP2C8 CYP2C9 CYP2C19 CYP2D6 CYP2E1 CYP3A4 CYP3A4	melatonin 2 mg nicotine 1 mg bupropion 37.5 mg repaglinide 0.25 mg losartan 12.5 mg omeprazole 10 mg dextromethorphan 30 mg chloroxazone 62.5 mg midazolam 1.85 mg omeprazole 10 mg	$AUC_{0-6\ h\ limited\ sampling}$, C_{max} and t_{max} and cumulative concentration in urine for probe drugs and metabolites calculated. 5-Hydroxyomeprazole indicative of CYP2C19 metabolism and omeprazole sulfone of CYP3A4 metabolism	β-glucuronidase hydrolysis for urine SPE, **PPT (method of choice)**, LLE LC-MS/MS – 3 separate runs	(164)
2017 Grangeon *et al.* *NOTE: chlorzoxazone administered separately to avoid interaction with CYP3A4*	Plasma and Urine	CYP1A2 CYP2B6 CYP2C9 CYP2C19 CYP2D6 CYP3A4 CYP2E1	caffeine 100 mg bupropion 100 mg tolbutamide 250 mg omeprazole 20 mg dextromethorphan 30 mg midazolam 2 mg chlorzoxazone 250 mg	Plasma and urinary concentrations of all probe drugs and metabolites were obtained in patients on polypharmacy.	β-glucuronidase / sulfatase hydrolysis PPT Three separate UPLC-MS/MS methods	(165)

Cocktail	Matrix	PKP	Probe drugs and doses	Phenotyping metrics	Analytical methods	Ref.
2018 Sao Paulo cocktail	Plasma	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4 P-gp	caffeine 10 mg losartan 2 mg omeprazole 2 mg metoprolol 10 mg midazolam 0.2 mg fexofenadine 10 mg	$AUC_{0-\infty}$ for all analytes except E-3174 where AUC_{0-12h} were used, C_{max} and Cl/F (L/h)	SPE, LLE, PPT Three separate UPLC-MS/MS methods	(154)

PKP = pharmacokinetic parameters; AUC = area under the plasma concentration time curve; UR = urinary recovery ratio; MR = metabolic ratio [parent]/[metabolite]; CYP = Cytochrome P450 enzyme; NAT2 = N-acetyltransferase 2; XO = Xanthine oxidase; OATP = Organic-anion-transporting polypeptide; UGT = Uridine diphosphate glycosyltransferase; P-gp = Permeability glycoprotein; par = paraxanthine; caf = caffeine; mep = *S*-mephenytoin; OH-mep = 4'-hydroxymephenytoin; met = metoprolol; OH-met = α-hydroxymetoprolol; OH-chlor = 6'-hydroxychloroxazone; chlor = chlorzoxazone; OH-mdz = 1'-hydroxymidazolam; mdz = midazolam; los = losartan; E 3174 = active losartan metabolite; OH-opz = 5'-hydroxy-omeprazole; opz = omeprazole; deb = debrisoquin; OH-deb = 4'-hydroxydebrisoquine; OH-qui = 3'-hydroxyquinine; 1X = 1-Methylxanthine; 1U = 1-methylurate; AFMU = 5-acetylamino-6-formylamino-3-methyluracil; 17U = 1,7-dimethylurate; dtp = dextrorphan; dex = dextromethorphan; COOH-tol+OH-tol = carboxytolbutamide + methylhydroxytolbutamide; tol = tolbutamide; dap-HA = dapsone hydroxylamine; dap = dapsone; OH-flb = hydroxyflurbiprofen; flb = flurbiprofen; MA-dap = dapsone; OH-dic = hydroxydiclofenac; dic = diclofenac; mel = melatonin; OH-mel = hydroxymelatonin; nic = nicotine; cot = cotinine; rep = repaglinide; OH-rep = hydroxurepaglinide; efv = efavirenz; OH-efv = hydroxy-efavirenz; OH-bup – hydroxy-bupropion; bup = bupropion; C_{max} = maximum plasma concentration; t_{max}, = time to reach maximum plasma concentration; $t_{1/2\,\lambda z}$ = terminal half-life; $F_{intestinal}$ = intestinal availability of midazolam; changes in intestinal CYP3A4 activity were calculated as the inverse of changes in $F_{intestinal}$; SPE = solid-phase extraction; LLE = liquid-liquid extraction; PPT = protein precipitation; MeOH = methanol; ACN = acetonitrile; HPLC-MS/MS = High performance liquid chromatography tandem mass spectrometry; HPLC-UV = High performance liquid chromatography ultraviolet detection; HPLC-FL = fluorescence detection; DBS – dried blood spots on Whatman filter paper 903.

During validation of phenotyping cocktails, pilot PK studies are conducted as a proof of concept for use in human populations, but most of the reviewed cocktails included only healthy non-smoking male subjects [37, 38, 143, 147, 151, 163] or healthy male and female cohorts[145, 150, 153, 154, 164], with the sample sizes varying from three to 33 (Table 1.3). Two groups tested their phenotyping cocktails on patient cohorts, namely Ghassabian *et al.*[157] on 11 patients with schizophrenia while Grangeon *et al.*[165] simultaneously assessed systemic and urinary clearance of a new drug using 30 patients on polypharmacy during a clinical trial.

Although some cocktail studies included genotyping (Table 1.3), the objective was not to infer genotype-phenotype relationships, but rather to exclude certain genotypes or as an exploratory analysis of interindividual variation. For the Pittsburg 2006 cocktail[152] for example, two of the volunteers were homozygous for the CYP2D6*4 allele and by removing their phenotypic data from the analysis the inter-subject CV % decreased from 44.8 to 31.9%

The absence of interactions between probe drugs is essential to accurately assign phenotype to specific enzymes or transporters. From these interaction studies it was determined that certain probes influenced the metabolic ratios or clearance of others and were therefore excluded in later cocktails or administered separately. A number of cocktails have been criticised for not evaluating interaction between probe drugs.[166] With advances in LC-MS/MS technology capable of higher detection limits, lower-dose cocktails have been validated, largely eliminating mutual drug interactions. A recent cocktail chose probes based on literature and used dosages up to 10 times less than the therapeutic doses, arguing less drug-drug interactions at lower doses, therefore no interaction study was deemed necessary[154]. Similarly, Puris *et al.* included 10 probe drugs in a low-dose cocktail based on recommendations by the EMA.[164] They site literature where different combinations of this cocktail was assessed for drug-drug interactions, but did not re-evaluate interactions in this cocktail combination. Not conducting interaction studies with low dose cocktails is strongly discouraged due to possible changes in substrate specificity at lower doses causing non-linear pharmacokinetics which will be discussed in detail in Section 1.3.2.1.

Interestingly, statistical analysis on the agreement between different sampling methods are lacking. In earlier cocktails both plasma and urine matrixes were used to assess phenotype simultaneously or separately based on the chosen metric for a specific enzyme (Table 1.2). Other cocktails assessed metabolic and transport activity in a single matrix.

Table 1.3: Evaluation of interaction studies, genotyping, sample size and statistical analysis to assess agreement between sampling methods in multi-drug phenotyping cocktails

Cocktail	I	G	N	Statistical analysis
‘‘GW cocktail’’	YES	NO	NS	N/A
Zhu *et al.*	YES	NO	14	N/A
Karolinska cocktail	YES	NO	24	N/A
Cooperstown 5+1 cocktail	YES	YES	12	N/A
Quebec cocktail	YES	NO	10	N/A
Loughborough -	YES	NO	12	N/A
Jerdi *et al.*	NS	NO	10	N/A
Yin *et al.*	NS	NO	16	N/A
Tomalik-Scharte *et al.*	NO	NO	16	N/A
Pittsburg +1	YES	YES	24	N/A
Darmstadt	YES	NO	18	N/A
Inje cocktail	YES	YES	12	Linear regression plotting the ratio AUC of parent/ metabolite to urinary excretion ratio in 0-4h urine
Petsalo *et al.*	NS	NO	NS	N/A
Ghassabian *et al.* (Inje)	YES	NO	11	N/A
Sanofi-Aventis cocktail	YES	NO	33	N/A
CIME cocktail	YES	YES	10	N/A
Inje – low dose	YES	NO	13	N/A
Wohlfarth *et al.*	NO	YES	14	N/A
Geneva cocktail	YES	YES	10	Linear regression plotting concentration of analyte in DBS vs its concentration in plasma
Basel cocktail	YES	YES	16	Linear regression of single point DBS and saliva ratios compared to AUC ratios
Lammers *et al.*	YES	NO	9	N/A
Puris *et al.*	NO	NO	4	N/A
Grangeon *et al.*	NS	NO	30	N/A
Sao Paulo cocktail	NO	YES	3	N/A

I – Interaction study; G – genotyping; N – sample size; NS – not stated; N/A – not applicable

Agreement between measurements in different matrixes were evaluated by linear regression in the Inje-, Basel- and Geneva cocktails. Important statistical considerations in extrapolating one measurement for another will be elucidated in Section 1.3.3. Other statistical analysis, conducted in phenotyping studies, were to evaluate correlation between single point metabolic ratio's against total AUC or analysis of variance (ANOVA) between treatment arms during interaction studies.

1.3.2 Current challenges limiting use of phenotyping cocktails in clinical practice

Since the first phenotyping cocktail strategy was proposed in 1990,[167] great progress has been made to address the challenges of simultaneous quantification of *in vivo* metabolic and transport phenotypes pointed out by earlier reviews of this approach.[168, 169]

Although most cocktails in Table 1.2 are fit for purpose when it comes to drug development and DDI studies of NCEs, their limitations of use in clinical phenotyping towards individualised therapy, could be summarised as follows;

- Multiple routes of administration[145, 147, 150, 158, 163]
- Use of both matrixes, urine and plasma, in the phenotype assessment[142-150, 152, 164, 165]
- Discontinuation of probes mephenytoin and debrisoquin in most countries[142-147, 149, 152]
- Use of therapeutic doses eliciting side-effects in earlier cocktails[142-146, 149-152, 155]
- Interaction between probe substrates requiring separate administration time points[142-145, 156, 164, 165]
- Extensive sampling procedures[38, 142-152, 154, 156, 160]
- Complicated sample workup or multiple extraction assays[144, 145, 147, 151, 154, 155, 157, 158]
- Impractical analytical procedures, i.e. multiple bioanalytical methods used in a single cocktail[154, 158, 163-165] whilst the use of detectors with low detection limits have become redundant [143, 144, 155, 170] due to advances in mass spectrometry with increased analyte specificity and sensitivity.

Despite the use of drug cocktails during drug development, routine phenotyping in clinical practice towards individualised pharmacotherapy has not yet become reality. The only example of routine phenotyping in clinical practice is the determination of phenylalanine in small volumes of blood (DBS) or urine in new born infants, for phenylketonuria screening.[171]

For clinical applicability, phenotyping cocktails are scrutinised for their ability to use probe drugs that are widely available with acceptable safety profiles, selective to specific CYP enzymes or P-gp and other transporters, well tolerated at the doses given to patients with an uncomplicated route of administration and sampling procedures. Herein a single matrix assay would promote implementation of phenotyping in routine practice, especially when coupled to limited sampling procedures. Phenotyping cocktails should also exhibit minimal PK or PD interaction (i.e. interference in absorption, metabolism or clearance or at the receptor site). Analytical interaction between multiple drugs administered together should be evaluated during sample preparation, detection and quantitation.[157] Fuhr *et al* made reference to the fact that chosen probe drugs and the phenotype identifying measurement, derived from assessing quantitative change in the biological response to the probe drug, must further provide an accurate estimate of the real-time *in vivo* biological activity, must be applicable to other substrates used to phenotype the same enzyme or transporter and should reflect changes in their biological activity in the presence of inhibitors or inducers.[35]

The main factors contributing to the challenge of implementing phenotyping in routine clinical practice are addressed below.

1.3.2.1 Selectivity of probe drugs for metabolising enzymes or drug transporters

The first main problem of current probes suggested by the FDA for phenotyping, is the fact that no probe drug is completely selective for a single metabolising enzyme or transporter. Nonetheless, the contribution of a specific pharmacokinetic pathway to the disposition of the probe drug should be primary and in addition must be indicative of changes in the phenotype when subject to an inducer or inhibitor.[141] For example, caffeine, a fully validated probe for CYP1A2, is also metabolised by CYP2E1, N-acetyl-transferase 2 (NAT2) and xanthine oxidase (XO) enzymes, but since CYP1A2 is the dominant metabolic pathway[172] most cocktails use the metabolic ratio of paraxanthine to caffeine plasma concentration[37, 38, 144, 148, 152, 157] as a CYP1A2 phenotype identifier. Alternatively, provided the phenotyping measurement is carefully chosen, all metabolites of caffeine could be quantified to assess NAT2 and XO activity simultaneously as in the Cooperstown[150] and Quebec[151] cocktails. Similarly, metabolism of omeprazole to its hydroxylated metabolite and sulfone metabolite have been used to simultaneously assess CYP2C19 and CYP3A4 metabolism respectively in a recent cocktail.[164] Tolbutamide is an almost exclusive probe for CYP2C9, but the proposed phenotyping measurement of 24-hour plasma concentration would restrict its usefulness in routine

phenotyping. Metoprolol has been studied as a selective probe for CYP2D6 metabolism, but correlation with other CYP2D6 probes could not be established in an African population from Tanzania carrying a population-specific CYP2D6*17 allele[173] questioning its usefulness as a probe. This discordance between genotype and observed phenotype with altered substrate specificity in African populations has been shown in a number of studies.[174-176] These findings confirm the need for further research on different population groups before routine phenotyping can be implemented in clinical practice.

Phenotyping drug transporter activity may also provide a useful metric to assess and predict drug absorption or excretion (depending on the location of the drug transporter protein) *in vivo*.[35] The role of transporters in drug-drug interactions and the clinical safety and efficacy of drugs has become the focus of the International Transport Consortium since 2010.[8] For the purpose of this thesis, only P-gp transport will be considered. In a review by Ma *et al.*, evaluating four P-gp probes, none met all the proposed validation criteria for an ideal probe drug.[177] Both digoxin and fexofenadine have overlapping substrate specificities with other transporters and their correlation with other P-gp probes have not been established, in addition digoxin has a narrow therapeutic window limiting its usefulness as a probe in patient populations. Despite the fact that no ideal P-gp probe exist, fexofenadine is safe and has been used in phenotyping drug cocktail studies[37, 154] and pharmacokinetic studies.[178-180]

Understanding the pharmacokinetic processes influenced by xenobiotic exposure, the site of exposure and the expression and distribution of metabolising enzymes and transporters at that site is imperative for assigning phenotype and making clinical decisions based on that assessment.

Chosen probe drugs should clearly elucidate the *in vivo* pharmacokinetic phenotype under investigation and overlapping substrate specificities between especially P-gp and CYP3A4 should be considered as discussed in Section 1.2.2.1. A higher expression of CYP3A4 in enterocytes will influence the first pass bioavailability of CYP3A4 substrates significantly and therefore if the objective is to phenotype hepatic CYP3A4 activity, probe substrates should be administered by intravenous route.[181]

Changes in substrate selectivity for metabolising enzymes and transporters when administered at lower subtherapeutic doses must be considered with validation of low dose cocktails. In most cases, a lower substrate dose will increase drug selectivity, however even validated cocktails

have to be re-evaluated when the dosages are lowered to ensure the applicability of the phenotype assessments.[181] An important factor to consider is dose-dependent plasma protein binding, as a result of saturation of the available binding sites, influencing the fraction of unbound drug in systemic circulation as explained by Macheras and Rosen.[182] Micro dosing strategies with phenotyping cocktails, containing dosages a 100-fold lower than the normal dosages have been proposed, but the authors stress that linear pharmacokinetics between normal and micro doses are required for correct prediction of enzyme or transport activity. This is due to the fact that protein binding may be dose-dependent and both decreased bioavailability or non-saturation of compartments during drug distribution may lead to non-linear pharmacokinetics. Furthermore, very precise and sensitive quantitation methods are required.[183]

1.3.2.2 Tolerability of drug doses used in phenotyping cocktails and safety profiles of some proposed probes

Secondly, earlier cocktails contained probe drugs at therapeutic doses (refer to Table 1.2), contributing to possible side-effects especially considering drugs with narrow therapeutic indexes, like tolbutamide, warfarin and digoxin. Any small variation in enzyme or transport activity could contribute greatly to the disposition of drugs with a narrow therapeutic index causing severe adverse reactions. Possible side effects with therapeutic probe drug doses included hypotension with debrisoquin (CYP2D6 probe), hypoglycaemia with tolbutamide[184] (CYP2C9 probe), bleeding risk with warfarin (CYP2C9 probe, requiring co-administration of vitamin K) and gastrointestinal side effects and sedation with mephenytoin (CYP2C19).[185]

The incidence of side-effects has been largely eliminated since the introduction of low dose phenotyping cocktails, however they present pharmaceutical complications, because probe drugs are not commercially available at these low doses and have to be compounded from available dosage forms. Pharmaceutical considerations of low dose cocktails will be discussed in Section 1.3.4. More importantly low dose phenotyping cocktails require optimised, sensitive bioanalytical methods to detect low concentrations of metabolites in biological matrixes, especially when probe drugs and their metabolites, all with different physicochemical properties, are to be simultaneously quantified in a single run. This will be discussed further in Section 1.4.

An example of an ideal probe drug is flurbiprofen for phenotyping CYP2C9. It is almost exclusively metabolised by this enzyme, has a wide therapeutic window and is not dependent on urinary conjugation for excretion, therefore it has a much better safety profile than tolbutamide and warfarin[186] justifying its incorporation into the Pittsburg cocktail.[152]

1.3.2.3 Sample collection protocols and corresponding phenotyping measurements chosen for phenotype assessment

A third main challenge of current proposed phenotyping cocktails is the inconvenient and impractical sample collection protocols. Multiple time point venous plasma sampling or collective urine sampling would not be feasible in a routine clinical environment. Use of a single or limited time-point sampling strategy to measure metabolic or transporter activity would be advantageous especially when coupled to probe drugs with short elimination half-lives to reduce the time patients have to spend at the clinic for observation. Studies comparing systemic clearance (AUC) of probe drugs or the clearance ratio of probe drug to metabolite, to limited AUC or single point metabolic ratios are currently underway.[38, 187-191] No consensus has yet been reached and results are conflicting. In validating their Basel phenotyping cocktail, Donzelli *et al.* correlated the AUC_{0-24h} ratios for probe versus metabolite to a number of single time point plasma metabolic ratios (see Table 1.2) including a 2-hour single timepoint midazolam metabolic ratio (r^2 of 0.959). Yang *et al.* on the other hand found a 4-hour limited sampling AUC for midazolam and a 4-hour single time point concentration to best fit a two-compartmental population PK model, derived from 2122 observations from 152 healthy subjects, for estimation of CYP3A4 metabolic activity.[190] A 5-hour single time point plasma midazolam concentration[192] and limited sampling at 0.5, 2 and 6 hours for midazolam[187] have also been suggested. Similarly, many single time point paraxanthine over caffeine metabolic ratios have been shown to correlate to systemic clearance of caffeine, ranging from 2-hours[37], 4-hours[157], and 8-hours[38] post oral dose. Care should be taken in choosing the phenotyping measurement to infer metabolic or transport activity in different patient populations. Chosen phenotyping measures should be validated, correlate with enzyme or transport activity and represent change clearly under induction or inhibition conditions, account for confounding factors such as glomerular filtration rate or urinary pH and have low intra-individual variability.[35, 181] Intraindividual variability is usually lower with plasma sampling rather than urinary sampling. Recently, low dose cocktails, with limited alternative sampling methods coupled with sensitive bioanalytical quantitation, using dried blood spots, saliva and hair were investigated and are discussed in Section 1.3.3.

1.3.2.4 Pharmacokinetic, pharmacodynamic and bioanalytical interaction between probe drugs in simultaneous assessment of phenotype

An understanding of the PK and PD interaction between probe drugs used together in a cocktail approach is essential. The mechanisms of PK interactions have been explained in Sections 1.2.1.1 and 1.2.2.1. Interactions at the target receptor sites (PD interactions) should also be considered, for example using antihypertensives losartan and debrisoquin together might cause hypotension. Each probe drug used in a proposed cocktail must be validated individually and then in combination to exclude interaction with other probe drugs. In the Basel cocktail, chlorzoxazone (a CYP2E1 probe) had to be excluded due to a significant interaction with CYP3A4, significantly increasing midazolam $AUC_{0–24h}$ when administered together.[38] To overcome this, Blakey *et al.* administered the midazolam intravenously to exclude this intestinal CYP3A4 interaction with chlorzoxazone.[145] Although separate intravenous dosing is feasible during drug interaction studies and during drug development, it would be difficult to implement in clinical practice. Chlorzoxazone also interacts with CYP1A2 and when administered together with caffeine caused a 16–20% decrease in caffeine metabolism in urine and plasma.[193] Simultaneous probe drug and metabolite quantitation using bioanalytical methods requires optimisation due to different physicochemical properties to reduce competition for charge and to optimise individual extraction recovery, ionisation efficiency and detection limits.

1.3.3 Sampling alternatives and appropriate statistical analysis to assesses agreement to plasma sampling

Non-invasive sampling would be advantageous for implementing phenotyping in routine practice to obtain an estimation of metabolic or transport activity at baseline or to continuously assess causes of unexpected drug plasma concentration during treatment. Multiple time-point plasma sampling remains the gold standard for obtaining quantitative drug concentrations, but requires a phlebotomist, is invasive and not suitable for home sampling in TDM. As discussed, urine sampling, proposed in many cocktails, are non-invasive but confounded by sampling errors, urinary pH and glomerular filtration rate attributing to the high intraindividual variability found in dextromethorphan [194] and caffeine[195] urinary metabolic ratios. Metabolite to parent single point ratio's in urine also proved to be problematic in clinical trials where extrapolation into sound dosing guidelines is a necessity. Other non-invasive sampling strategies include measurement of the metabolic ratio between caffeine and paraxanthine in hair[196] or measuring xenobiotics in saliva as an alternative to plasma and serum in TDM[197]. Many studies have also

been published comparing minimal invasive sampling with a finger-prick and measurement in DBS instead of conventional plasma and urine sampling, for example measuring single point DBS metabolic ratios of flurbiprofen and its metabolite[198] or using DBS sampling for TDM of antiepileptic drugs[43] and antipsychotics.[199]

With regards to phenotyping cocktails, Bosilkovska *et al.* have shown that a dried blood spots (DBS) sampling technique can be used to simultaneously assess P-gp and CYP activity with a low-dose phenotyping cocktail and limited sampling to measure pharmacokinetic markers to measure phenotype.[162] Donzelli *et al.* further explored phenotyping using saliva in their Basel cocktail.[38] When using alternative sampling strategies in phenotyping metabolic or transport activity, it is important to consider the distribution of the expressed enzymes or transporters under investigation and the pharmacokinetic processes involved, i.e. absorption or excretion rates and drug distribution in different physiological compartments (influenced by physiological pH and dose-dependent protein binding as well as blood to plasma ratio in the case of DBS).

For DBS sampling, the question remains as to whether the quantitative bioanalysis of drugs in a capillary whole blood matrix correlates to that of the standard plasma/serum matrixes used as a reference in the current clinical environment. The composition of capillary blood differs from venous whole blood, with lower total protein, bilirubin, calcium and electrolytes measured in capillary blood.[200] Capillary blood is also routinely contaminated with interstitial fluid and intra-cellular fluid as a result of trauma at the puncture site. Quantitative concentrations between venous and capillary blood could be further influenced by excess squeezing of the finger after puncture. Small volume DBS samples also require sensitive analytical instruments for quantitation, such as LC-MS/MS and optimal extraction recovery. More importantly the partitioning of drugs between the cellular and water compartments in blood and plasma protein binding influence the measured unbound fraction in plasma and the erythrocyte-to-plasma concentration ratio.[201] Another major drawback of dried blood spot sampling (DBS) is haematocrit bias impacting quantitation,[202] requiring additional methods be put in place to correct and control for haematocrit bias.[45] The pharmacokinetic relationship at equilibrium between these factors is given by Equation 1.[201]

$$C_{plasma} = \frac{C_{blood}}{[(1 - H) + H.\rho.f_u]} \quad \text{(Equation 1)}$$

Where: C_{plasma} is the concentration of the drug in total plasma
C_{blood} is the concentration of the drug in total blood
H is the haematocrit
ρ is the erythrocyte-to-unbound plasma concentration ratio
f_u is the fraction unbound drug in plasma

Consider that: $$C_{plasma} = \frac{C_{unbound}}{f_u} \quad \text{(Equation 2)}$$

From Equations 1 and 2 it is clear that when f_u, ρ and H are constant, C_{blood} will be proportional to $C_{unbound}$ and for C_{plasma} to be proportional to $C_{unbound}$, f_u needs to be constant. Under these circumstances, measurements in either plasma or blood would be representative of the PK of a specific drug. It is the concentration of the unbound drug that drives the PK and PD of drugs.

Specifically, for a dried blood spot matrix, considering that the fraction of a given analyte bound to the erythrocyte is concentration-dependant (or change over exposure time), the relationship would be given by Equation 3.[203]

$$\left(\frac{DBS_{[analyte]}}{(1 - H)}\right) x\ (1 - f_{BC}) = Plasma_{[analyte]} \quad \text{(Equation 3)}$$

Where: $DBS_{[analyte]}$ is the concentration of the analyte/drug in the total blood spot
f_{BC} is the fraction of the analyte/drug bound to the erythrocytes
$Plasma_{[analyte]}$ is the concentration of the analyte/drug in plasma ($C_{unbound}$)

Rowland and Emmons describe a number of circumstances under which this hold true and outlines the importance of correcting for or accommodating the resulting variability when correlating data between different matrixes and ultimately relating them to $C_{unbound}$.[201]

However, the blood to plasma partitioning of analytes is rarely concentration or time dependant unless the drug is pharmacodynamically directed at the erythrocyte, for example antimalarials. Then a linear relationship exists between the concentrations of the analyte in blood and plasma and is given by Equation 4;

$$\left(\frac{DBS_{[analyte]}}{(1 - H)}\right) = Plasma_{[analyte]} \quad \text{(Equation 4)}$$

When choosing alternative sampling methods, care should be taken to ensure that the quantitative results obtained are reproducible, i.e. provides the same quantitative answer as measurements obtained from plasma sampling. A simple regression model between the two different sampling techniques will only explain a proportion of the variance between measurements and cannot account for missing values, will be greatly influenced by single outliers and is unable to predict whether the change in the dependant variables are a true reflection of the change in the independent variable.[204] When comparing two alternative clinical sampling methods to measure a phenotype, determining the correlation coefficient (r) or the coefficient of determination (r^2) between the two methods of measure may be misleading, because it does not assess statistical agreement between them.[205] Use of the Pearson correlation only explains how close the measurements taken with different methods best fit the regression line, but does not indicate agreement, i.e. whether the data fits to a line of equality (a 45-degree line through the origin).[206] Lin's concordance correlation coefficient corrects this by assessing both closeness of fit but also how far the regression line is from the line of true equality. Bland and Altman have shown the inappropriate use of correlation coefficient in a data set measuring peak expiratory flow rate using two different peak flow meters. Although a high correlation (r) between the two peak flow meters was evident, plotting the difference in measurement between the two peak flow meters against their mean measurement showed a considerable lack of statistical agreement between the two meters.[207]

The International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) founded a Microsampling Working Group (MWG). They recommend that a simple plot of DBS versus plasma concentration be done as an initial evaluation in bridging studies from *in vitro* to *in vivo* as the slope of the linear regression line should be in good agreement with the erythrocyte-to-unbound plasma concentration ratio found *in vitro*. Where the erythrocyte-to-unbound plasma concentration ratio is concentration or time dependant, this could be also visualised.[208] For different formats of the same matrix, i.e. DBS and "wet" whole blood a perfect correlation (1:1) is expected with Lin's concordance coefficients greater than 0.99, where the analyte recovery from the DBS is optimised and the analyte is stable in the DBS.[208] This is important during phenotyping cocktail validation where whole blood, collected via venepuncture from volunteers, is spiked with analyte before DBS sampling whereas blood will be collected peripherally during application of the cocktail in a patient cohort. Differences between the composition of venous and capillary blood, could be visualised by applying a Bland-Altman plot of the difference in quantitative measurement between the two sampling

methods (i.e. venous sampling versus peripheral sampling) against the mean obtained from both methods.[208] When perfect agreement exists between sampling methods the result should be zero.

Another index used to assess between-method agreement (reproducibility of results from two different sampling methods for example) is the intraclass correlation coefficient (ICC).[206] It represents the variance between two methods of measure proportional to the total variability in the measure and accounts for systematic error. A value between 0 and 1 is obtained and indicate no agreement (0) to full agreement (1).

Other statistical approaches recommended to graphically visualise agreement between plasma and DBS sampling are[208];

(a) plotting the percentage difference between the predicted analyte concentration in plasma, based on DBS measurements, against the true observed concentration in plasma. A difference larger than 20% indicates non-agreement and the necessity to implement a non-linear model in predicting plasma concentrations from DBS sampling.

(b) Plotting the ratio of analyte concentration in DBS and plasma against the true observed concentration in plasma. The resultant plot should be centered around a horizontal line consistent with the analyte erythrocyte-to-unbound plasma concentration ratio.

1.3.4 Rational for use of the Geneva phenotyping cocktail in a South African cohort and important considerations for use

Taking into account the advantages of using DBS in a South African context, unique population demographics and the ideal criteria phenotyping cocktails have to fulfil for clinical application, the already validated Geneva phenotyping cocktail was selected for this study. The cocktail consists of seven probe drugs that are easily available in South Africa, safe and tolerable at validated dosages with no interaction between probe drugs[209], contains two probes already applied to South African cohorts in a previous study[91, 210], established limited PK sampling and straightforward phenotyping measures[162] and with established enzyme and P-gp selectivity. The probe drugs and metabolites for six CYP450 metabolic pathways and the probe drug for studying P-gp activity are depicted in Figure 1.4.

The probe drugs however, have different physico-chemical properties that need to be considered during quantitative bioanalytical method validation, whilst compounding a low-

dose phenotyping cocktail capsule from therapeutic commercially available drugs pose further pharmaceutical challenges.

1.3.4.1 Bioanalytical considerations for implementation of Geneva cocktail

Differences in the physicochemical properties of probe drugs present a challenge in developing a single extraction and injection method for analysis with LC-MS/MS. In the 7-drug Geneva cocktail, for example, omeprazole will degrade at acidic pH levels[211], bupropion in its free base form undergoes first order catalysis by the hydroxide ions present in aqueous solution at pH above 5[212] and midazolam undergoes ring closure rendering it more lipophilic at pH above 4.[213] This will have an influence on the sample storage conditions, sample preparation and LC-MS/MS conditions. Some of the physicochemical properties of the Geneva cocktail probe drugs and their metabolites are given in Table 1.4

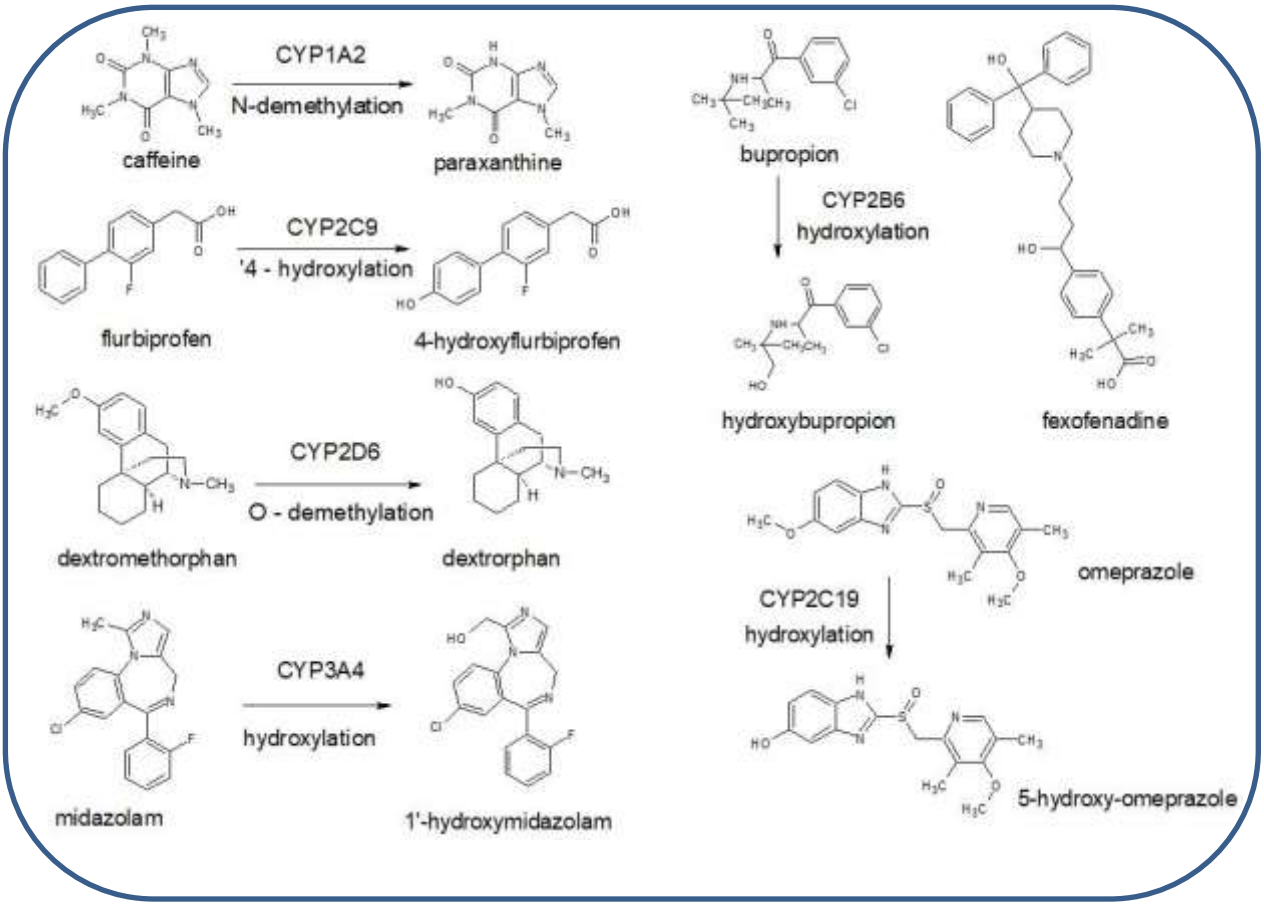


Figure 1.4: Metabolic pathways of the 7-drug Geneva phenotyping cocktail (Structures drawn with ChemSketch[214])

Table 1.4: Physicochemical properties of the Geneva phenotyping cocktail probe drugs and their metabolites

Probe drugs and metabolites	Log P	pKa	Proton acceptor count	Proton donor count
Caffeine	-0.07	14.00	3	0
Paraxanthine	0.24	10.76	3	1
Bupropion	3.28	8.22	2	1
Hydroxybupropion	2.22	14.79	3	2
Flurbiprofen	3.94	4.42	2	1
4'-Hydroxyflurbiprofen	3.64	4.24	3	2
Dextromethorphan	3.49	9.85	2	0
Dextrorphan	2.90	10.46	2	1
Omeprazole	2.43	7.40 & 14.7	5	1
5'-Hydroxyomeprazole	1.15	4.80	6	2
Midazolam	3.33	5.50	2	0
α-Hydroxymidazolam	2.48	13.95	3	1
Fexofenadine	2.94	4.04	5	3

The log P value is an indication of the hydrophobicity of an analyte, whilst the pK_a value indicate the relative acid strength of the analyte in solution. In the 2004 Geneva phenotyping cocktail that consisted of 5 probe drugs, caffeine (neutral drug), flurbiprofen (acidic drug), omeprazole, dextromethorphan and midazolam (basic drugs), four different extraction methods (protein precipitation (PPT) or liquid-liquid extraction (LLE)) were used to improve the extraction efficiencies[155] of all analytes based on their physicochemical properties, however in their latest 7-drug cocktail a single non-discriminating PPT method with ACN for plasma samples and MeOH for DBS, containing no buffers or additives was used. The authors reported sufficient selectivity with a 5500 Qtrap® mass spectrometer. However, when working with ionisable analytes, the development of a rugged and reproducible method with consistent resolution, peak shape and selectivity warrants control of mobile phase pH. Availability of new instrumentation is also limited in the developing world and often older, less sensitive instruments are used for analysis.[215]

1.3.4.2 Pharmaceutical considerations in compounding a low-dose cocktail capsule

Phenotyping cocktail probe drugs are not available at the low dosages necessary for phenotyping and have to be compounded from commercially available dosage forms. This presents difficulties with dosage homogeneity, interference by excipients in commercial dosage forms, differences in release kinetics, potential chemical degradation or incompatibilities of drugs in combination and differences in the pharmaceutical properties of the probe drugs. Additionally, hand-filling is used to produce small batches of capsules for use, with larger variances in dosage homogeneity than with automated filling. It is important to assess the quality of any compounded cocktail capsule before administration to patient cohorts.

1.4 LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS) FOR THE SIMULTANEOUS QUANTIFICATION OF METABOLITES IN BIOLOGICAL MATRICES

Liquid chromatography tandem mass spectrometry is an analytical research methodology that has shown tremendous growth in published papers in recent years with wide practical applications such as pharmaceutical metabolite analysis in urban waste water[216], food safety[217], toxicology[218], metabolomics[219] and proteomics[220]. Multidrug cocktails often consist of related substituted aromatic and polycyclic pharmaceutical analytes that may require more selective separation to avoid isobaric interferences and matrix effects. Improved analytical sensitivity is required for low-dose cocktails when coupled with low volume dried blood spot sampling procedures, to accurately detect and quantify a given drug-to-metabolite concentration ratio or drug AUC related to *in vivo* metabolic or transport phenotype. Most importantly the bioanalytical method has to be optimised to maximise process efficiency of all analytes, with different physicochemical properties, combined in a multidrug cocktail. LC-MS/MS is the quantitative method used in most recent cocktails due to its sensitive, selective and high-throughput capabilities.[221]

Chromatography is a separation methodology where analytes in a mixture are separated based on their distribution between a stationary phase and liquid mobile phase due to differences in their physicochemical properties. It also allows for separation of poorly retained matrix components from the analytes under investigation. Reversed phase chromatography, where the stationary phase is non-polar and the mobile phase more polar, has been widely applied to the

separation of small molecules including pharmaceuticals, peptides and proteins.[222] The principles of reversed phase liquid chromatography will be discussed in Section 1.4.1.

Mass spectrometry has the power of identifying analytes based on their mass fingerprint with a high degree of certainty and when coupled to upstream chromatographic separation could successfully quantify different analytes in a mixture. In tandem mass spectrometry (MS/MS or MS^e) experiments more than one step of mass fingerprinting is used to identify analytes with higher selectivity and sensitivity, monitoring the transition of a precursor mass ion from a given analyte to its unique product ion mass fragments. Different types of MS instruments, with MS/MS capability, have been developed for quantitation, including triple quadrupole, high resolution ion trap and quadrupole time of flight instruments.[223] Although the latter two provide excellent resolution, they lack the sensitivity when compared to triple quadrupole instruments. Sargent *et al.* compared the main types of LC-MS instruments showing all the benefits and features as well as the disadvantages of different instruments.[221]

The two interfaces, liquid chromatography and mass spectrometry, are linked by the ionisation source of the mass spectrometer, that simultaneously desolve the liquid phase by added heat or a nebulising gas and ionises (adds charge to) the analytes for detection by the mass spectrometer. Different ionisation sources have been developed, atmospheric pressure chemical ionisation, atmospheric pressure photo-ionisation and electrospray ionisation (ESI).[223] ESI is a sensitive technique for the ionisation of polar analytes with the added advantage of yielding multiply charged ions useful for the analysis of larger peptides and proteins. The mobile phase is sprayed into a strong electrostatic field to create charged droplets[224] and the ionisation efficiency is enhanced when analytes are already present in their ionised form in solution prior to introduction to the ESI source.[225] The major draw-back of ESI is that co-eluting compounds in the matrix might enhance or suppress the ionisation of the analyte to be quantified, but by optimising the chromatographic separation gradient to shift retention times of interfering matrix components, matrix effects could be largely overcome. Alternatively, sample clean-up, addition of an internal standard (IS) or matrix matched calibration curves could compensate for an observed matrix effect.[226] APCI on the other hand is less sensitive to matrix effects than ESI, but generates only singly charged ions. It is useful for the ionisation of less polar analytes. APPI has limited clinical applicability, but could be used for non-polar analytes that cannot be ionised by either ESI or APCI.[223]

From the LC-MS/MS experiment a total ion chromatogram is obtained in arbitrary units of signal intensity over time. Each chromatographic peak represents an analyte detected by the mass spectrometer at a given time based on its mass fingerprint.[221] This is visualised in Figure 1.5. The mass spectrum of each detected analyte represents the mass to charge ratio (m/z) of the precursor and product ions at specific mass spectrometric conditions. If one charge is added to a precursor ion the m/z value is written as M+1, where M is the molecular mass of the analyte. Mass spectrometers are also able to distinguish between co-eluting analytes based on their unique mass fingerprints and mass transitions.

Before the application of LC-MS/MS to quantify *in vivo* drug concentrations (to be used for assessing phenotype) in biological matrices is discussed (Section 1.4.3), an overview of the basic chromatographic parameters and principles of retention in reversed phase chromatography will be emphasised (Section 1.4.1). Thereafter the importance of choosing the optimal stationary and mobile phase composition in separation of a diverse mixture of analytes with different physicochemical properties will be considered (Section 1.4.2).

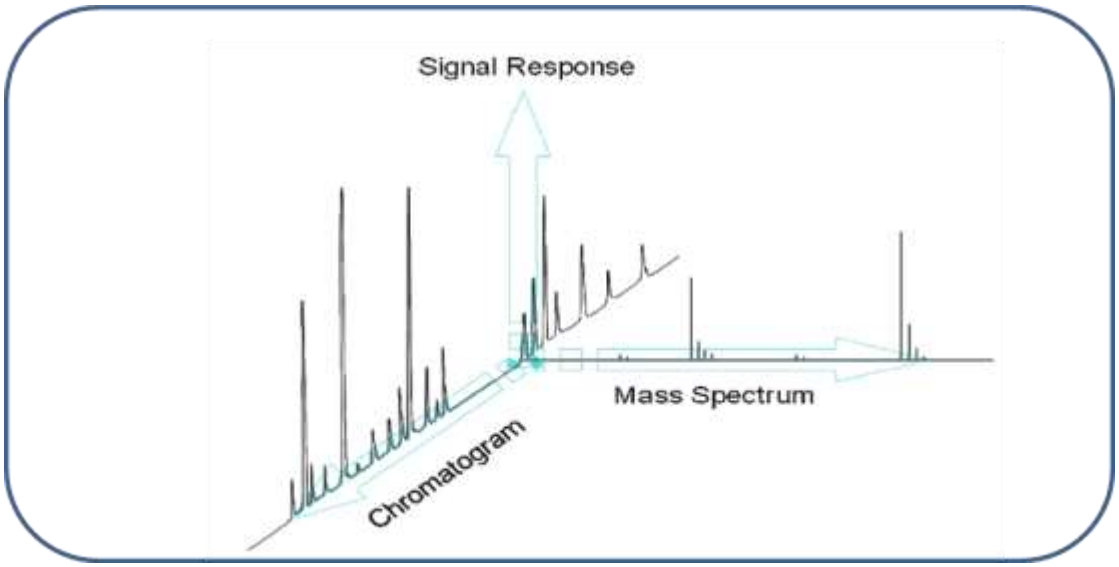


Figure 1.5: Graphical presentation of liquid chromatography tandem mass spectrometry detection. Copyright LCGC Limited © 2013. Used with permission.[221]

1.4.1 Theory and principles of retention in reversed phase chromatography

In chromatographic terms, optimum resolution should be achieved with baseline separation between analytes to eliminate false positives or negatives during quantitation. The resolution of an analyte is influenced by many factors and the resolution equation is given by[227]:

$$R_s = 1/4\sqrt{N}x\frac{\alpha - 1}{\alpha}x\frac{k}{1+k} \qquad \text{(Equation 5)}$$

Where: **R_s** is the resolution between any two peaks on a chromatogram (i.e. degree of differentiation between them)
N is the number of theoretical plates measured for an analyte (efficiency of a chromatographic peak) and indicates the analyte's dispersion through the system
α is the selectivity (separation) between two adjacent peaks and indicates capability of the column to chemically differentiate between two analytes
k is the retention (capacity) factor measured as the retention time (t_R) of an analyte relative to an unretained analyte or the baseline disturbance observed in the chromatogram as the solvent passes through the mass detector. (t_0)

The impact of each parameter on the overall resolution is shown in Figure 1.6. To accurately quantify the amount of an analyte, present in a mixture, it is preferable that it be separated from all other analytes of interest in the mixture. All of the factors in the resolution equation could be manipulated to obtain the desired resolution. In general, longer well packed columns will increase N, whereas k could be altered by changing the polarity of the mobile phase. Ultimately the quality of the separation is optimal where k is between 1 and 5 as this is where k has greatest effect on the overall resolution. The retention factor range is, however, extended to between 2 and 10 for complex mixtures. Still, the most effective tool for changing and optimising resolution is by altering the selectivity factor, since it is dependent on many different factors, i.e. analyte chemistry, choice of both stationary and mobile phase (especially the organic phase), solvent pH (applicable to ionisable analytes), solvent strength and composition and column temperature.[222] Optimisation of selectivity by changing stationary or mobile phase type, composition and pH will be discussed further in Section 1.4.2.

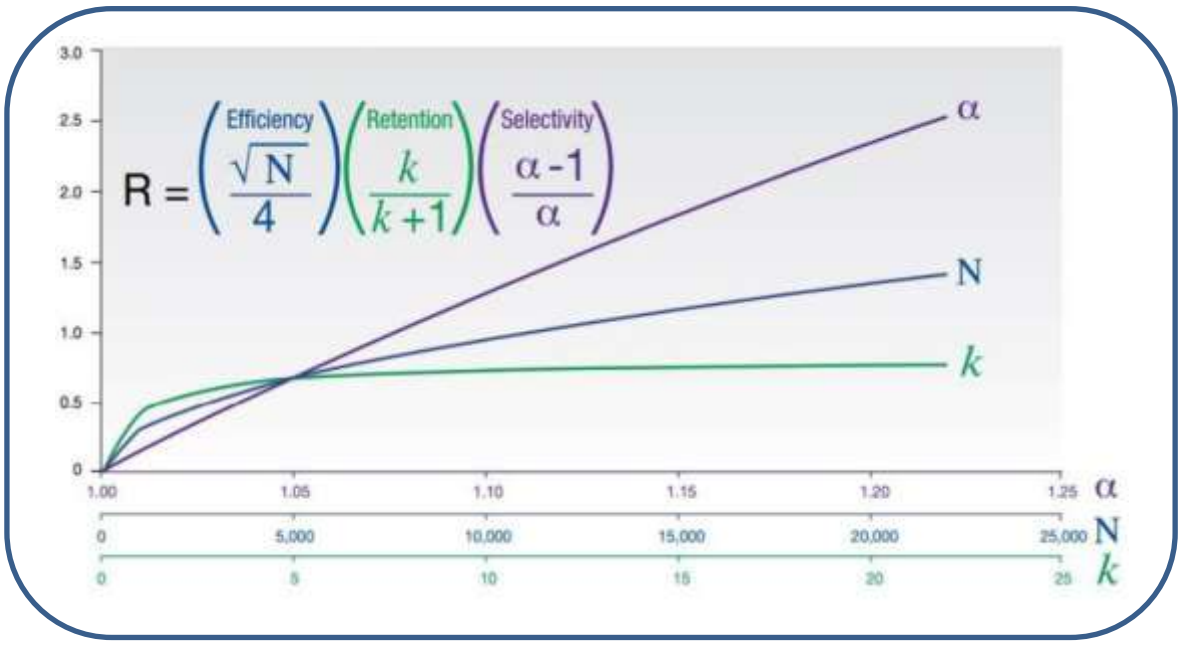


Figure 1.6: Impact of efficiency, retention and selectivity on the overall resolution of the chromatographic separation. Copyright © www.chromacademy.com. Used with permission.

In reversed phase chromatography the main criterion driving retention is the hydrophobicity of an analyte, which is determined by the degree of partitioning of an analyte between two immiscible solvents. Under standard conditions, using water and octanol, it is given by Equation 6.[228]

$$\boldsymbol{Log\ P_{octanol/water} = log\left(\frac{[solute]_{octanol}}{[solute]_{deionised\ H2O}}\right)} \qquad \text{(Equation 6)}$$

Where: $Log\ P_{octanol/water}$ is the logarithm of the partitioning of an analyte in its neutral form between octanol and water
$[solute]_{octanol}$ is the concentration of the analyte dissolved in octanol
$[solute]_{deionised\ H2O}$ is the concentration of the analyte dissolved in the aqueous phase, deionised water

Thus, a non-polar stationary phase (bonded to surface silica particles) will have greater affinity for more hydrophobic analytes, with higher Log P values, which will be better retained. On the other hand, less hydrophobic analytes will partition into a more polar mobile phase and they will be less retained on the stationary phase. The elution strength of the mobile phase will increase with decreased polarity. Water (the aqueous phase) is most polar and will repel hydrophobic analytes into the stationary phase, but as the mobile phase composition becomes

more non-polar (most commonly with methanol and/or acetonitrile) partitioning into the mobile phase will increase, the analyte will spend less time partitioned into the stationary phase and will elute earlier. The retention of an analyte can be altered by changing the elution strength of the mobile phase. On the other hand, less hydrophobic analytes will partition into a more polar mobile phase and they will be less retained on the stationary phase. The elution strength of the mobile phase will increase with decreased polarity. Water (the aqueous phase) is most polar and will repel hydrophobic analytes into the stationary phase, but as the mobile phase composition becomes more non-polar (most commonly with methanol and/or acetonitrile) partitioning into the mobile phase will increase, the analyte will spend less time partitioned into the stationary phase and will elute earlier. The retention of an analyte can thus, be altered by changing the elution strength of the mobile phase.

Other indicators of retention behaviour in reversed phase chromatography include analyte chemical structure and polarity. In general, elution order from most retained to least retained, is aliphatic compounds (with hydrophobicity increasing with carbon content), followed by compounds containing dipoles, weak Lewis bases, strong Lewis bases, weak Lewis acids and lastly strong Lewis acids. Weak Lewis bases (electron pair donors) include ketones, aldehydes and esters, whereas amines are examples of strong Lewis bases. Weak Lewis acids (electron pair acceptors) are phenols and alcohols, whereas carboxylic acid is a strong Lewis acid. Alternative column chemistries may significantly influence the elution order based on orthogonal interaction mechanisms between analytes and the stationary phase (See Section 1.4.2).

It is also important to note that the retention of ionisable analytes will change as a function of the pH of the mobile phase as they contain functional groups that easily dissociate to yield positive or negative ions. For any given analyte, when it is in its ionised state, it will be more polar and will be less retained on a reversed phase column. The state of ionisation will dependent on the pK_a of a given analyte. The relationship between analyte pK_a and pH is described by the Henderson-Hasselbach equation.[229] Ideally, the pH of the mobile phase should be 2 pH units above the pK_a for weak bases or 2 pH units below the pK_a for weak acids in order to force the drug to be in its unionised state rendering better retention times on reversed phase columns in HPLC analysis. Figure 1.7 gives a schematic representation of the degree of ionisation of acids and bases at different pH conditions. Basic analytes are neutral when deprotonated [B] and acids when protonated [HA] and will be more retained on a reverse phase column due to their more hydrophobic nature in this state. When a base is protonated [BH^+]

and an acid deprotonated [A^-] on the other hand, they are more hydrophilic in nature, will be less retained on a reverse phase column and elute earlier.[230]

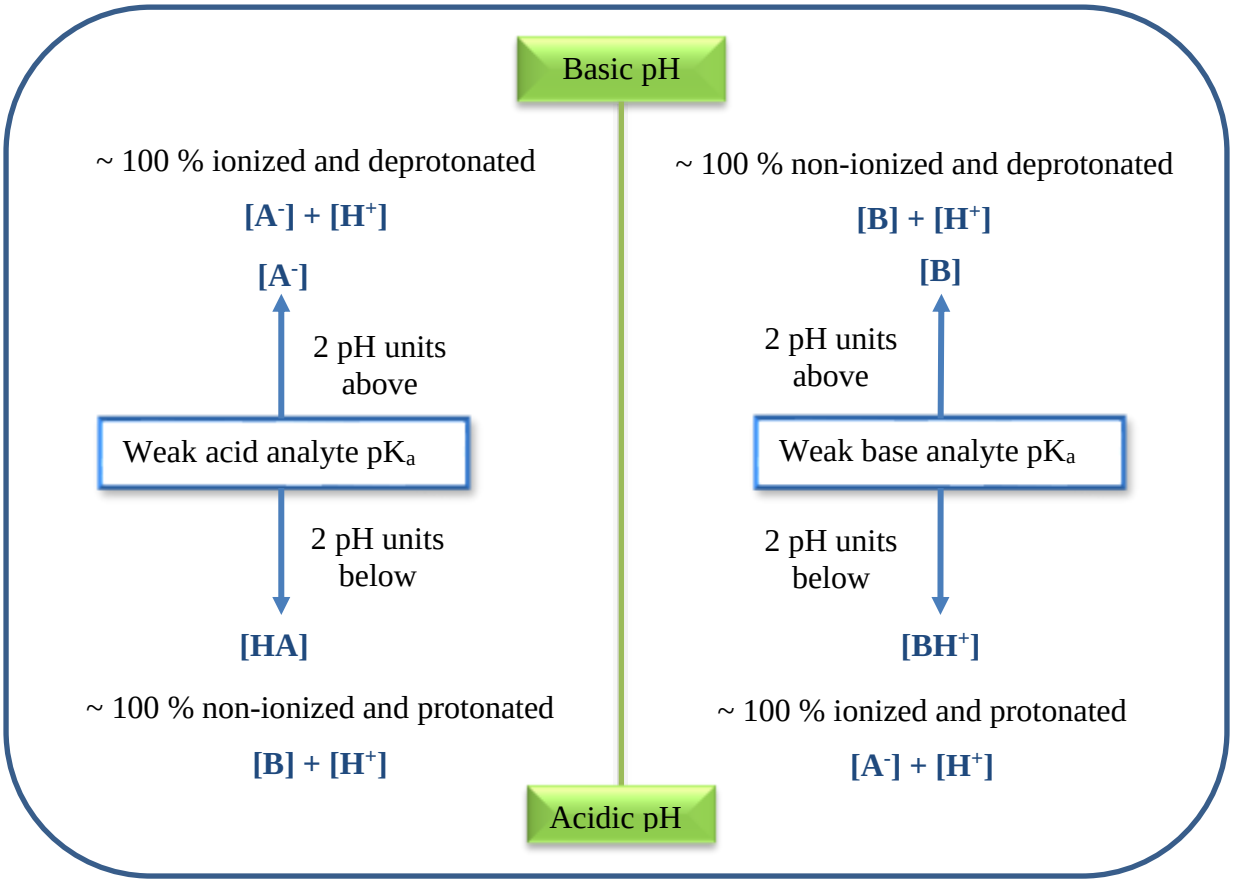


Figure 1.7: Ionisation of weak acids and bases as a function of change in pH

When ionisable analytes are present the pH of the mobile phase needs to be controlled to ensure reproducible retention times and to improve peak shape. However, when combining a number of analytes with markedly different physicochemical properties this becomes more difficult. To optimise the separation selectivity in a complex analyte mixture of ionisable analytes, retention factors at different mobile phase pH conditions may be plotted during method development. Where pK_a values differ, the retention versus pH plots will vary from one analyte in the mixture to the next and significant changes in selectivity and retention might be possible with a small change in the pH of the mobile phase, particularly if the pH is within 1 unit of the analyte pK_a.

1.4.2 Stationary and mobile phase considerations during liquid chromatography tandem mass spectrometry method optimisation

Analyte selectivity and retention will be affected by both the choice of organic eluent (type and composition) and column (stationary phase) selection and must be considered during development of a new method for quantitation. During method optimisation the ruggedness/robustness of the method may be assessed by altering the stationary phase or mobile phase pH and composition.[231, 232] When changes in these analytical conditions cause statistically significant variation in the retention behaviour, selectivity, efficiency and sensitivity of the analytes of interest, they should be suitably controlled to yield reliable, reproducible quantitative results.

In reversed phase chromatography the ideal retention factor range for all analytes present in a complex mixture should be between two and 10 as discussed above. The reason for this being that analytes with retention factors below two risk co-elution with poorly retained matrix components, whereas analytes with retention factors above ten have increased band-broadening with accompanying reduction in column efficiency. The type and composition of the organic eluent, chosen for the separation, should deliver k' values within the preferred range. In general analyte retention time will be reduced with an increase in organic eluent percentage (eluent strength). The three main organic eluents used in LC-MS/MS are acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF). All three are miscible with water and volatile, increasing their usefulness in ESI-LC-MS/MS, but they differ according to their elution strength, with THF > ACN > MeOH, which is inversely proportional to their polarity, as well as their solubility and viscosity. Aqueous mixtures of each of the three organic phases have altered viscosity that must be taken into account in a LC system as it increases system backpressure. Furthermore, ACN contains a triple bond between the carbon and nitrogen atom resulting in a molecular dipole preferable for the retention of polarisable analytes. Methanol contains an alcohol functional group increasing its acidic nature, preferable for the retention of basic analytes, but with the risk of forming methyl esters with Lewis acids. Use of THF has been limited by its potentially hazardous nature (forms explosive peroxide species in reaction with oxygen) and carcinogenicity. Organic eluents may also be combined in different combinations to alter selectivity. If a complex analyte mixture includes ionisable analytes the pH of the mobile eluent, that affects the state of ionisation, should also be considered (see Section 1.4.1). The pH of the eluent may be stabilised by adding buffers. [231] The first-choice mobile phase additive recommended as a starting point for LC-MS method development is 0.1% formic acid (pH 2.7),

providing robust methods with stable selectivity and retention times. Additionally, at this pH the free silanol groups in silica phase columns are neutralised, improving peak shape and reducing peak tailing (this is especially important for basic analytes). At this pH, acidic analytes are in their unionised form, increasing retention and reducing interference with early eluting matrix components.

Retention and selectivity may also be altered by choosing a different column chemistry during LC-MS/MS method development.[233] The three main interactions driving separation between analytes and a specific stationary phase, are dispersive, ionic and polar interactions. The most significant interaction is dispersion, driving separation to some extend for most organic analytes where the hydrophobicity of the molecule dictates its retention time on the column. Alkyl phases, such as C18 and C8 mostly use dispersion as their retention mechanism. These are temporary weak van der Waals interactions that collectively drive partitioning between the stationary and mobile phase. Ionic interactions on the other hand provide strong longer lasting interactions between the stationary phase and interacting analyte. Polar interactions stem from π-π interactions, dipole interactions or hydrogen bond formation between the stationary phase and analyte. Polar steric interactions also provide spatial selectivity since the spatial arrangement of the analyte will influence its capacity to form hydrogen bonds with the stationary phase especially with rigid aromatic embedded stationary phases such as phenyl phases.

C18 columns have been the cornerstone of most pharmaceutical separations, but when the desired resolution cannot be obtained, alternative column chemistries may be explored to better define the differences between analytes and change their selectivity. Traditional alkyl phases perform well for separation of mixtures that vary marginally in polarity, but falls short when dispersive interactions are insufficient to separate mixture of highly variable polarities[233], such as mixtures of lipophilic pharmaceutical probe drugs and their more polar metabolites present in the same biological matrix.

Most pharmaceutical drugs and their metabolites have aromatic structures that differ in their pK_a values and structure that might benefit from orthogonal column selectivity such as aromatic phases. For most aromatic analytes charge transfer interactions or π-π interactions will drive the retention profile when a phenyl phase column is used. When combined with a methanolic mobile phase, the π-π interactions are favoured and enhanced. Other interactions are electrostatic or dipole interactions and hydrogen bonding driving the separation.[234] Table 1.5

summarises the main types of column chemistries based on their retention mechanisms and their relative contribution to the separation.[235]

Table 1.5: Comparison of retention mechanisms of different column chemistries

Column type		**Retention mechanism**	**Contribution to separation**
Linear alkyl silane	C4, C8, C18	Hydrophobic interactions	Strong to very strong for C18
		Ion exchange interactions where free silanols are negatively charged (usually at higher pH).	Moderate
Polar embedded	Carbamate, amide, urea etc.	Hydrophobic interactions	Strong
		H-bond formation	Strong acceptor
		Dipolar interactions	Moderate
Cyano		Hydrophobic interactions	Moderate
		Dipole-dipole interactions	Strong
Aromatic	Phenyl (Lewis base)	Hydrophobic interactions	Strong
		π-π Interactions	Strong donor
		Steric interactions	Strong (rigid)
	Pentafluoro phenol (PFP) (Lewis acid)	Hydrophobic interactions	Moderate
		H-bond formation	Moderate acceptor
		Dipole-dipole interactions	Strong
		π-π Interactions	Strong acceptor
		Steric interactions	Strong (rigid)
		Ionic interactions with free silica surface and Lewis acid PFP ring	Strong

A simple approach to explore alternative column selectivity is by plotting the logarithm of the retention factors, obtained under the same conditions, obtained on an alternative column against the reference column, usually C18.[236] The degree of scatter around the regression line as well as the slope of the regression line demonstrate orthogonality between the two different column chemistries with a high degree of scatter indicative of greater selectivity.

A number of models have been developed to predict similarities or orthogonality between different columns to assist chromatographers in initial column selection during method development, i.e. the hydrophobic subtraction model (HSM), quantitative structure retention relationship model, linear solvation energy relationship test, Engelhardt's and Tanaka's test which have been reviewed recently.[236]

1.4.3 Application of orthogonal column chemistry and different mobile phase compositions to the Geneva cocktail

The analytes in the chosen Geneva phenotyping cocktail are all related substituted aromatic analytes with both electron withdrawing and electron donating functional groups that render them amphoteric and should affect their ionisation behaviour at different pH levels and hence their chromatographic selectivity. The aromatic ring is electron dense and acts as an electron donor (Lewis base). A biphenyl stationary phase has the advantage of secondary pi-pi interactions that occur between the pi electrons of the phenyl group and those of the solute species such as aromatic pharmaceutical molecules. This can be advantageous in optimisation of methods when considering the physical and chemical characteristics of the analytes of interest.

CHAPTER 2: AIMS AND OBJECTIVES, STUDY DESIGN, CHEMICALS AND REAGENTS AND INSTRUMENTATION

2.1 INTRODUCTION

The purpose of the current study was to compound the validated "Geneva phenotyping drug cocktail", from available API sources and develop a validated analytical method for testing the seven drugs and their respective metabolites in limited plasma volumes when using the Mitra™ volumetric absorptive microsampling device for blood collection. This phenotyping assay is to be used in future studies of South African patient cohorts to assist clinicians in identifying patients susceptible to adverse drug effects or therapeutic failure, getting closer to practical personalised medicine.[237]

2.2 AIM OF THE STUDY

The aim of the study was to assess inter-method agreement of measured probe drug and metabolite concentrations (to be used to assess phenotype) between a low sample volume from a dried blood spot equivalent, known as a volumetric absorptive microsampling device, and conventional plasma sampling when administering the "Geneva phenotyping cocktail". A cocktail approach is used to simultaneously quantifying drug to metabolite ratios to assess *in vivo* CYP450 metabolic activity of the CYP1A2, -2B6, -2C9, -2C19, -2D6 and -3A4 enzymes and P-gp transport activity.

2.3 OBJECTIVES

The objectives of the study were to:

- Optimise an LC-MS/MS method using an alternative biphenyl stationary phase analytical column, comparing analyte selectivity of a biphenyl stationary phase analytical column to that of a traditional C18 stationary phase used traditionally in phenotyping assessments (Chapter 3).
- Evaluation of the influence of different solvents, different mobile-phase compositions and different pH on the ionisation efficiency of the analytes and their metabolites during separation on a biphenyl stationary phase (Chapter 3).

- Compound the phenotyping cocktail into capsules containing 6 of the seven probe drugs from available active pharmaceutical ingredients (API) or available drug formulations on the market (where API's were not available as pure compounds), with a hand-filler after homogenisation, followed by weight and content uniformity assays using an ultra-performance liquid chromatography tandem quadrupole time of flight mass spectrometry (UPLC-qTOF-MS/MS) method (Chapter 4).

- Validate the newly optimised LC-MS/MS method according to ICH[231] guidelines in dried blood spots, sampled with the Mitra™ DBS sampling device and human plasma using an HPLC system coupled to a mass spectrometer (Chapter 5).

- Graphically visualise agreement between plasma and DBS sampling, under different DBS extraction conditions, using known spiked concentrations across the three non-sequential validation days (Chapter 5).

2.4 STUDY DESIGN

The experimental study design, statistical data analysis, results and discussion and conclusion for each of the study objectives in Section 2.3, are outlined in Chapters 3 - 5, followed by an overall conclusion of the study including perspectives for further research and study limitations in Chapter 6.

2.5 ETHICAL AND LEGAL CONSIDERATIONS

The University of Pretoria Research and Ethics Committee, Faculty of Health Sciences, granted written approval for this study (Annexure I). An outline of the ethical and legal aspects pertaining to the proposed study is given below and deals with informed consent, risk and benefit analysis and regulatory considerations.

2.5.1 Informed consent

All participants were recruited on a voluntary basis from apparently healthy males and females from all race groups, older than 18 years but less than 65 years of age who were deemed to understand the study procedures and outcomes. The only exclusion criterium was that volunteers had to abstain from drinking any caffeine containing substances 72 hours prior to sampling. Voluntary informed written consent was obtained from all research participants prior

to the collection of donor blood samples used for method development and validation. The information leaflet and consent form are provided in Appendix II.

The compounded phenotyping cocktail was administered to one patient volunteer in a proof of concept pilot study. The pilot study objectives and procedures including the drug names and their effects and how the pharmacokinetic sampling was to take place were discussed with the volunteer, after which a copy of the information leaflet was given to the volunteer and the opportunity provided to ask questions. The information leaflet and consent form for the pilot PK study are provided in Appendix III.

2.5.2 Risk/benefit analysis

The probe drug concentrations incorporated into the validated phenotyping cocktail are used internationally [37], are well below therapeutic doses (Table 2.1) and subsequently have a low potential side-effect profile.

Table 2.1 Phenotyping probe drug doses, metabolites, suggested phenotyping metrics used to predict *in vivo* activity and normal daily doses used during treatment.

Probe drug	Oral Dosage	Metabolite	Phenotyping metric	Normal daily doses
CAF	50 mg	PAR	[par]/[caf] at 2.5 hours	Up to 400 mg per day
BUP	20 mg	OH-BUP	[OH-bup]/[bup] at 3 hours	150 – 300 mg
FLB	10 mg	OH-FLB	[OH-flb]/[flb] at 3 hours	200 – 300 mg
DEX	10 mg	DTP	[dor]/[dem] at 3 hours	90 – 120 mg
OPZ	10 mg	OH-OPZ	Limited sampling $AUC_{1,2.5,3,3.5OH\text{-}opz}$/ $AUC_{1,2.5,3,3.5\ opz}$	10 – 20 mg
MDZ	1 mg	OH-MDZ	[OH-mdz]/[mdz] at 2.5 hours	7.5 – 15 mg
FEX	25 mg	Not applicable	limited-sampling $AUC_{1,2.5,3,3.5}$	120 – 180 mg

Where CAF – caffeine, BUP – bupropion, FLB – flurbiprofen, DEX – dextromethorphan, OPZ – omeprazole, MDZ – midazolam, FEX – fexofenadine, AUC – Area under the plasma concentration-time curve.

Table 2.2 summarises the probe drug doses administered in mg per kilogram body mass per day and is based on an average body weight of 70 kg.

Table 2.2 Oral phenotyping dosages in mg/kg/day compared to normal daily therapeutic doses

Probe drug	Probe for	Oral Phenotyping dose in mg/kg/day	Normal daily dose in mg/kg/day
Caffeine (CAF)	CYP 1A2	0.715	5.71
Bupropion (BUP)	CYP 2B6	0.286	2.14 – 4.29
Flurbiprofen (FLB)	CYP 2C8	0.143	2.85 – 4.29
Dextromethorphan (DEX)	CYP 2D6	0.143	1.28 – 1.71
Omeprazole (OPZ)	CYP 2C19	0.143	0.14 – 0.29
Midazolam (MDZ)	CYP 3A4	0.014	0.11 – 0.21
Fexofenadine (FEX)	P-gP	0.357	1.71 – 2.57

2.5.3 Regulatory considerations

Probe drug "cocktails" are not registered as drug cocktails or for any indications but fall into the therapeutic drug class due to the APIs in the combined mixture and as such, they are validated in literature and scrutinised for potential pharmacokinetic DDI's, with no reported interactions found in the Geneva phenotyping cocktail used in this study. Interactions between probe drug substrates are minimised by administering low doses and using very sensitive analytical methods for simultaneous quantification of all drug precursors and metabolites (discussed in Chapter 1). No fatalities have ever been reported in literature when using validated phenotyping cocktails to assess *in vivo* phenotypes for CYP metabolic activity and P-gp transport. [36-38, 152, 153, 157, 161]. The rational for choosing this cocktail for future application in a South African cohort is discussed in Chapter 1 (see Section 1.3.4).

2.6 CHEMICALS AND REAGENTS

Reference standards caffeine (batch # BCBR6677V), bupropion as hydrochloric salt (batch # 063M4707V), flurbiprofen (batch # SLBD4598V), hydroxy-omeprazole (batch # BCBS0382V), dextromethorphan (batch # SLBQ0513V) and dextrorphan (batch # 065K3257) were purchased from Sigma Aldrich (Pty) Ltd. (Johannesburg, South Africa), paraxanthine (batch # FN11121501), hydroxy-bupropion (batch # FN0213150Z), omeprazole (batch #

FN02201501) and α-hydroxy-midazolam (batch # FN02041502) from Cerilliant (Pty) Ltd. (Texas, USA) supplied by Sigma-Aldrich (Cambridge, UK) and 4-hydroxy-flurbiprofen (batch # CRC-0151-048-F) and fexofenadine (batch # S-FF-516) from Clearsynth (Pty) Ltd. (Mumbai, India). Midazolam (batch # F1058F03, Roche) was obtained as Dormicum™ 15 mg.3 mL^{-1} ampoules from a local hospital pharmacy. Internal standards (IS) imipramine (batch # 107K0697) for positive mode was supplied by Sigma Aldrich (Pty) Ltd. (Johannesburg, South Africa) and probenecid as European Pharmacopoeia standard by Cayman Chemicals (Ann Harbour, MI, USA)

All solvents used during sample preparation and chromatography were HPLC grade. Acetonitrile (Romil® purity >99.9%), methanol (Romil® purity >99.9%) and Romil® HPLC-water were purchased from Microsep (Pty) Ltd. (Johannesburg, South-Africa). Analytical grade formic acid (purity ≥98%), ammonium formate (batch # MKCF2569), ammonium acetate (batch # 15398/4773) and ammonium bicarbonate (batch # 060M0177V) were obtained from Sigma Aldrich (Pty) Ltd. (Johannesburg, South Africa). In-house double deionised pyrogen-free water (>18 MΩ and < 5 ppm TOC), used during sample preparation, was produced using an ELGA Genetics water purification unit (ELGA, Wycombe, UK) housed in the Department of Pharmacology.

The phenotyping cocktail capsules, containing six of the seven probe drugs were compounded from dextromethorphan, caffeine and flurbiprofen available as analytical standards and commercially available pharmaceuticals supplied by a local pharmacy as follows:

Bupropion as bupropion hydrochloride in Wellbutrin XL™ 300 mg tablets (batch # B00618F, Glaxo), midazolam as midazolam maleate in Dormicum™ 15 mg tablets (batch # X4996B01, Roche) and fexofenadine as fexofenadine hydrochloride in Fexo™ 180 mg tablets (batch # 77083, Cipla). Omeprazole was administered separately as commercially available 10 mg Omez™ capsules, due to problems with stability in combined formulations.

Kinetex™ C18 and Kinetex™ Biphenyl 100 mm × 2.1 mm, 2.6-µm columns were purchased from Phenomenex Inc. (Johannesburg, South-Africa). The Mitra™ (CE/IVD) microsampling devices (batch # 60709A-P5L3-16238) were imported from Neoteryx (Torrance, CA, USA).

2.7 INSTRUMENTATION

Both a triple quadrupole liquid chromatography tandem mass spectrometry (LC-MS/MS) system and an ultra-performance liquid chromatography tandem quadrupole time of flight mass spectrometry (UPLC-QTOF-MS/MS) system were used during this study. The triple quadrupole LC-MS/MS system were used during development and validation of the quantitative method for the detection of low concentrations of phenotyping probe drugs and their metabolites in low volume plasma and dried blood spot samples, whereas the UPLC-QTOF-MS/MS system was used to determine the content uniformity of the phenotyping cocktail capsules after compounding. Both mass spectrometer systems were co-funded by the National Research Foundation (NRF) of South Africa.

2.7.1 Triple quadrupole high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) system

The LC-MS/MS system consisted of an Agilent 1100/1200 combined series HPLC system (Agilent Technologies, Palo Alto, CA, USA), including a solvent vacuum degasser, binary pump, temperature-controlled column oven and autosampler coupled to an ABSciex 4000 triple quadrupole mass spectrometer, equipped with a Turbo-V® electrospray ionisation (ESI) source (Sciex, Concord, Canada). Analyst™ Software, version 1.5.2 (Sciex, Concord, Canada), was used to operate the system and manage the optimisation, data acquisition and perform the data analysis. The triple quadrupole LC-MS/MS system is housed at the Department of Pharmacology at the University of Pretoria.

2.7.2 Ultra-performance liquid chromatography tandem quadrupole time of flight mass spectrometry (UPLC-QTOF-MS/MS) system

The UPLC-QTOF-MS/MS system is comprised of a Waters AQUITY UPLC™ system equipped with a binary solvent manager, temperature-controlled autosampler compartment, column oven and degasser coupled to a Waters Synapt G2 QTOF mass spectrometer (Milford, MA, USA) equipped with a Z-spray ESI source. MassLynx™ software (Milford, MA, USA), version 4.1, was used for system control and data acquisition and TargetLynx™ for quantification. The system is housed at the Department of Chemistry at the University of Pretoria.

CHAPTER 3: LC-MS/MS METHOD OPTIMISATION USING AN ALTERNATIVE COLUMN CHEMISTRY AND MOBILE-PHASE COMPOSITIONS

3.1 INTRODUCTION

LC-MS/MS analytical procedures for simultaneous quantification of the physico-chemically diverse, structurally related aromatic probe drugs were optimised using an alternative column chemistry and mobile-phase compositions. A triple quadrupole high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) system (Section 2.7.1) was used for method optimisation. Firstly, the mass spectrometric detection parameters for all probe drugs in the Geneva phenotyping cocktail and their metabolites were individually optimised by manual tuning. Next, the selectivity of the probe drugs and their metabolites on a biphenyl column in comparison to a traditional C18 phase were evaluated. The aromatic structures of the pharmaceutical drugs and their metabolites vary in polarity and acid dissociation constants and could benefit from an alternative column selectivity. Thereafter, the influence of changes in the analytical conditions involving mobile phase pH and solvent mixture composition as well as the solvent type, used to reconstitute the sample and present in the autosampler vial, on analyte selectivity, retention and stability were assessed, using a biphenyl column as the stationary phase. This was done to assess the robustness of the method, assaying multiple ionisable analytes from a single sample, where the reproducibility of the final method could be influenced.

3.2 MATERIALS AND METHODS

3.2.1 Optimisation of mass spectrometric detection parameters of the Geneva phenotyping probe drugs and their metabolites

Targeted multiple reaction monitoring (MRM), using a triple quadrupole mass spectrometer, was used for quantitation of the 15 analytes in biological matrices. Each of the 7 probe drugs present in the Geneva cocktail and 6 corresponding CYP450 metabolites as well as the positive mode IS, imipramine and negative mode IS, probenecid, were individually tuned to determine their optimal mass spectrometric detection parameters with the manual tuning function on Analyst™ 1.5.2 software.

Stock solutions at 1 $mg.mL^{-1}$ (m/v) in methanol were prepared by accurately weighing off approximately 1 mg of each analyte on a small foil weighing boat with a Sartorius microbalance (Zeiss West Germany Pty. Ltd.) and quantitatively carrying it over to 2 mL volumetric flasks to make up the final stock solution, except for paraxanthine, hydroxy-bupropion, omeprazole and hydroxy-midazolam that were bought as 1 $mg.mL^{-1}$ (m/m) solutions and midazolam solution diluted to 1 $mg.mL^{-1}$ (v/v) from sterile Dormicum™ 5 $mg.mL^{-1}$ ampoules. After vortex mixing, aliquots of 100 µL were transferred to Eppendorf vials and stored at -20°C. Working standard dilutions of 1 $\mu g.\mu L^{-1}$ (v/v) in 50:50 methanol: water containing 0.1% formic acid, for positive ESI mode optimisation and in 50:50 methanol: water containing 0.025% ammonium formate for negative ESI mode optimisation were prepared by volumetrically carrying over 10 µL of the stock solutions, using a calibrated Eppendorf pipette, to a 10 mL A grade volumetric flask and making up to volume. Further dilutions to approximately 100 $ng.mL^{-1}$ were prepared and infused directly into the ESI source of the Sciex 4000 QTrap mass spectrometer at a constant flow rate of 10 or 20 $\mu L.min^{-1}$ using a Harvard syringe pump (Harvard Apparatus, Hollinston, MA, USA).

Both positive and negative ESI mode precursor ion (Q1) scans were performed for all analytes with initial source/gas parameters set as follows: The ion spray voltage at +/- 4500 V, curtain gas set to 23 psi, turbo heater temperature at 200°C with the nebuliser gas flow rate kept at 30 psi and the heater gas flow rate at 35 psi. The declustering potential (DP) was ramped from 5 to 100 volts for ESI+ mode and from - 5 to - 100 volts for ESI- mode.

The Q1 scan with the greatest scan intensity in either positive $[M + 1]^+$ or negative mode $[M - 1]^-$ were then chosen for further optimisation. Next, enhanced resolution (ER) scans to optimise the analyte detection and sensitivity was performed, followed by product ion (MS2 and EPI) scans, whilst ramping the collision energy (CE) in Q2 to determine the most abundant product ions (detected in Q3) for each precursor ion at optimised conditions. Final targeted MRM scans, precursor (Q1) to product ion (Q3) transitions for each analyte was carried out whilst optimising source/gas parameters, DP, CE and collision cell exit potential (CXP) for each transition.

3.2.2 Assessing alternative column selectivity on the separation of the Geneva phenotyping cocktail probe drugs and metabolites

An analyte mixture in methanol was prepared from 1 $mg.mL^{-1}$ (m/v) stock solutions volumetrically by adding appropriate volumes of the analytes to an Eppendorf vial and making it up to a final volume of 1000 µL. Final concentrations of the analytes were 7.5 $\mu g.\mu L^{-1}$ (v/v) for midazolam, 15 $\mu g.\mu L^{-1}$ (v/v) for bupropion, hydroxy-bupropion, hydroxyflurbiprofen, omeprazole, hydroxy-omeprazole, dextromethorphan, dextrorphan and hydroxy-midazolam, 30 $\mu g.\mu L^{-1}$ (v/v) for fexofenadine and 75 $\mu g.\mu L^{-1}$ (v/v) for caffeine, paraxanthine and flurbiprofen. Blank human plasma and solvent (190 µL) were spiked with 10 µL of the analyte mixture and extracted by a simple 3 step protein precipitation procedure. During the first step 200 µL of acetonitrile was added to the spiked plasma and solvent mixtures, vortex mixed for 5 minutes (Lasec Vortex Genie2) and sonicated for 5 minutes (Bran Sonic 52 ultrasonicator) followed by two more additions of 100 µL acetonitrile each and the vortex mixing and sonication steps repeated. After protein precipitation the mixtures were centrifuged (Beckman Coulter Microfuge™ 16 centrifuge) at 14,000 *xg* for 10 minutes to remove the precipitated proteins. The supernatant (80 µL) was pipetted (Eppendorf pipette) into clean amber 2 mL LC vials, containing 200 µL glass tapered autosampler vial inserts, with 20 µL of IS mix, containing 0.75 $\mu g.\mu L^{-1}$ (v/v) probenecid and imipramine. The final solution was made up to 200 µL by adding 100 µL of pyrogen free double deionised water to make up a 50:50 methanol: aqueous mixture at 4 different conditions, namely 0.1% formic acid, 5 mM ammonium bicarbonate, 10 mM ammonium acetate and 10 mM ammonium formate just prior to LC-MS/MS analysis.

Isocratic chromatographic separation of all analytes and IS was achieved on two different columns with similar column dimensions, a Kinetex™ C18 and Kinetex™ biphenyl column (100 mm × 2.1 mm, 2.6 µm particle size) at two different mobile phase conditions. The mobile phases consisted of methanol: water (60:40) or acetonitrile: water (40:60) with the flow rate set at 100 $\mu L.min^{-1}$ and the sample injection volume 10 µL. The column temperature was controlled at 40°C ± 3°C. Each injection was done in triplicate and average retention times recorded for all analytes under different conditions. Retention factors were calculated and scatter plots were drawn comparing the logarithm of the retention factors (log k') for all analytes on the biphenyl column against their respective log k' values on the C18 column for both mobile phase conditions. The slopes and correlation coefficients were determined from linear regression analysis to expose alternative column selectivity.

3.2.3 Effect of altered mobile phase and solvent composition on analyte resolution, stability and peak area

Spiked plasma and solvent samples were prepared, extracted and made up in four different solvent conditions in LC vials according to the experimental method described above in Section 3.2.2. An isocratic separation was achieved on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm particle size) with methanol and LC-MS grade water in a ratio of 60:40 as the mobile phase. The mobile phase composition was altered with four different additives and the pH of the aqueous phase measured.

The purpose was to study the effect of different mobile phase conditions on the state of ionisation and retention behaviour of the analytes with markedly different physicochemical properties aiming to optimise the separation selectivity and stability of the acid-label omeprazole. The column temperature was kept constant at 40°C ± 3°C, with the mobile flow rate at 100 µL.min^{-1} and the sample injection volume 10 µL. The four different mobile phase conditions and measured pH are given in Table 3.1. The average of the retention factors (k'), from triplicate injections, of all the analytes were plotted against the four different mobile phase conditions at different pH.

The effect of the composition of the solvent in the LC vial and the effect of the mobile phase composition on the analyte peak area (signal intensity) and the interaction between these two independent variables on the signal intensity were evaluated with a repeated measures two factor ANOVA with Geisser-Greenhouse correction to determine statistical significance. The distribution was determined with a Shapiro-Wilk normality test and data represented graphically with a QQ plot. Normal and lognormal distribution were compared and data transformed where a lognormal distribution was more likely, before the ANOVA test was performed. Matched values were both spread and stacked across a row and simple effects compared within rows. The effect was deemed significant if the F statistic was greater than the critical F value ($\alpha < 0.05$). Post hoc Tukey tests were conducted to establish the source of variability by multiple comparisons using hypothesis testing. The statistical analysis was carried out with GraphPad Prism version 8.0.2 statistical software for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Table 3.1: Mobile phase and sample vial composition with measured pH used to study analyte retention behaviour and sensitivity.

	Mobile phase composition	Measured pH
A	0.1% formic acid	2.7
B	10 mM ammonium formate (NH_4COOH) acidified with 1 M solution of formic acid	3.9
C	10 mM ammonium acetate (NH_4COOCH_3)	6.5
D	5mM ammonium bicarbonate (NH_4HCO_3)	8.3

3.3 RESULTS AND DISCUSSION

3.3.1 Optimisation of mass spectrometric detection parameters

Negative ionisation mode showed greater sensitivity for the acidic flurbiprofen and its hydroxylated metabolite, which could be expected from the presence of the carboxylic acid-group in their chemical structures. Probenecid was used as the internal standard for negative mode acquisitions. The optimised mass transitions for flurbiprofen, hydroxy-flurbiprofen and probenecid are presented in Table 3.2. All other analytes in the Geneva phenotyping cocktail and their respective metabolites, yielded highest sensitivity in positive ionisation mode and imipramine was used as the internal standard for these positive mode acquisitions. Two MRM transitions were monitored for fexofenadine and dextromethorphan while single precursor to product ion transitions were monitored for all other analytes. These details are displayed in Table 3.3. All mass transitions detected corresponded to those reported in literature.

Table 3.2: Optimised mass spectrometric fragmentation parameters for flurbiprofen, hydroxy-flurbiprofen and internal standard probenecid in negative mode $[M - H]^-$

Analyte	CYP probe	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP (V)
Flurbiprofen	**CYP2C9**	243.20	199.30	-12	-14	-12
Hydroxyflurbiprofen		259.20	215.20	-40	-11	-4
Internal Standard						
Probenecid		284.30	240.30	-50	-24	-10

CYP = Cytochrome P450; m/z – mass to charge ratio; DP = declustering potential in volts; CE = collision energy in volts; collision cell exit potential in volts.

Table 3.3: Optimised mass spectrometric fragmentation parameters for analytes and internal standard imipramine in positive mode $[M+H]^+$

Analyte	Probe	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP (V)
Fexofenadine	**P-gp**	502.7	466.6; 484.7	100	38	12
Caffeine	**CYP1A2**	195.3	138.2	20	25	6
Paraxanthine		181.1	124.2	70	27	5
Bupropion	**CYP2B6**	240.4	131.3	20	50	11
Hydroxybupropion		256.4	103.1	50	52	3
Omeprazole	**CYP2C19**	346.3	198.1	25	30	10
Hydroxyomeprazole		362.1	214.4	50	15	10
Dextromethorphan	**CYP2D6**	272.4	147.4; 171.5	90	50	10
Dextrorphan		258.4	157.2	80	45	4
Midazolam	**CYP3A4**	326.3	291.4	80	35	14
Hydroxymidazolam		342.2	324.1	89	29	18
Internal Standard						
Imipramine		281.5	86.1	50	50	10

P-gp = permeability glycoprotein; CYP = Cytochrome P450; m/z – mass to charge ratio; DP = declustering potential in volts; CE = collision energy in volts; collision cell exit potential in volts.

3.3.2 Comparison of analyte selectivity between a C18 and Biphenyl column

Detection of the analytes was achieved with an ABSciex 4000QTrap triple quadrupole mass spectrometer with separate acquisitions performed for negative and positive polarity mode respectively. During the initial method development, separation of the Geneva cocktail probe drugs and their metabolites was performed on a commonly used small particle size C18 column (Kinetex™ C18, 100 mm × 2.1 mm, 2.6 µm) with a binary gradient elution using different mobile phase compositions. An extracted ion chromatogram (XIC), following optimisation of the chromatographic conditions, showing the elution order of analytes acquired in positive ESI mode, with optimised gradient elution, is depicted in Figure 3.1.

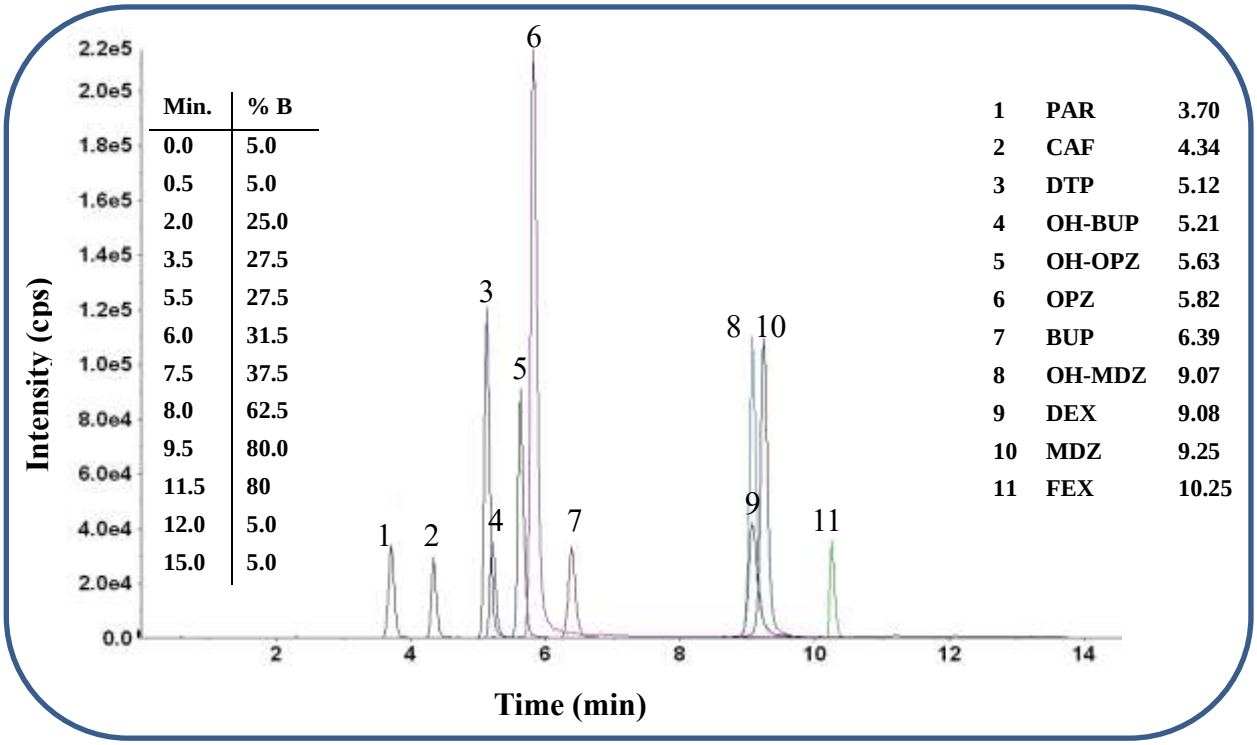


Figure 3.1: XIC of analytes in positive ESI mode, separated on a Kinetex™ C18 column (100 mm × 2.1 mm, 2.6 µm). Flow rate: 300 µL. min^{-1}, with mobile phase A: H_2O 0.1% formic acid and mobile phase B: 50:50 MeOH: CAN with gradient elution. Identities and retention times are shown in the table.

The initial aim was to develop a method with optimal separation and resolution of all analytes in a run time maintaining retention factors between 2 and 10. Despite optimisation of solvent type and gradient adjustment of both slope and timing, it is clear that dextrorphan (5.12 ± 0.02 min.) and hydroxy-bupropion (5.21 ± 0.01 min.) and dextromethorphan (9.08 ± 0.01 min.), hydroxymidazolam (9.07 ± 0.02 min.) and midazolam (9.25 ± 0.02 min.) were difficult to

separate on a C18 column where the separation was driven mainly by differential solubility (Figure 3.1). These analytes are all aromatic compounds with similar hydrophobicity and show similar retention behaviour on the alkyl C18 column. This lack of resolution of these compounds led to exploring the influence of an alternative column chemistry on the selectivity and resolution of the analytes under different isocratic mobile phase conditions using acetonitrile: water (40:60) or methanol: water (60:40) (flow rate 100 $\mu L.min^{-1}$), with similar elution strength. A biphenyl phase stationary phase column was selected to separate the mixture of probe drugs and their metabolites and the selectivity compared to a traditional C18 column. The average retention times (t_R) of all the analytes, their respective retention factors (k') and the calculated percentage variance for k' for each analyte are summarised in Tables 3.4 and 3.5.

Where 40% acetonitrile was used as the eluent, the results show good correlation between the separation of the analytes on the biphenyl and the C18 columns. Under these conditions the interaction of analytes with the stationary phases were probably controlled by a common separation mechanism. Acetonitrile suppresses the π-π interaction between the analytes and the biphenyl groups present in the stationary phase as a result of its C-N triple bond; an effect that is well documented.[234, 240] This is highlighted when comparing the extracted ion chromatograms of the analytes on the different columns, as shown in Figure 3.2.

Fexofenadine was the only analyte with a percentage variance for k' increase of greater than 10%. Using XIC to compare the separation of the analytes between the different columns with 60% methanol as the eluent, showed significant differences in the retention behaviour of caffeine, 5'-hydroxy-omeprazole, dextromethorphan, midazolam, fexofenadine and α-hydroxymidazolam (Table 3.5). When using a methanol containing mobile phase, the non-polar π-π interactions between the analytes and the stationary phenyl groups were favoured and possibly enhanced. Figure 3.3 highlights the alternative selectivity differences of the analytes on the two different columns, showing the analytes most affected by the enhanced π-π interactions.

Table 3.4: Average retention times (t_R) and retention factors (k') of analytes separated on a Kinetex™ C18 or Kinetex™ Biphenyl column (+ESI) with acetonitrile: water (40:60) as the mobile phase.

Analyte	Kinetex™ C18		Kinetex™ Biphenyl		% variance for k'
	Average t_R minutes	k'	Average t_R minutes	k'	
Paraxanthine	3.44 (±0.002)	0.274	3.28 (±0.003)	0.215	0.18%
Dextrorphan	3.45 (±0.002)	0.278	3.53 (±0.010)	0.307	0.04%
5'-Hydroxyomeprazole	3.47 (±0.005)	0.285	3.50 (±0.007)	0.296	0.01%
Hydroxybupropion	3.54 (±0.003)	0.311	3.64 (±0.009)	0.348	0.07%
Caffeine	3.58 (±0.002)	0.326	3.53 (±0.003)	0.307	0.02%
Omeprazole	3.73 (±0.047)	0.381	3.99 (±0.019)	0.478	0.46%
Bupropion	3.93 (±0.003)	0.456	4.14 (±0.011)	0.533	0.30%
Dextromethorphan	4.34 (±0.003)	0.607	4.94 (±0.014)	0.830	2.47%
α-Hydroxymidazolam	4.37 (±0.009)	0.619	4.69 (±0.015)	0.737	0.70%
Midazolam	4.38 (±0.005)	0.622	4.98 (±0.014)	0.844	2.47%
Fexofenadine	5.86 (±0.006)	1.170	7.52 (±0.019)	1.785	18.90%

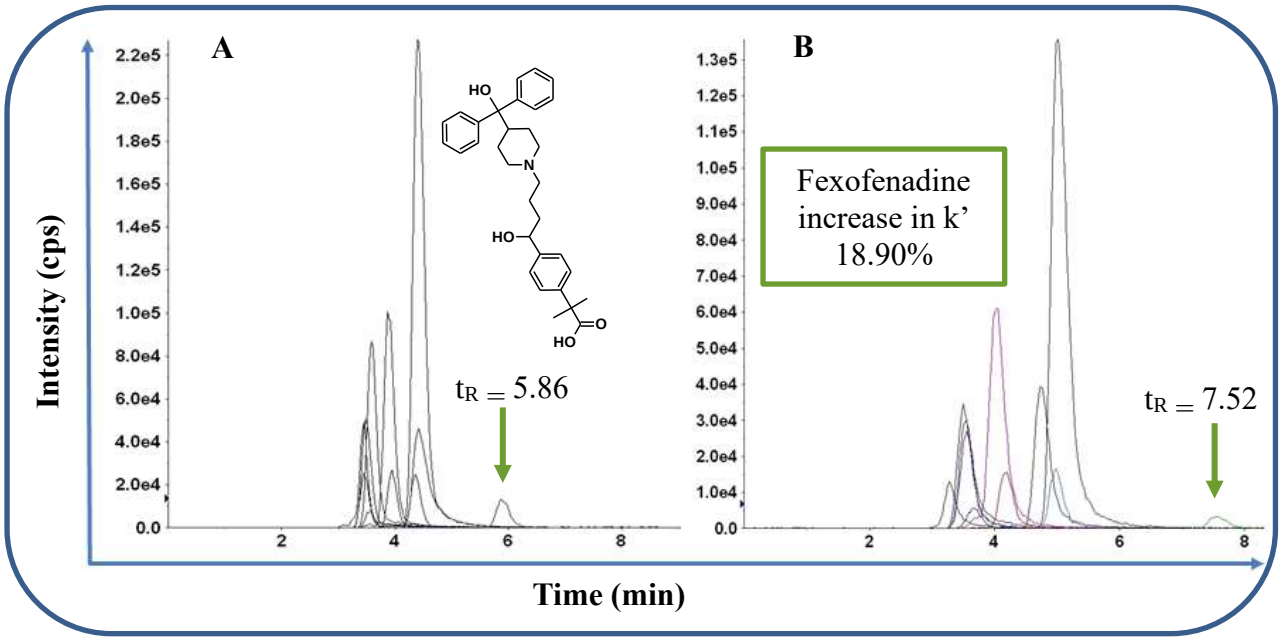


Figure 3.2: XIC of MRM+ transitions on (A) Kinetex™ C18 and (B) Kinetex™ Biphenyl column with isocratic acetonitrile: water (40:60) as the mobile phase at a flow rate of 100 µL.min^{-1}.

Table 3.5: Average retention times (t_R) and retention factors (k') of analytes separated on a Kinetex™ C18 or Kinetex™ Biphenyl column (+ESI) with methanol: water (60:40) as the mobile phase.

Analyte	Kinetex C18 column		Kinetex Biphenyl column		% variance for k'
	Average t_R minutes A	k'	Average t_R minutes B	k'	
Dextrorphan	3.49 (± 0.015)	0.293	4.02 (± 0.013)	0.489	1.93%
Paraxanthine	3.59 (± 0.006)	0.330	4.20 (± 0.009)	0.556	2.55%
Hydroxybupropion	3.75 (± 0.011)	0.389	4.17 (± 0.012)	0.544	1.21%
Caffeine	3.87 (± 0.005)	0.433	5.69 (± 0.007)	1.107	22.72%
Bupropion	3.93 (± 0.011)	0.456	4.66 (± 0.011)	0.726	3.66%
5'-Hydroxyomeprazole	4.04 (± 0.027)	0.496	5.58 (± 0.020)	1.067	16.27%
Dextromethorphan	4.35 (± 0.014)	0.611	8.42 (± 0.015)	2.119	113.61%
Midazolam	4.41 (± 0.013)	0.633	9.04 (± 0.013)	2.348	147.03%
Omeprazole	4.71 (± 0.029)	0.744	5.25 (± 0.009)	0.944	2.00%
Fexofenadine	4.83 (± 0.009)	0.789	13.37 (± 0.027)	3.952	500.22%
α-Hydroxymidazolam	5.04 (± 0.029)	0.867	10.49 (± 0.026)	2.885	203.72%

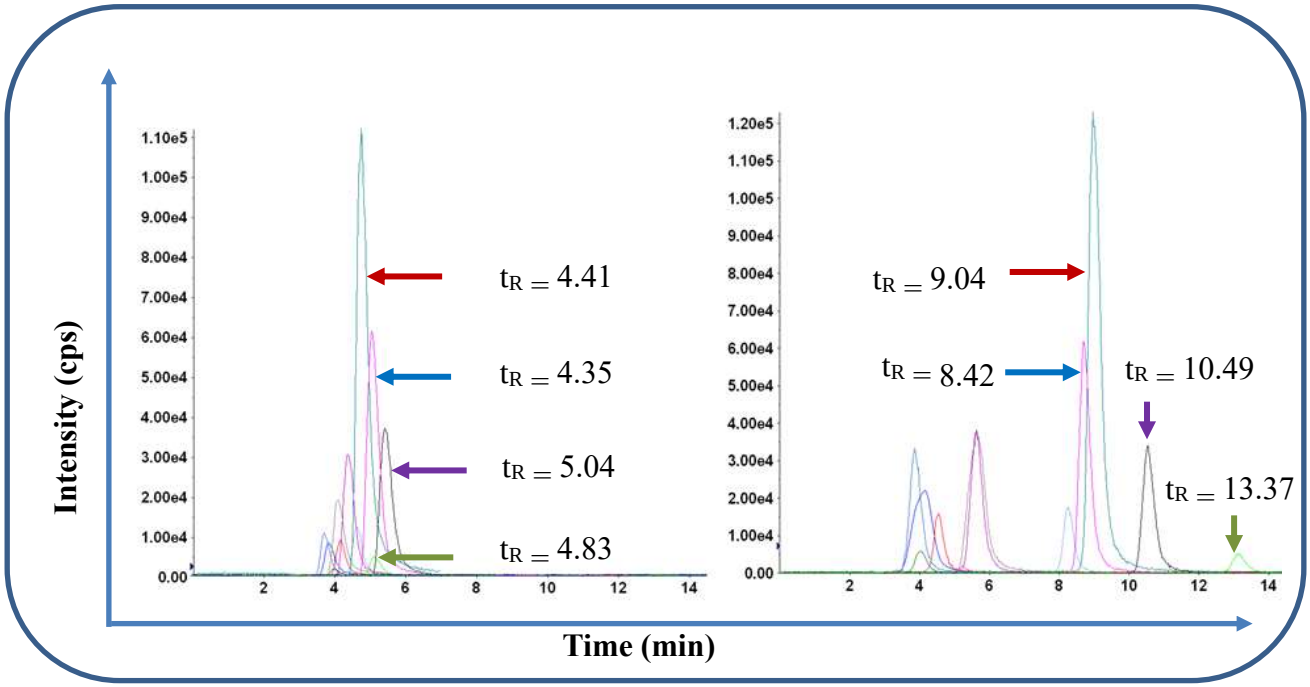


Figure 3.3: XIC of MRM+ transitions on (A) Kinetex™ C18 and (B) Kinetex™ Biphenyl column with isocratic methanol: water (60:40) as the mobile phase at a flow rate 100 µL.min^{-1}. Increase in retention times are indicated by the coloured arrows; midazolam (red), dextromethorphan (blue), α-hydroxymidazolam (purple) and fexofenadine (green).

These differences in analyte-stationary phase interaction were explored further by plotting the logarithm of the retention factors, obtained from separation on the biphenyl column against those measured on the C18 column for each of the analytes. The resulting scatter plots for both mobile phase conditions (40% acetonitrile and 60% methanol respectively), are shown in Figure 3.4. The comparison of the scatter plot data infer that the biphenyl phase has more non-polar interactions when methanol is used compared to acetonitrile as a mobile phase. The slope of the respective linear regression analysis is indicative of the relative strengths of these separation interactions. The correlation coefficients (r^2) when using acetonitrile was 0.97 indicating a high degree of similarity between the interactions involved in the separation on the two stationary phases. The graph on the right shows more differentiation in retention when methanol was used with more scattered data and a flatter slope with a correlation coefficient of 0.74. Despite the slopes appearing approximately similar note that the y-axis scale is different. The compounds below the trendline, including all the hydroxylated metabolites are influenced by the electron donating effects of the biphenyl stationary phase and are thus better retained resulting in the improved resolution.

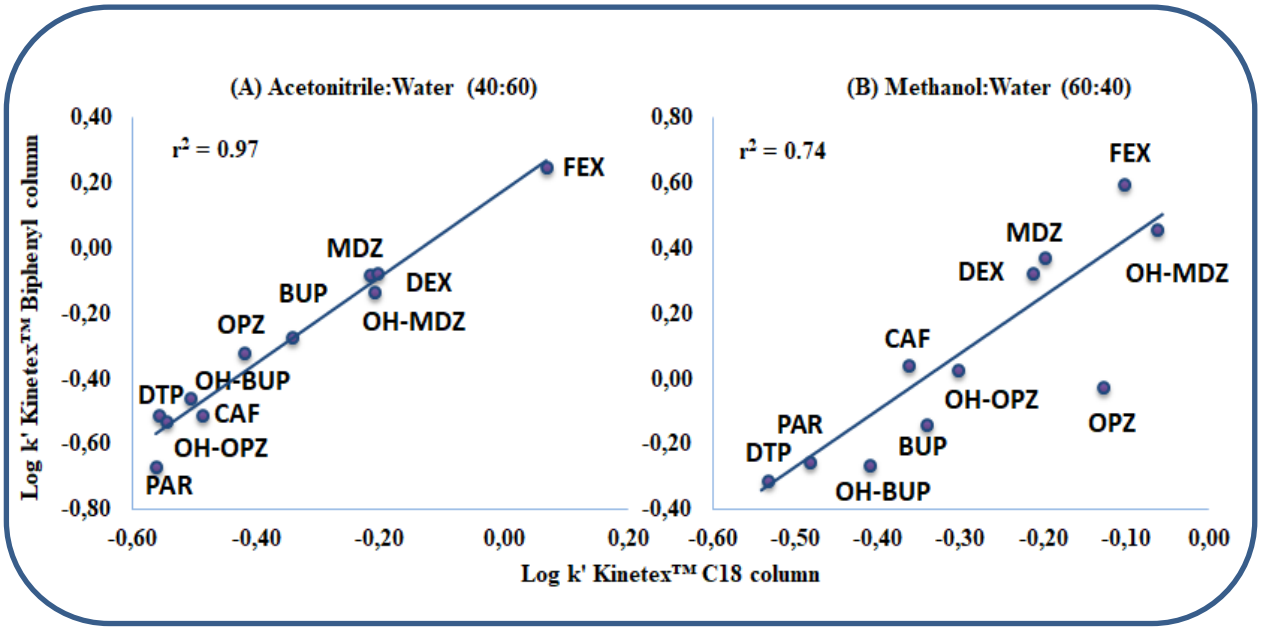


Figure 3.4: Scatter plots of the analyte retention factors on the biphenyl column against their retention factors on the C18 column for (A) acetonitrile: water (40:60) and (B) methanol: water (60:40) isocratic elution.

When using gradient elution with a Kinetex™ Biphenyl column (Figure 3.5), analytes that were difficult to separate on the C18 column were slightly better resolved, indicating improved selectivity offered by the biphenyl stationary phase. Methanol proved to be a good choice of mobile phase solvent when paired with the biphenyl column offering increased selectivity in part from π-π interactions in addition to hydrophobic interactions which dominate reverse phase high performance liquid chromatography. These interactions are reduced by using increasing percentages of acetonitrile, which interferes with π-π interactions due to competition from the acetonitrile triple bond (nitrile group). While these π-π interactions are not the only parameter controlling the retention on phenyl based stationary phase columns in methanol, they do provide a slight enhancement to complicated separations of closely related compounds with wide applicability to quantitative methods. Despite the slightly longer run time on the biphenyl column, the improved resolution allows for accurate quantitative pharmacokinetic analysis. Flurbiprofen and hydroxy-flurbiprofen were the only analytes measured in negative ionisation mode and baseline separation was easily achieved using a shorter optimised gradient elution on both column types.

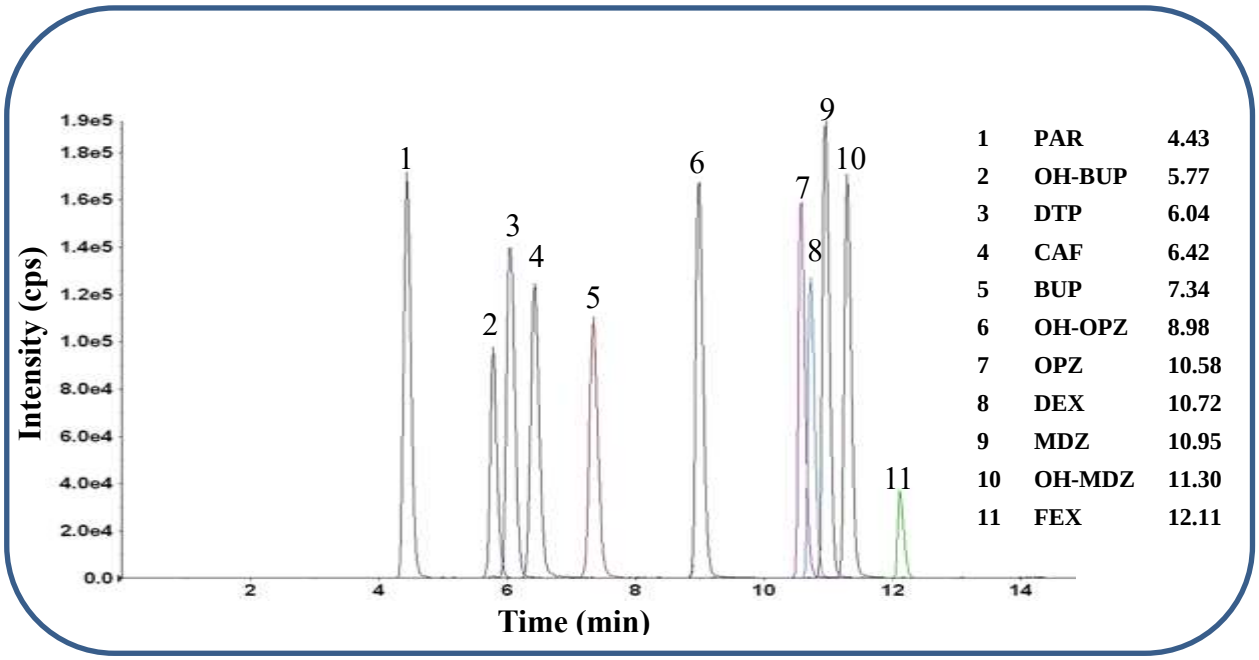


Figure 3.5: XIC of analytes in positive ESI mode, separated with a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm). Flow rate: 300 µL. min^{-1}, with mobile phase A: H_2O 0.1% formic acid and mobile phase B: MeOH 0.1% formic acid. Identities and retention times are shown in the table.

3.3.3 Effect of altered mobile phase and solvent composition on the stability, selectivity and sensitivity of the Geneva cocktail probe drugs and metabolites

Ionisation efficiency could be increased by pairing appropriate mobile phase additives at the molar concentrations that optimise the pH so that analytes are already charged in solvent before LC separation.[241] Most of the pharmaceutical analytes in the Geneva cocktail and their metabolites will be ionic at acidic pH. During the initial method development, it was however observed that omeprazole and its hydroxylated metabolite degrade rapidly when the composition of the sample solvent in the sample vial is acidic to match the mobile phase (0.1% formic acid) conditions. The lability of omeprazole under acidic conditions is well known.[211] This was problematic, since the first-choice additive in LC-ESI-MS separations is formic acid, especially in analysis involving basic pharmaceutical analytes. At this pH, basic analytes will be fully ionised and adsorption interactions with free silanol groups can be controlled, thereby improving peak shape and symmetry. The acidic flurbiprofen and hydroxylated metabolite, will also be completely un-ionised at a low pH, avoiding early elution with matrix components. A set of experiments to evaluate the influence of mobile phase composition and pH, in the sample vial on analyte stability, sensitivity, selectivity and retention behaviour was carried out. The aim was to determine the best combination of sample reconstitution solution and mobile phase to optimise the stability while maintaining ionisation efficiencies of all analytes with different physicochemical properties and maintaining optimum retention and selectivity during the chromatographic separation.

As expected, the quality of the separation was optimal with mobile phase pH of 2.7 (0.1% formic acid), where k' was between 1.19 and 10.99 with isocratic elution (Table 3.6). When the mobile phase pH is increased to 3.9 (containing both 0.1% formic acid and ammonium formate) retention factors increased to between 1.01 and 23.09. Figure 3.6 shows two XIC's of the analytes at identical sample vial conditions with mobile phase pH 2.7 (A) and 3.9 (B) respectively with altered elution order due to different degrees of ionisation of the analytes during the chromatographic separation at altered pH.

Table 3.6: Mean retention factors (k') ± the standard deviation (SD) of analytes and their coefficient of variation (CV %) in extracted human plasma from triplicate injections over time at the same sample vial condition and within different conditions at four different mobile phase conditions and pH levels.

pH	Composition of solution in LC-vial	CAF			PAR			BUP			OHBUP		
		Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)
2.70	0,1% formic acid	2.38	0.02	0.64	1.11	0.01	1.04	1.71	0.01	0.58	1.23	0.01	0.47
	10 mM NH_4COOH	2.39	0.04	1.74	1.13	0.04	3.36	1.66	0.02	1.26	1.20	0.01	0.83
	10 mM NH_4COOCH_3	2.35	0.15	6.38	1.12	0.12	10.97	1.67	0.11	6.28	1.19	0.10	8.01
	5mM NH_4HCO_3	2.31	0.02	0.90	1.08	0.06	5.16	1.61	0.03	1.80	1.14	0.02	1.52
	Average	2.36			1.11			1.66			1.19		
	CV (%)	1.44			1.86			2.58			3.05		
3.90	0,1% formic acid	2.37	0.16	6.85	0.99	0.13	12.98	2.45	0.17	6.92	1.71	0.13	7.53
	10 mM NH_4COOH	2.35	0.05	1.95	1.01	0.05	4.90	2.47	0.07	2.64	1.72	0.05	2.99
	10 mM NH_4COOCH_3	2.24	0.17	7.78	0.94	0.14	15.41	2.34	0.21	8.89	1.64	0.16	9.90
	5mM NH_4HCO_3	2.42	0.06	2.51	1.11	0.07	6.35	2.51	0.05	1.99	1.77	0.05	2.89
	Average	2.35			1.01			2.44			1.71		
	CV (%)	3.25			7.00			2.96			3.28		
6.50	0,1% formic acid	2.47	0.05	2.01	1.14	0.08	6.85	2.78	0.04	1.27	1.83	0.08	4.26
	10 mM NH_4COOH	2.49	0.04	1.41	1.20	0.05	4.29	2.97	0.04	1.36	1.99	0.04	2.01
	10 mM NH_4COOCH_3	2.47	0.03	1.24	1.19	0.03	2.56	3.01	0.03	0.84	2.01	0.02	0.76
	5mM NH_4HCO_3	2.54	0.12	4.69	1.20	0.10	7.94	3.03	0.07	2.20	2.03	0.06	3.01
	Average	2.49			1.18			2.95			1.96		
	CV (%)	1.41			2.57			3.97			4.72		
8.30	0,1% formic acid	2.68	0.08	2.89	1.30	0.08	6.42	7.35	0.53	7.18	2.90	0.14	4.83
	10 mM NH_4COOH	2.68	0.12	4.33	1.30	0.08	6.43	9.40	0.26	2.81	3.72	0.10	2.81
	10 mM NH_4COOCH_3	2.68	0.05	1.94	1.27	0.04	2.84	9.30	0.17	1.86	3.71	0.13	3.37
	5mM NH_4HCO_3	2.59	0.13	5.03	1.25	0.03	2.58	9.33	0.21	2.23	3.69	0.13	3.41
	Average	2.66			1.28			8.85			3.51		
	CV (%)	1.76			2.03			11.30			11.49		

pH	Composition of solution in LC-vial	OPZ			OHOPZ			DEX			DTP		
		Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)
2.70	0,1% formic acid	-	-	-	-	-	-	5.61	0.04	0.78	1.01	0.00	0.00
	10 mM NH_4COOH	6.45	0.06	0.93	2.77	0.01	0.42	5.57	0.05	0.90	0.96	0.02	2.08
	10 mM NH_4COOCH_3	6.49	0.12	1.83	2.79	0.11	3.94	5.62	0.14	2.49	0.98	0.08	8.35
	5mM NH_4HCO_3	6.37	0.05	0.81	2.73	0.04	1.60	5.53	0.01	0.21	0.91	0.03	3.16
	Average	6.44			2.77			5.58			0.97		
	CV (%)	0.90			1.17			0.75			4.20		
3.90	0,1% formic acid	-	-	-	-	-	-	8.30	0.20	2.41	1.43	0.13	9.01
	10 mM NH_4COOH	11.90	0.40	3.36	3.84	0.12	3.13	8.17	0.25	3.08	1.43	0.04	3.05
	10 mM NH_4COOCH_3	11.70	0.36	3.08	3.69	0.23	6.36	8.03	0.29	3.59	1.36	0.16	11.46
	5mM NH_4HCO_3	11.80	0.10	0.85	3.83	0.02	0.45	8.13	0.06	0.71	1.52	0.06	4.24
	Average	11.80			3.79			8.16			1.43		
	CV (%)	0.85			2.27			1.35			4.48		
6.50	0,1% formic acid	-	-	-	-	-	-	8.60	0.14	1.64	1.50	0.07	4.71
	10 mM NH_4COOH	12.47	0.06	0.46	3.96	0.05	1.24	8.77	0.06	0.66	1.73	0.03	1.85
	10 mM NH_4COOCH_3	12.50	0.17	1.39	3.95	0.03	0.77	8.83	0.15	1.73	1.74	0.01	0.57
	5mM NH_4HCO_3	12.60	0.20	1.59	3.99	0.09	2.18	8.90	0.20	2.25	1.80	0.06	3.47
	Average	12.52			3.97			8.78			1.69		
	CV (%)	0.55			0.48			1.47			7.81		
8.30	0,1% formic acid	-	-	-	-	-	-	13.83	0.40	2.92	2.41	0.11	4.55
	10 mM NH_4COOH	13.57	0.38	2.79	4.27	0.14	3.19	15.93	0.23	1.45	3.23	0.02	0.64
	10 mM NH_4COOCH_3	13.43	0.15	1.14	4.26	0.12	2.83	15.93	0.40	2.54	3.23	0.14	4.19
	5mM NH_4HCO_3	13.37	0.23	1.73	4.16	0.16	3.87	15.90	0.30	1.89	3.22	0.11	3.42
	Average	13.46			4.23			15.40			3.02		
	CV (%)	0.76			1.45			6.78			13.45		

NB: The – indicate that no peaks were detected for these analytes at this sample vial condition.

pH	Composition of solution in LC-vial	MDZ			OHMDZ			IMIP			FEX		
		Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)	Mean k'	SD	CV (%)
2.70	0,1% formic acid	6.37	0.04	0.55	8.63	0.06	0.67	8.27	0.06	0.70	11.03	0.15	1.38
	10 mM NH_4COOH	6.41	0.05	0.83	8.70	0.10	1.15	8.20	0.10	1.22	10.93	0.15	1.40
	10 mM NH_4COOCH_3	6.46	0.13	1.97	8.70	0.17	1.99	8.23	0.21	2.53	11.03	0.31	2.77
	5mM NH_4HCO_3	6.37	0.02	0.24	8.60	0.00	0.00	8.17	0.06	0.71	10.97	0.15	1.39
	Average	6.40			8.66			8.22			10.99		
	CV (%)	0.62			0.58			0.52			0.45		
3.90	0,1% formic acid	23.20	0.40	1.72	19.87	0.35	1.77	12.13	0.21	1.72	15.87	0.35	2.21
	10 mM NH_4COOH	23.20	0.56	2.40	19.83	0.65	3.28	12.00	0.40	3.33	15.73	0.75	4.77
	10 mM NH_4COOCH_3	23.00	0.56	2.42	19.53	0.60	3.09	11.83	0.40	3.42	15.43	0.60	3.91
	5mM NH_4HCO_3	22.97	0.35	1.53	19.53	0.31	1.56	11.90	0.20	1.68	15.47	0.35	2.27
	Average	23.09			19.69			11.97			15.63		
	CV (%)	0.54			0.93			1.09			1.34		
6.50	0,1% formic acid	39.50	0.00	0.00	21.45	0.07	0.33	12.65	0.07	0.56	12.95	0.07	0.55
	10 mM NH_4COOH	39.90	0.62	1.57	21.53	0.23	1.07	12.93	0.06	0.45	13.03	0.06	0.44
	10 mM NH_4COOCH_3	39.87	0.81	2.03	21.53	0.32	1.49	12.97	0.21	1.61	13.00	0.17	1.33
	5mM NH_4HCO_3	40.10	0.44	1.09	21.80	0.30	1.38	13.17	0.23	1.75	13.17	0.23	1.75
	Average	39.84			21.58			12.93			13.04		
	CV (%)	0.63			0.71			1.65			0.71		
8.30	0,1% formic acid	45.93	0.47	1.03	22.57	0.35	1.56	27.53	0.76	2.77	10.43	0.06	0.55
	10 mM NH_4COOH	48.37	1.16	2.40	23.83	0.49	2.07	28.23	0.51	1.82	10.83	0.32	2.97
	10 mM NH_4COOCH_3	47.87	0.65	1.36	23.53	0.25	1.07	28.00	0.35	1.24	10.70	0.17	1.62
	5mM NH_4HCO_3	47.60	1.00	2.10	23.37	0.42	1.78	28.00	0.24	0.84	10.67	0.23	2.17
	Average	47.44			23.33			27.94			10.66		
	CV (%)	2.22			2.32			1.05			1.56		

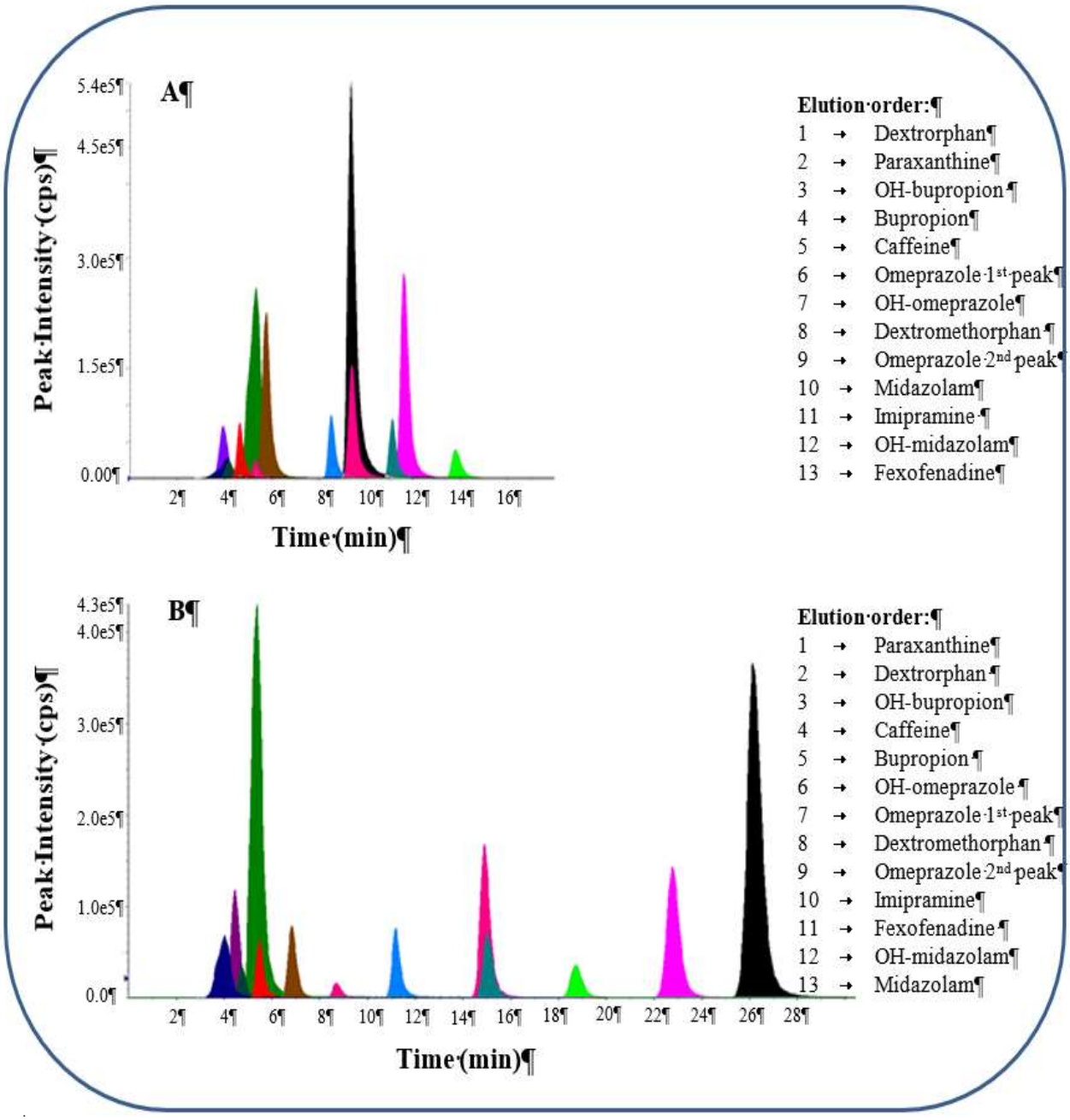


Figure 3.6: XIC of analytes (100 ng.mL^{-1}) from a sample in 50:50 methanol: water containing 10 mM ammonium formate separated by isocratic (100 µL.min^{-1}) 60:40 methanol: water containing (A) 0.1% formic acid, pH 2.7 and (B) 0.1% formic acid with ammonium formate adjusted to pH 3.9 on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm).

Plotting the mean retention factors (k') of the analytes (obtained from the four different sample vial conditions) against the pH of the mobile phase, gives a graphical representation (Figure 3.7) of the relationship between the retention behaviour at different mobile phase pH. Isocratic elution was necessary to avoid the pH shift observed during changes in organic mobile eluent under gradient elution conditions.[230] The error bars represent the standard deviation of the mean retention factor observed between the four sample vial conditions. For neutral analytes, caffeine and paraxanthine, changes in mobile phase pH had a minimal effect on their retention behaviour as expected, however, for the ionisable basic analytes, midazolam, hydroxymidazolam, bupropion, omeprazole, and dextromethorphan, significant changes in retention were observed as a result of their degree of ionisation at different pH.

Most of the analytes carry more than one functional group in their chemical structure, rendering them amphoteric with varied degrees of ionisation depending on the pK_a of the basic or acidic functional groups. Fexofenadine for example has a carboxyl (strong acid), two alcohol functional groups (weak acid) and a nitrogen atom (weak base) with an overall predicted pK_a value of 4.04, a proton acceptor count of 5 and proton donor count of 3. This would explain why the retention times change significantly in pH ranges between 2.7 and 6.5. At a pH of 2.7, fexofenadine would have a positive charge on the nitrogen atom and would be ionised, rendering it more polar with a shorter retention time on the column. When the mobile phase pH increases to 3.9, the molecule would be 50% ionised since it is near the pKa value, thus increasing its retention time. This could also explain the peak broadening seen at this pH. At two pH units above the pK_a (at pH 6.5), the molecule would be 100% in its ionised form due to the loss of the hydrogen atom on the carboxyl group, again with a shorter retention time. Typical behaviour was observed for the other basic analytes where the pH of the mobile phase increased their degree of ionisation at lower pH, decreasing hydrophobicity and showing shorter retention factors. Large changes in the selectivity were observed for dextromethorphan, bupropion and midazolam when the pH of the mobile phase changed from 6.5 to 8.3, with large changes in retention times. At basic pH the acid functional groups would be deprotonated and the degree of ionisation determined by how close the pH is to the pK_a of the analyte. Similarly, a change in pH from 2.7 to 3.9 changed the retention times of midazolam, OH-midazolam omeprazole, OH-omeprazole, bupropion, OH-bupropion, dextromethorphan and dextrorphan. This could be explained by the fact that the nitrogen atoms in their chemical structures are protonated at low pH. The formation of intermolecular hydrogen bonds should also be considered since their presence increases the hydrophobicity of the molecule and might influence the degree of net

ionisation. This is the case with omeprazole, for example, where an intermolecular hydrogen bond is formed between the oxygen atom on the sulfoxide group and the hydrogen atom on the nitrogen present in the 5-methoxybenzimidazole ring.[242] The authors have also shown that there are three possible acid/conjugate base pairs for omeprazole with the possibility of two protonations, however the first acid/conjugate base pair from the di-cation to the cation was found to be very unstable and the pK_a for this species could not be determined with UV-Vis spectrophotometry.

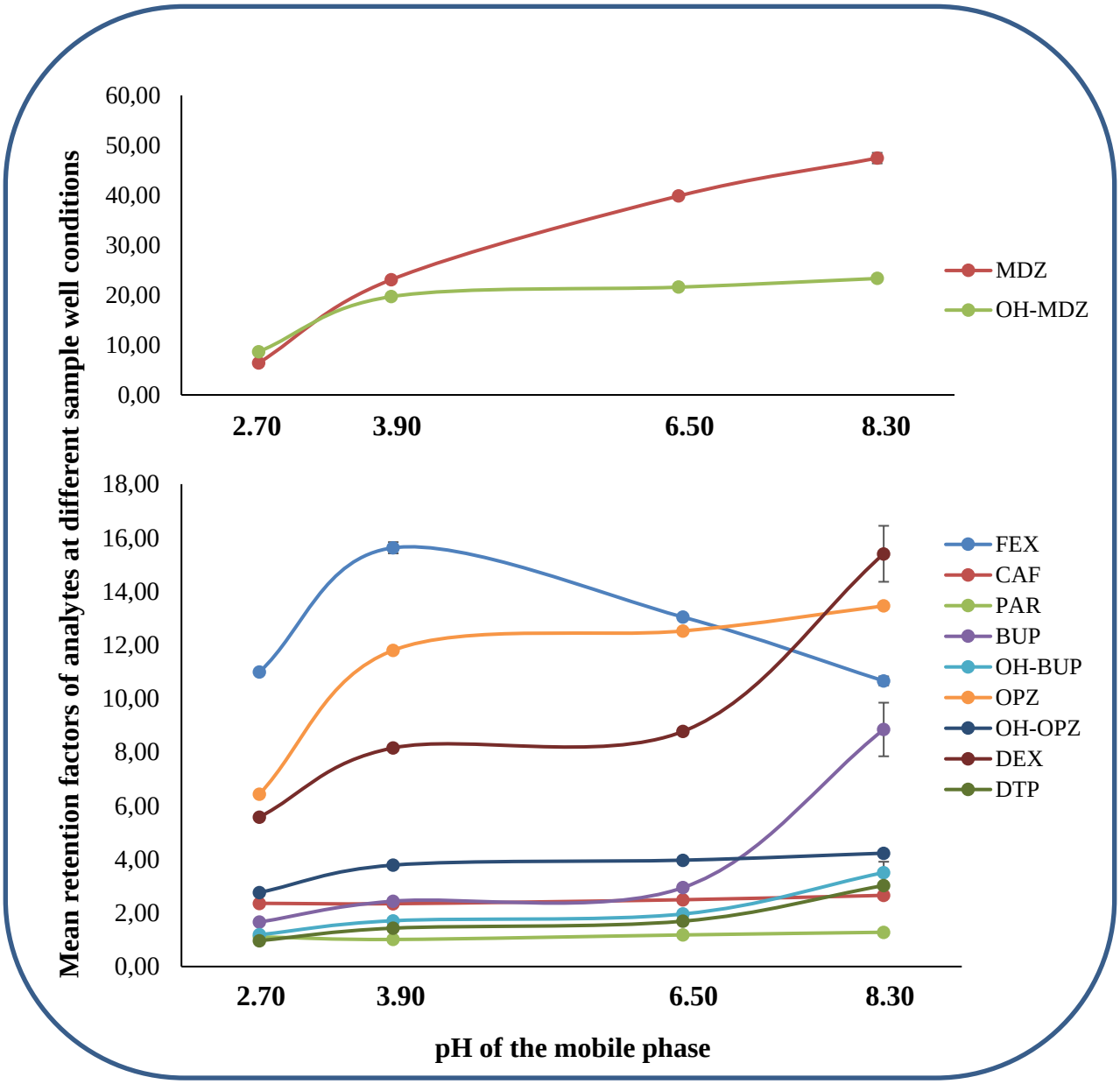


Figure 3.7: Relationship between retention behaviour and the mobile phase pH under isocratic elution conditions, flow rate: 100 µL.min^{-1} with methanol: water (60:40) on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm).

During the LC-MS/MS analysis in this study two distinct peaks at different retention times were observed for omeprazole with the same MRM transitions. When the analyte solution was kept in acidic 0.1% formic acid 50:50 methanol: water solution within the sample vial, omeprazole degraded completely over time and no peaks were observed at these conditions after as little as one hour. This was attributed to the fact that omeprazole undergoes conversion to a cyclic sulphonamide under acidic conditions.[161] When the chromatographic analysis was carried out immediately after extraction, before the acid hydrolysis could reach completion, omeprazole was observed at only the first of the two recorded retention times. Figure 3.8 shows overlaid XIC of omeprazole (100 ng.mL^{-1}) from the four different mobile phase compositions and pH. The peaks were colour coded to depict the different mobile phase conditions.

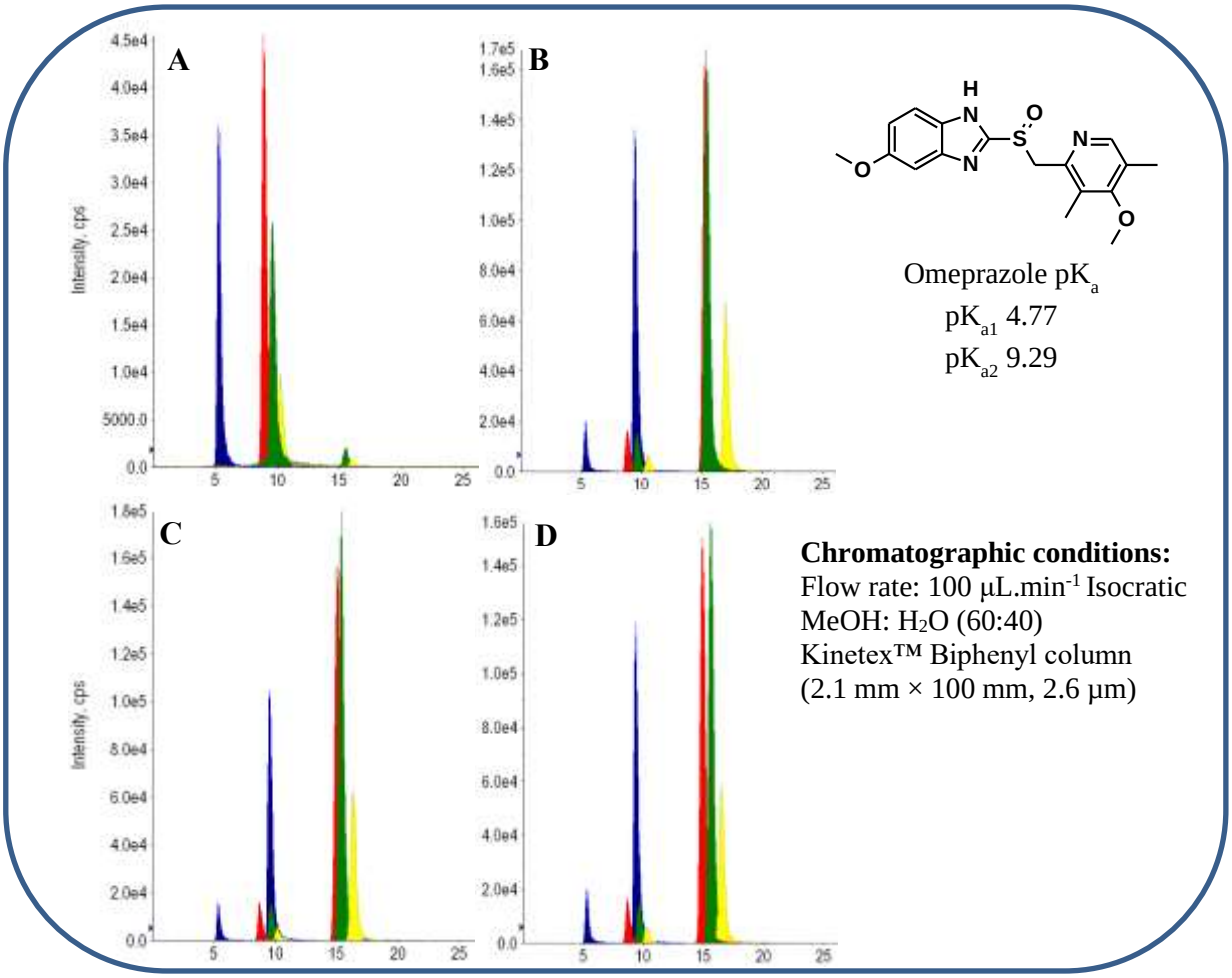


Figure 3.8: Overlaid XIC of omeprazole (100 ng.mL^{-1}) in 50:50 methanol: water containing 0.1% formic acid (A), 10 mM ammonium formate (B), 10 mM ammonium acetate (C) and 5 mM ammonium bicarbonate (D) at different mobile phase conditions; pH 2.7 (blue peak), pH 3.9 (red peak), pH 6.5 (green peak) and pH 8.3 (yellow peak).

Chromatograms A-D, represent the four different sample vial conditions. In chromatogram A, the peaks represent omeprazole kept at 50:50 methanol: water containing 0.1% formic acid in the sample vial, when the solution was made up immediately before analysis and the analysis carried out within one hour. When omeprazole was kept at acidic conditions for longer times prior to injection, no peaks were detected at any mobile phase condition due to its acid hydrolysis. This confirms earlier findings by Wohlfardt *et al.*[161] For sample vial conditions containing ammonium formate, ammonium acetate and ammonium bicarbonate, the second eluting omeprazole peak had higher intensity at all four mobile phase conditions and pH. Omeprazole stability was found to be affected by the pH and composition of the sample vial and possibly different acid-base pairs forming at different pH. The peak intensities and analyte peak areas were stable under conditions containing ammonium formate, ammonium acetate and ammonium bicarbonate, despite the observation of a consistent small peak at an earlier retention time. This finding warrants further investigation to confirm whether the observed two peaks were the result of the simultaneous presence of both cation and unstable di-cation or the presence of an omeprazole impurity. During final method development, working standard solutions of omeprazole were freshly prepared from a commercially available capsule on each validation day, where only one peak was observed, consistent with the second eluting and most prevalent peak from analytical standards.

Although the retention times for all analytes except omeprazole and its hydroxylated metabolite, were stable at the four different sample vial conditions, the ESI efficiency and hence the analyte sensitivity was influenced by both the composition of the solvent in the sample vial and the mobile phase used. The measured mean peak areas from triplicate injections ± the standard deviation, in counts per second, at each of the 16 possible combinations are presented in Figure 3.9. When developing a quantitative analytical method for the simultaneous low concentration detection of analytes with different physicochemical properties, at low concentrations, from low volume biological samples (like a 20 µL DBS), it is important that the sensitivity and detection limits are optimised. The ESI source produces charged molecules which are then detected by the mass spectrometer. This ionisation technique is a complex process influenced by many parameters, including solvent ratios, boiling temperatures and additive characteristics (ionic strength, volatility, viscosity, pH, electrolyte concentration etc.), analyte physicochemical properties (pKa, Log P, ion solvation energy, proton affinity etc.) and operational parameters (voltage, flow rate, desolvation temperature etc.).[241]

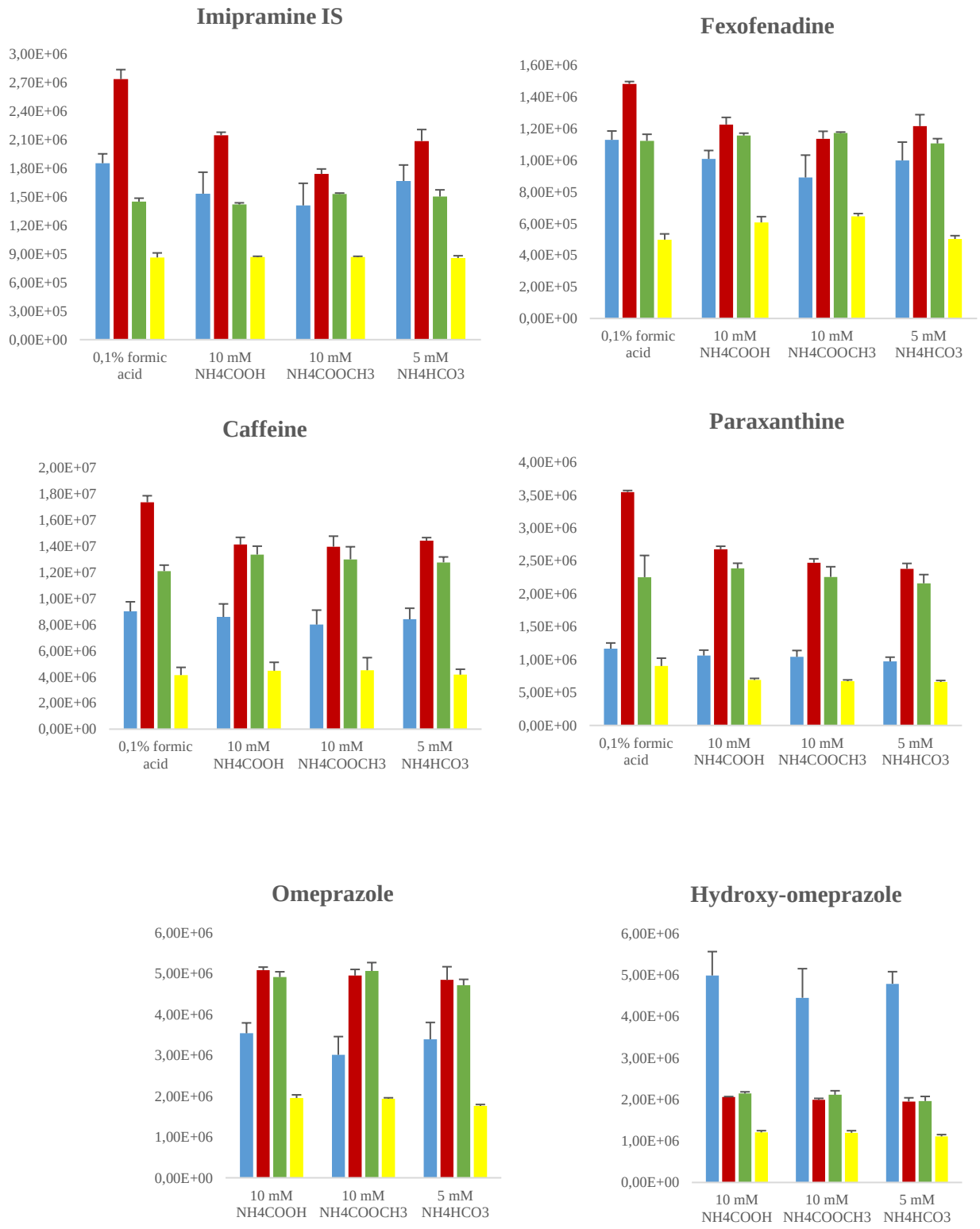


Figure 3.9: Comparison of the average analyte peak area (cps) (n = 3), measured in human plasma at four different mobile phase conditions and pH; blue (pH 2.7), red (pH 3.9), green (pH 6.5) and yellow (pH 8.3), with the analytes at different sample vial conditions. Error bars represent the SD between triplicate analytical measurements.

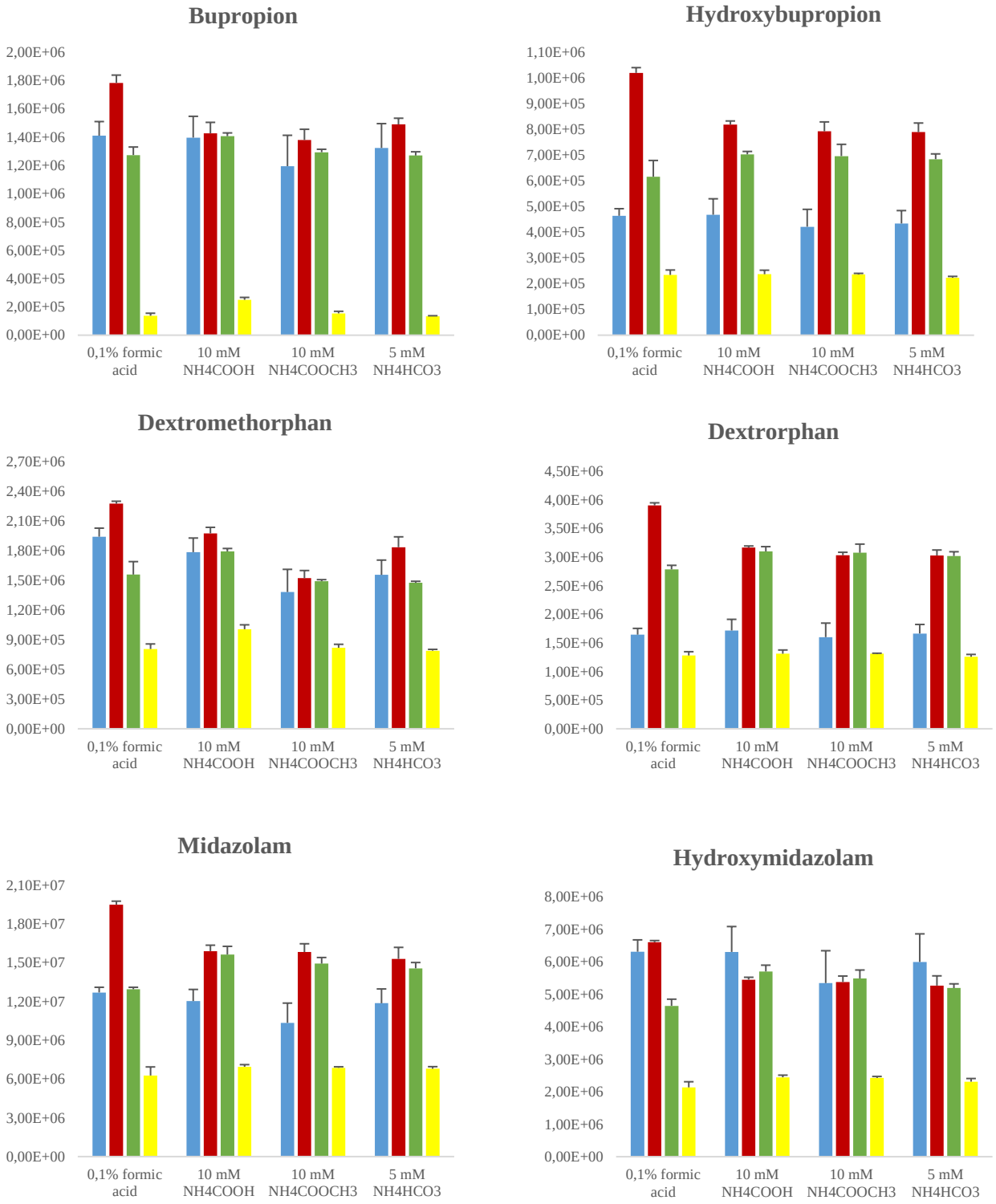


Figure 3.9 (continued): Comparison of the average analyte peak area (cps) (n = 3), measured in human plasma at four different mobile phase conditions and pH; blue (pH 2.7), red (pH 3.9), green (pH 6.5) and yellow (pH 8.3), with the analytes at different sample vial conditions. Error bars represent the SD between triplicate analytical measurements.

From Figure 3.9 it is clear that the signal response or sensitivity (and ultimately the ESI ionisation efficiency of the analytes) varied greatly for the same concentration injected into the ESI source after chromatographic separation using mobile phases of different pH. The flow rate, desolvation temperature and ESI voltage were kept the same under isocratic mobile phase conditions. From first visual observations it was concluded that ammonium bicarbonate at 5 mM caused signal suppression (reduced ionisation efficiency) of all analytes, regardless of the sample vial conditions. Extensive peak tailing for the internal standard imipramine and dextromethorphan could be anticipated from the incompatibility of this mobile phase (5 mM ammonium bicarbonate) when paired with the basic properties of these pharmaceutical analytes.

The greatest overall sensitivity was achieved with a mobile phase at pH 3.9 (consisting of both formic acid and ammonium formate), despite the conditions of the sample vial, except for omeprazole and its hydroxylated metabolite that degraded in the sample vial consisting of 0.1% formic acid. The repeated measures two factor ANOVA revealed interaction effects between the conditions of the sample vial when paired with different mobile phase pH (Table 3.6), affecting the analyte peak areas and thus the detection limits, sensitivity and overall ESI efficiency of the analytes in a plasma matrix.

A repeated measures two factor ANOVA, with Geisser-Greenhouse correction, tested both the effect of the two independent variables, i.e. the composition of the solvent in the sample vial and the mobile phase conditions and pH, on the analyte sensitivity (peak area measured in cps) and the interaction effect between the two, on analyte sensitivity and ultimately ESI efficiency. Normal distribution was confirmed for most analytes at all four mobile phase conditions with the Shapiro-Wilk normality test ($\alpha = 0.05$). Normal and lognormal distribution were compared and data transformed where a lognormal distribution was more likely, before the ANOVA test was performed. For the most, data conformed to a Gaussian distribution (40/48) and in which cases it did not, results for a repeated measures ANOVA, were confirmed using a repeated measures ANOVA for ranks.

The effect of the sample vial composition on the analyte peak areas of bupropion ($p = 0.0130$*), hydroxy-bupropion ($p = 0.0462$*), dextromethorphan ($p < .001$**) and internal standard imipramine ($p < .001$**) were found to be a source of variance, however, effect sizes were small (< 1% of the total variance for bupropion and hydroxy-bupropion; 5.68% for internal standard imipramine; 11.11% for dextromethorphan). Acidic conditions (0.1% formic acid) in the

sample vial clearly influenced omeprazole and hydroxy-omeprazole, due to rapid acid hydrolysis and was subsequently excluded from further analysis to test the interaction of the other sample and mobile phase parameters.

The mobile phase composition was found to be the largest source of variance on analyte peak areas with the percentage contribution to overall variance 88.02% for fexofenadine (p < .001**), 95.08% for caffeine (p = 0.003**), 91.24% for paraxanthine (p < .001****) , 97.20% for bupropion (p < .001***), 93.82% for hydroxy-bupropion (p < .001***), 96.52% for omeprazole (p = 0.0014**), 96.40% for hydroxy-omeprazole (p = 0.007**), 78.49% for dextromethorphan (p = 0.002**), 94.04% for dextrorphan (p = 0.0013**), 93.67% for midazolam (p = 0.0023), 88.64% for hydroxymidazolam (p = 0.0122*) and 82.33% for imipramine (p = 0.0048**).

The interaction effect between both the sample vial condition and the mobile phase pH were a source of variance for the following analytes: 6.99% for fexofenadine (p = 0.0043**), 2.73% for caffeine (p = 0.0047**), 4.24% for paraxanthine (p = 0.0421*), 1.60% for bupropion (p = 0.0131*), 3.91% for hydroxy-bupropion (p = 0.0132*), 7.22% for dextromethorphan (p = 0.0278*), 4.34% for dextrorphan (p = 0.0065**), 3.93% for midazolam (p 0.0169*), 5.64% for hydroxymidazolam (p = 0.0077**) and 9.15% for imipramine (p = 0.0046**).

From the repeated measure two factor ANOVA, it was concluded that both the composition and pH of the mobile phases were the main source of variance on analyte peak area for all the analytes. Tukey's multiple comparison tested multiple hypothesis with pair-wise comparisons between the mean difference of each of the different mobile phases for each independent sample vial condition. As expected, a mobile phase pH of 8.3 had a significant effect on the retention behaviour and the sensitivity of the basic analytes (visualised in Figure 3.9 – details on Tukey's multiple comparison test not shown here). Other significant sources of variance were acidic conditions in the sample vial on the stability of omeprazole and hydroxy-omeprazole.

More importantly, the choice of mobile phase pH significantly influenced the retention behaviour of the analytes resulting in unacceptable retention factors. Figure 3.10 is a XIC chromatogram depicting retention factors well in excess of 10, for midazolam, hydroxymidazolam and dextromethorphan at basic pH.

It should be noted that the flow rate may also influence ESI efficiency and retention behaviour as could be seen when higher flow rates were used during final validation assays. Considering

all factors influencing the resolution of the analytes, i.e. the retention, selectivity, stability and sensitivity, a 10 mM ammonium formate in the sample vial with a mobile phase containing 0.1% formic acid (pH 2.7) were chosen for method validation using a Kinetex biphenyl column as the stationary phase. With this combination, analyte sensitivity was sacrificed for a higher throughput method and better peak shape (column efficiency). The method was still sensitive enough with detection limits acceptable for all analytes of interest, in both plasma and DBS to enable low dose administration for phenotyping despite small volume dry blood spot sampling.

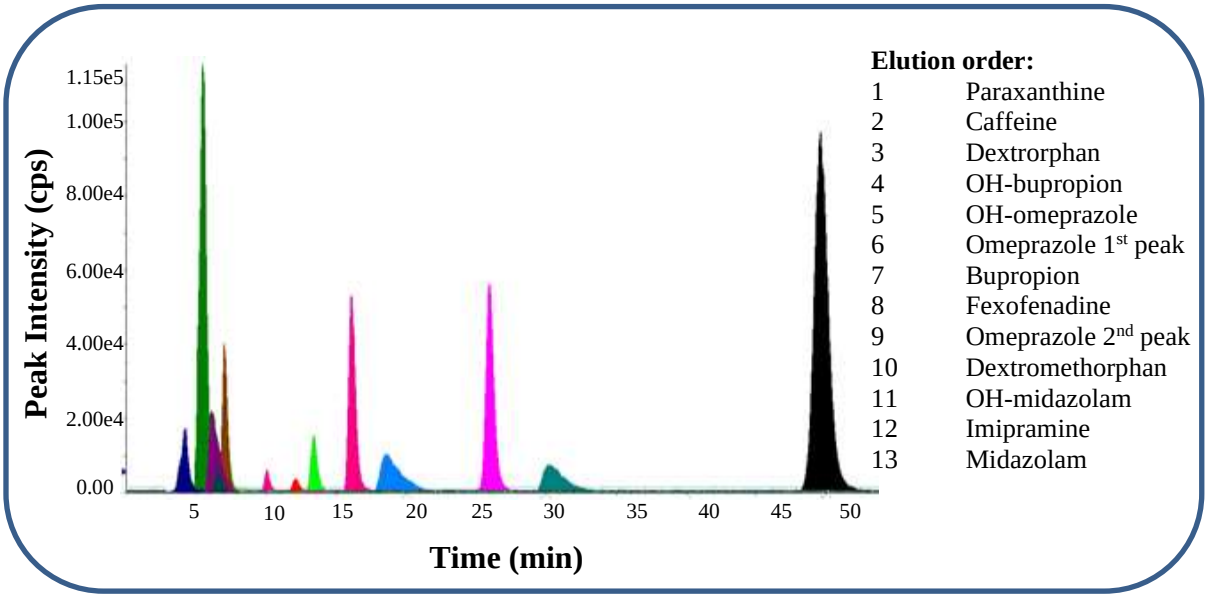


Figure 3.10: XIC of analytes (100 ng.mL^{-1}) from a sample in 50:50 methanol: water 5 mM ammonium bicarbonate separated by isocratic (100 µL.min^{-1}) 60:40 methanol: water containing 5 mM ammonium bicarbonate on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm).

3.4 CONCLUSION

In conclusion, the method was assessed for optimal resolution of all the analytes by altering the stationary phase and mobile phase composition and pH. Changes in these analytical conditions caused statistically significant variation in the retention behaviour, selectivity, column efficiency and sensitivity of the analytes of interest and therefore these parameters were controlled for reliable, reproducible quantitative results during method validation.

CHAPTER 4: COMPOUNDING PHENOTYPING COCKTAIL CAPSULES AND ASSESSMENT OF WEIGHT AND CONTENT UNIFORMITY

4.1 INTRODUCTION

The validated Geneva phenotyping cocktail were compounded from commercially available dosage forms which introduce challenges with regards to dosage homogeneity, chemical interference or degradation and possible incompatibilities of drugs when used in combination. USP assays were performed to determine the weight and content uniformity of the compounded phenotyping cocktail containing six of the seven probe drugs. Content uniformity was evaluated with an Acquity UPLC system coupled to a Synapt G2 QTOF mass spectrometer (Section 2.7.2).

4.2 MATERIALS AND METHODS

4.2.1 Ingredients used for compounding of the phenotyping cocktail capsules

A 6-drug phenotyping cocktail capsule was compounded from three Sigma USP analytical standards; caffeine, flurbiprofen and dextromethorphan HBr salt and from three commercially available drug formulations; bupropion as bupropion hydrochloride in Wellbutrin™ XL 300 mg tablets, midazolam as midazolam maleate in Dormicum™ 15 mg tablets and fexofenadine as fexofenadine hydrochloride in Fexo™ 180 mg tablets. Due to problems with stability, omeprazole should be given as a single 10 mg Omez™ capsule, equivalent to a therapeutic paediatric dose, and did not form part of the phenotyping cocktail capsule. Other excipients present within the commercially available formulations are given below:

- Wellbutrin™ XL 300 mg tablets: polyvinyl alcohol, glyceryl behenate in the Tablet core and ethyl cellulose, povidone, polyethylene glycol 1450, methacrylic acid copolymer dispersion, silicone dioxide and triethyl citrate
- Dormicum™ 15 mg tablets: carmellose sodium, lactose anhydrous, hypromellose, indigo carmine (CI73015), macrogol, magnesium stearate, maize starch, microcrystalline cellulose, polyacrylate dispersion, talc and titanium dioxide (CI 77891)
- Fexo™ 180 mg tablets: powdered cellulose, mannitol (E421), maize starch, croscarmellose sodium, colloidal anhydrous silica, magnesium stearate, hypromellose (E464), titanium dioxide (E171), macrogol 400, allura red AC (E129), and iron oxide.

4.2.2 Compounding procedures and assessment of weight and content uniformity

Ten individual tablets of each commercially available formulation were weighed on a Precisa XT 120A™ precision balance to determine the average weight, standard deviation and CV per Tablet (Table 4.1), containing the formulated amount of active ingredient.

All ten tablets, of each formulation, were pooled, grinded to a homogenous powder using a mortar and pestle and the required amounts weighed out to constitute the final active ingredient mix. Table 4.2 summarises the calculated weight content per capsule, active ingredient weight per capsule and the total weight of each analyte carried over to a Kenwood blender for homogenisation of the active phenotyping cocktail blend.

Table 4.1: Average weight per Tablet for each of the commercially available analytes to be used in the compounded phenotyping cocktail.

Analyte	Average weight per Tablet (mg)	Std dev.	CV (%)
Fexo™ 180 mg tabs	627.95	7.26	1.16
Wellbutrin™ XL 300 mg tabs	355.58	4.66	1.31
Dormicum™ 15 mg tabs	211.16	1.31	1.1

Table 4.2: Compounded phenotyping cocktail capsule active mix, active amount per capsule and total weighed out for homogenisation.

Ingredient	Content per capsule (mg)	Active per capsule (mg)	Total weighed out (mg)
Caffeine (CAF)	50.00	50.00	10 000.40
Flurbiprofen (FLB)	10.00	10.00	2 000.10
Dextromethorphan (DEX)	10.00	10.00	2 000.10
Bupropion (Wellbutrin™) (BUP)	23.86	20.00	4 771.60
Midazolam (Dormicum™) (MDZ)	14.01	1.00	2 801.00
Fexofenadine (Fexo™) (FEX)	87.70	25.00	17 540.60

A Precisa XT 120A balance were used to weigh out the analytes in mg for a total of 200 capsules, since the hand-filler could compound a minimum 150 capsules at a time. The extra 25% allowed for experiments to determine the fill volume per capsule. Compounding was done by the Department of Pharmaceutics at the School of Pharmacy on the Potchefstroom campus of the University of the North-West.

After the compounding of the phenotyping capsules, weight and content uniformity assays were performed before the cocktail could be used for oral administration.

4.2.2.1 Weight uniformity of the compounded phenotyping cocktail capsules

The accurate weight of 20 capsules from the compounded batch were recorded with a Scientech ZSA 120™ balance, in accordance with the United Stated Pharmacopoeia (USP) standards. Individual capsules were emptied and the powdered content of each capsule accurately weighed and recoded to determine the actual content weight and reported as the average ± the standard deviation. The calculated coefficient of variation was deemed acceptable within 10% of the calculated mean.

4.2.2.2 Content uniformity of the compounded phenotyping cocktail capsules

The content uniformity of the 6 analytes in the phenotyping cocktail was measured with a UPLC-QTOF-MS/MS system (Section 2.7.2). A standard calibration curve in methanol was prepared by weighing out, in triplicate, amounts of each analyte equal to the active probe drug present in the cocktail. Working standard solutions were diluted (1:100) from the stock standards with methanol. The working standard solutions were then pooled and an analyte mix prepared containing all analytes in 1:1 methanol: water containing 0.1% formic acid and 2 mM ammonium formate. The final 7-point calibration concentrations, in $\mu g.mL^{-1}$, are given in Table 4.3.

The content of twenty compounded capsules were volumetrically carried over to 10 mL volumetric flasks (A-grade) with methanol and after 20 minutes of sonication and 15 minutes of vortex mixing the solutions were made up to volume with methanol and left at 4ºC for one hour to equilibrate. The supernatant was diluted (1:1000) in a mixture of 1:1 methanol: water containing 0.1% formic acid and 2 mM ammonium formate, using Eppendorf pipettes. The final solution was centrifuged at 14,000 *xg* for 10 minutes to remove any magnesium stearate

particles used as the filler in the formulated phenotyping capsules. The solution was carried over to autosampler vials with a 0.2 µm syringe filter for analysis.

The probe drugs in the phenotyping cocktail were separated on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm particle size), with the column temperature controlled at 40°C. A gradient "inlet method" was set up using LC-MS grade water containing 0.1% formic acid and 2 mM ammonium formate as aqueous mobile phase and an 80:20 MeOH: ACN organic mix, containing 0.1% formic acid.

Table 4.3: Calibration curve concentrations in (µg.mL^{-1}) for content uniformity assay

Analyte	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7
CAF	0.500	1.251	2,502	3.753	5.003	7.505	10.007
BUP	0.200	0.499	0.999	1.498	1.997	2.996	3.995
FLB	0.1003	0.2508	0.5017	1.1250	1.0033	1.5050	2.0067
DEX	0.0997	0.2493	0.4985	0.7478	0.9971	1.4956	1.9941
MDZ	0.0100	0.0250	0.0500	0.0749	0.0999	0.1499	0.1998
FEX	0.2504	0.6259	1.2519	1.8778	2.5038	3.7557	5.0076

The optimised inlet method (Waters AQUITY UPLC system) was programmed, starting with 25% mobile phase B going up to 48% by 0.10 minutes, with a shallow gradient from 48% to 62% over the next 3.9 minutes at a fixed flow rate of 350 µL.min^{-1} and the upper UPLC pressure limit set at 8000 psi. The gradient was further extended to high organic for 0.25 minutes, to wash off any lipophilic analytes remaining on the column, before re-equilibrating the column to starting conditions for one minute for a total UPLC run time of 5.50 minutes. A needle wash step was included between each injection to limit sample carry-over. Sample temperature was controlled with the Waters AQUITY Sample Manager at 4°C, ensuring analyte stability and the sample injection volume was set at 5 µL.

Quantitative data-independent acquisition (DIA) was performed with a Water Synapt G2 QTOF mass spectrometer in positive and negative ESI resolution mode. Leucine enkephalin (50 pg. mL^{-1}) was used as the lock mass calibrant, ensuring accurate mass acquisition. ESI source parameters were optimised by infusing the analyte mix directly into the Z-spray source. The optimal source conditions used in the acquisition MS tune file were: capillary voltage 3.0 kV,

source temperature 120°C, sampling cone voltage 30.0 V, extraction cone voltage 4.0 V and the cone gas flow set at 20 $L.Hr^{-1}$. The desolvation temperature was 450°C with the desolvation gas flow 300 $L.Hr^{-1}$. An MS^e-experiment, acquiring data in both low (10 V) and high (30 V) collision energy functions simultaneously, allowed alignment of precursor and fragment ions of all analytes at their respective retention times within the acquisition mass range set at 100 – 600 m/z with a scan time of 0.1 sec.

4.3 RESULTS AND DISCUSSION

Producing a homogenous analyte powdered mix proved to be a challenge, probably due to interference or possible incompatibilities of the different excipients present in the commercial dosage forms which complicated calculations of fill volume and final volume of magnesium stearate to be added as filler. Additionally, the low flowability of the final powder mix affected the ease of hand-filling of the capsules.

4.3.1 Weight uniformity

The average weight content of the phenotyping capsules and nett content (in mg ± standard deviation) with the percentage coefficient of variation (% CV) are summarised in Table 4.4. The % CV fell within 10% of the calculated mean and this was deemed acceptable for the phenotyping drug cocktail capsules for use in participants of a pharmacokinetic trial.

Table 4.4: Average weight (in mg ± standard deviation) of the compounded phenotyping drug cocktail capsules and the nett dry powdered content.

	Weight uniformity of compounded phenotyping cocktail capsules (n=20)	
	Weight of Capsule (mg)	**Weight of dry content (mg)**
Average	432.505	331.42
stdev	21.676	21.585
% CV	5.012	6.513

4.3.2 Content uniformity

The UPLC-QTOF-MS/MS acquisition mode, retention time (with % CV) of each analyte, standard curve calibration equations and linearity are given in Table 4.5. A typical ESI+ XIC chromatogram is shown in Figure 4.1. The standard curves for each individual drug were used

to calculate the content of each analyte in a sample of 20 randomly selected capsules after filling.

Table 4.5: UPLC-Q-TOF acquisition mode, retention time and calibration equation of all analytes compounded in the phenotyping cocktail.

	Acquisition mode	Retention time (min)		Calibration equation		
		Average	%CV	linearity (r^2)	m	c
CAF	+	1.37	0.32	0.9952	234.889	-186.548
BUP	+	1.48	0.50	0.9971	5.303	0.736
FLB	-	1.89	0.22	0.9994	40.177	-3.680
DEX	+	2.28	0.77	0.9942	4263.360	0.443
MDZ	+	2.41	0.47	0.9940	4162.770	-34.401
FEX	+	3.40	0.53	0.9944	1034.250	-157.075

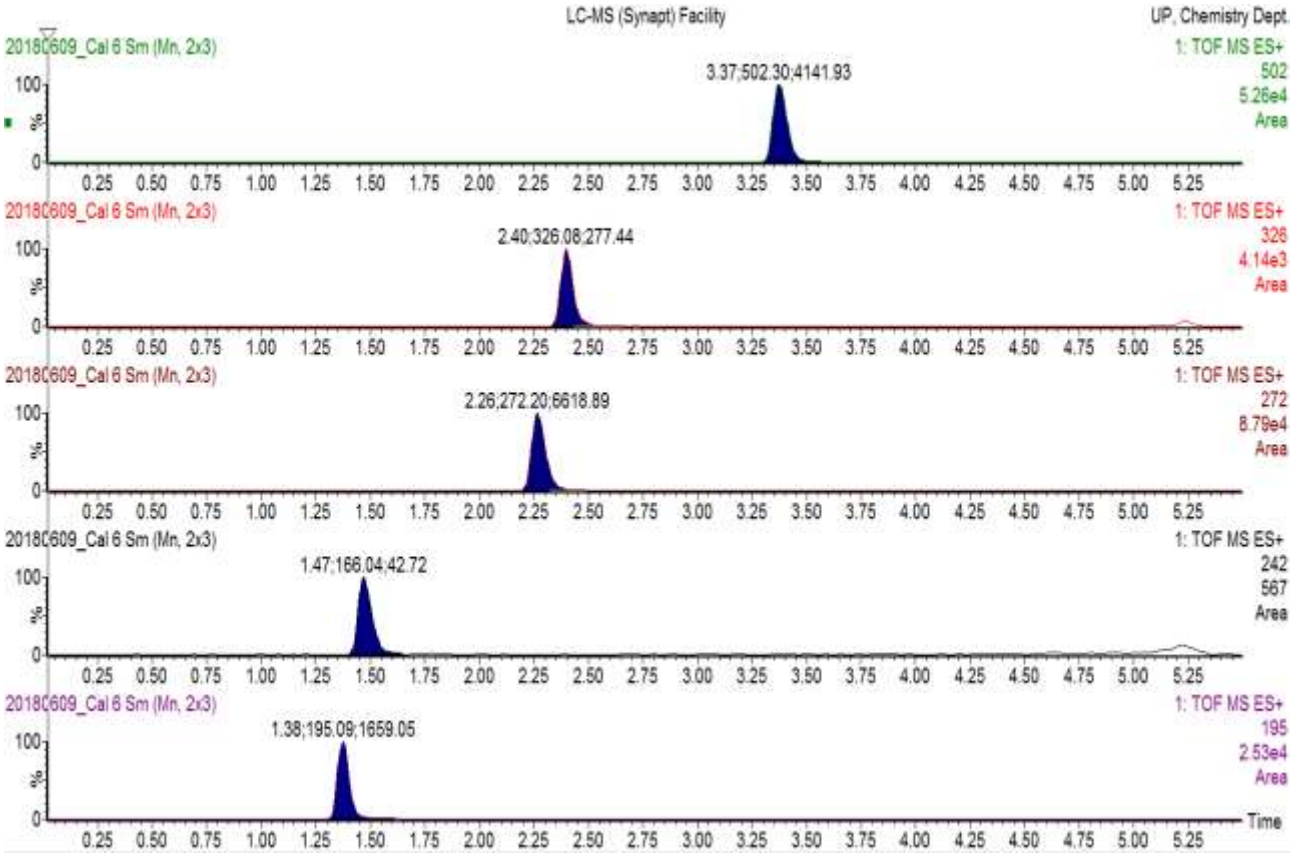


Figure 4.1: XIC chromatogram obtained from a standard calibrant depicting analyte elution order and retention times in positive ESI resolution mode.

The expected and found concentrations (mean in µg.mL^{-1} ± standard deviation) of all analytes with calculated precision (% CV) and percentage recovery for all analytes are summarised in Table 4.6.

Table 4.6: Content uniformity of phenotyping cocktail analytes with percentage recovery and precision.

	Capsule content (n=20) made up to 10 mL each			
	Expected (µg.mL^{-1})	**Found (mean in µg.mL^{-1} ± SD)**	**CV (%)**	**Recovery (%)**
CAF standard	5.00	5.386 ± 0.285	5.29	107.72
BUP as BUP.HCl	1.75	1.357 ± 0.095	7.01	77.68
FLB standard	1.00	0.822 ± 0.115	14.04	82.15
DEX as DEX.HBR	1.00	0.890 ± 0.084	9.47	88.95
MDZ as MDZ maleate	0.07	0.057 ± 0.004	7.69	78.09
FEX as FEX.HCl	2.51	3.230 ± 0.147	4.55	128.51

According to the USP, the acceptance value for dosage forms containing less than 25 mg active ingredient should be ≤ 15%. In the compounded phenotyping capsules, content uniformity fell outside the allowed 15%. Most notable was that this high error occurred for all the purchased commercially available formulations that were re-homogenised using a mortar and pestle. The stipulated dose of fexofenadine was over estimated by 28.51% and under estimated by 21.91% for midazolam and 22.32% for bupropion, although this was a consistent trend throughout the content uniformity assay with % CV between 4.55 and 7.69%. The largest variation was found with analytical standard flurbiprofen that had a % CV value of 14.04%. Variances and calculated analyte content should be kept in mind when phenotyping patient cohorts with the current cocktail capsule. In their article: "CombiCap: A novel drug formulation for the Basel phenotyping cocktail"[247], the authors emphasise suboptimal phenotyping with previous drug cocktails due to non-standardised pharmaceutical drug formulation. The results of this study support their report. Their platform loads all cocktail analytes into di-calcium phosphate particles to produce individual analyte immediate release mini-tablets compounded into one capsule.

4.4 CONCLUSION

Standard requirements for diagnostic phenotyping cocktail formulations are recommended to ensure uniform release kinetics with consistent inactive excipients and chemical stability of multidrug cocktails. Although the compounded cocktail in this study did not meet the required USP standards for pharmaceutical products, it could still be used to assess individual patient metabolic and transport phenotypes, with individual micro-dosing and use of metabolite to parent drug ratios.

CHAPTER 5: LC-MS/MS METHOD VALIDATION AND ASSESSMENT OF METHOD AGREEMENT BETWEEN DRIED BLOOD SPOT AND PLASMA SAMPLING

5.1 INTRODUCTION

A triple quadrupole high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) system (Section 2.7.1) were used for method validation and assessment of agreement between the two sampling methods. Optimised MS fragmentation source conditions (Table 3.2 and 3.3) were used to monitor the most abundant MRM transitions for all analytes. After optimisation of the chromatographic separation method, the method was validated according to ICH guidelines for matrix effects, recovery, linearity, limits of quantitation and detection, carry-over, inter and intraday precision and accuracy and analyte stability in plasma and whole blood matrixes. The data from the validation experiment was used to assess *in vitro* agreement between DBS and plasma sampling using a statistical approach as recommended by the International Consortium Microsampling Working Group.

5.2 MATERIALS AND METHODS

5.2.1 Optimisation of the chromatographic separation method

Chromatographic separation of all probe drug analytes and their metabolites was achieved on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm particle size) with the column temperature set at 40°C ± 3°C to reduce system backpressure in combination with a viscous methanolic mobile phase. A standard gradient elution program, starting with high aqueous ratio changing to a high organic eluent ratio over time, was used as a starting point to determine initial elution order and co-elution with different organic mobile phase compositions containing mostly methanol to enhance the π-π interactions of the stationary phase with the aromatic analytes. The organic phases consisted of 100% methanol, 90:10 methanol: acetonitrile and 80:20 methanol: acetonitrile for which the system backpressure, analyte retention and selectivity under a mobile flow rate of 300 µL.min^{-1} was assessed. The aqueous and organic mobile phases all contained 0.1% formic acid to improve analyte peak shape. Gradient elution programs were manipulated to optimise analyte separation and to determine the best separation within an acceptable separation time frame. An analyte mixture dissolved in 10 mM NH_4COOH 50:50 methanol: deionised water was prepared just prior to sample injection and made up

freshly on each day of the analysis. The sample injection volume for all injections was set at 10 µL. Based on the optimised chromatographic separation, the final method with gradient elution was set up with "scheduled" MRM with a 60 second detection window for each analyte and a scan time of 0.1 second ensuring more data points collected over each analyte chromatographic peak for accurate peak integration and quantitation.

5.2.2 Human plasma and whole blood collection and storage

Caffeine-free human blank whole blood was collected by individual venepuncture from healthy volunteers into four purple 4 mL ethylene-diamine-tetra acetic acid (EDTA) vacutainers (batch # 8004813) for each volunteer. The whole blood was stored immediately at 4°C and used within four hours after collection to prepare blank and spiked dried blood spot samples for method validation. Blank plasma was obtained from separate EDTA tubes by centrifugation (1500 *xg* for 15 minutes) at room temperature. Aliquoted human plasma samples were stored at -80°C and were allowed to thaw at room temperature and vortex mixed before sample preparation and extraction.

5.2.3 Sample preparation procedures

5.2.3.1 Preparation of stock and working standard solutions

Standard stock solutions of all seven probe drugs, six CYP450 metabolites and IS at concentrations of 1 $mg.mL^{-1}$ were prepared in methanol. Paraxanthine, hydroxy-bupropion, omeprazole and hydroxy-midazolam were bought as 1 $mg.mL^{-1}$ (m/m) solutions from Cerilliant and midazolam was diluted (1:5) volumetrically to 1 $mg.mL^{-1}$ (v/v) from sterile Dormicum™ 5 $mg.mL^{-1}$ ampoules. The powdered analytes (caffeine, bupropion, flurbiprofen, hydroxy-flurbiprofen, hydroxy-omeprazole, dextromethorphan, dextrorphan, fexofenadine, imipramine and probenecid) were accurately weighed with a Sartorius microbalance (Zeiss West Germany Pty. Ltd.) on small pieces of foil and carried over to 1 mL volumetric flasks. Final solutions were prepared gravimetrically by adding accurate masses of methanol (density 0.79 $mg.mL^{-1}$) to make up 1 $mg.mL^{-1}$ (m/m) solutions using a Precisa XT 120A™ balance. Working standard solutions were diluting from stock solutions, in methanol (v/v), on each day of the validation, using Thermo Scientific™ eVol™ dispensing system syringes. Final working standard solutions were stored at -20ºC. The working standard concentrations were 20 $\mu g.mL^{-1}$ and 2 $\mu g.mL^{-1}$ for caffeine and flurbiprofen, 10 $\mu g.mL^{-1}$ and 1 $\mu g.mL^{-1}$ for paraxanthine, bupropion, hydroxy-bupropion, hydroxyflurbiprofen, omeprazole, hydroxy-omeprazole, dextro-

methorphan, dextrorphan and fexofenadine, 4 µg.mL^{-1} and 0.4 µg.mL^{-1} for midazolam and hydroxy-midazolam, taking into account the signal response of the mass spectrometer and the expected *in vivo* concentrations in human whole blood and plasma after administration of phenotyping doses.

5.2.3.2 Preparation of spiked plasma, whole blood and solvent samples

Plasma, whole blood and solvent were spiked from the working standard solutions to form an initial 9-point calibration curve, including the blank, on the first day of the validation experiment, to assess limits of detection (LOD), limits of quantitation (LOQ), range, linearity, recovery and absolute matrix effects. This resulted in a total experimental run time of 42 hours, with triplicate injections. Due to time constraints, a 5-point calibration was used for further assessment of linearity, recovery, accuracy and precision on the second and third days of validation. Relative matrix effects were assessed from three different batches at three concentrations levels. Finally, matrix effects were evaluated with the direct post column infusion method. The final calibration ranges for all analytes, after spiking human blank plasma, whole blood or solvent before extraction are given in Table 5.1 and Table 5.2 summarises the three concentration levels spiked after extraction.

5.2.3.3 Protein precipitation extraction from human plasma

To assess analyte recovery, linearity, precision and accuracy, appropriate volumes from working standard analyte mixture containing all probe drugs and metabolites were added to 2 mL Eppendorf tubes, using Thermo Scientific™ eVol™ dispensing system syringes, to make up the calibration range, covering the expected *in vivo* concentrations. The solvent was evaporated under vacuum at 30°C (Centrivap™, Labconco, Kansas City, MO, USA) and reconstituted with 100 µL of thawed plasma followed by 10 minutes of vortex mixing before a 3-step protein precipitation extraction. Blank plasma samples were also prepared and extracted with the same method. The extraction was performed by adding 100 µL of acetonitrile to both blank and spiked plasma samples, followed by sonication (Bran Sonic 52 ultrasonicator) for 15 minutes and 5 minutes vortex mixing (Lasec Vortex Genie2). The sonication and vortex mixing steps were repeated twice more after further additions of 100 µL acetonitrile each. After protein precipitation the mixtures were centrifuged (Beckman Coulter Microfuge™ 16 centrifuge) at 14,000 *xg* for 10 minutes to remove all precipitated plasma proteins.

Table 5.1: Calibration curve concentrations (9 point excluding the blank for initial assessment and 5 point highlighted in yellow) for the Geneva phenotyping cocktail probe drugs and metabolites after spiking plasma, whole blood or solvent before extraction and IS concentrations.

Analyte	Working std ($\mu g.mL^{-1}$)	Concentrations ($ng.mL^{-1}$) prepared from low concentration working standard solution			Calibration concentrations ($ng.mL^{-1}$) prepared from high concentration working std. (specific volumes evaporated and reconstituted given in blue)				
		3.0 µL	7.5 µL	15 µL	3.0 µL	7.5 µL	15 µL	30 µL	45 µL
Caffeine	2; 20	60	150	300	600	1500	3000	6000	9000
Paraxanthine	1; 10	30	75	150	300	750	1500	3000	4500
Bupropion	1; 10	30	75	150	300	750	1500	3000	4500
OH-Bupropion	1; 10	30	75	150	300	750	1500	3000	4500
Flurbiprofen	2; 20	60	150	300	600	1500	3000	6000	9000
OH- Flurbiprofen	1; 10	30	75	150	300	750	1500	3000	4500
Omeprazole	1; 10	30	75	150	300	750	1500	3000	4500
OH- Omeprazole	1; 10	30	75	150	300	750	1500	3000	4500
Dextromethorphan	1; 10	30	75	150	300	750	1500	3000	4500
Dextrorphan	1; 10	30	75	150	300	750	1500	3000	4500
Midazolam	0.4; 4	12	30	60	120	300	600	1200	1800
OH- Midazolam	0.4; 4	12	30	60	120	300	600	1200	1800
Fexofenadine	1; 10	30	75	150	300	750	1500	3000	4500
Internal standards									
Imipramine	0.5								
Probenecid	0.25								

Table 5.2: Concentration levels for the Geneva phenotyping cocktail probe drugs and metabolites in plasma, whole blood or solvent spiked after extraction.

Analyte	Concentrations ($ng.mL^{-1}$) for matrix effect assessment prepared from working standard solutions given in Table 3.2					
	Plasma			DBS		
	Low	Medium	High	Low	Medium	High
Caffeine	37.50	187.50	375.00	15.00	75.00	150.00
Paraxanthine	18.75	93.75	187.50	7.50	37.50	75.00
Bupropion	18.75	93.75	187.50	7.50	37.50	75.00
OH-Bupropion	18.75	93.75	187.50	7.50	37.50	75.00
Flurbiprofen	18.75	93.75	187.50	7.50	37.50	75.00
OH- Flurbiprofen	18.75	93.75	187.50	7.50	37.50	75.00
Omeprazole	18.75	93.75	187.50	7.50	37.50	75.00
OH- Omeprazole	18.75	93.75	187.50	7.50	37.50	75.00
Dextromethorphan	18.75	93.75	187.50	7.50	37.50	75.00
Dextrorphan	18.75	93.75	187.50	7.50	37.50	75.00
Midazolam	7.50	37.50	75.00	3.00	15.00	30.00
OH- Midazolam	7.50	37.50	75.00	3.00	15.00	30.00
Fexofenadine	18.75	93.75	187.50	7.50	37.50	75.00

A 100 µL aliquot of the supernatant was transferred, with an Eppendorf pipette, to glass tapered autosampler vial inserts in 2 mL amber glass LC vials and diluted with 100 µL of a 10 mM ammonium formate aqueous solution that contained the IS for positive and negative ESI mode acquisition respectively, just prior to the LC-MS/MS analysis. The same procedure was followed for blank and spiked solvent samples. For the assessment of matrix effects blank plasma and solvent samples were extracted as described above and spiked post extraction with 3 different concentrations of all the analytes with the same IS concentration by adding 100 µL of a spiked solution of 10 mM ammonium formate aqueous solution.

5.2.3.4 Methanol/acetonitrile extraction from Mitra™ tips

Similar to the protein precipitation method above, appropriate volumes from an analyte mixture containing all probe drugs and metabolites were added to 2 mL Eppendorf vials to make up calibration curves over the expected concentration range for the assessment of analyte recovery, linearity, precision and accuracy. After evaporation of the solvent under a vacuum at 30°C (Centrivap™, Labconco, Kansas City, MO, USA), the samples were reconstituted with a 100 µL of whole blood and vortex mixed at low speed for 5 minutes. Mitra™ tips were used at an angle of 45°, to volumetrically soak up exactly 20 µL of blank or spiked whole blood taking care not to submerge the tip into the samples. The sample filled Mitra™ tips were allowed to air dry at room temperature for approximately 2 hours and then stored in closed containers at room temperature until extraction. Blank whole blood samples were prepared in a similar way. The spiked and blank sample filled Mitra™ tips were inserted into clean 2 mL Eppendorf vials and analytes extracted with an organic 1:1 mixture of methanol and acetonitrile according to the method by Ye and Gao[239] reported to allow better extraction of hydrophobic analytes with a more consistent recovery across a wide haematocrit range.[239] The extraction was carried out in two steps by adding 100 µL of a 1:1 methanol: acetonitrile mixture to the Eppendorf vial containing the Mitra™ tip followed by ultrasonication for 30 minutes initially, which was later extended to 60 minutes and then vortex mixing at maximum rpm for another 15 minutes to accelerate the extraction of the analytes. The sonication and vortex mixing steps were repeated once more after addition of another 100 µL of the 1:1 methanol: acetonitrile mixture. The final extracts were combined and then centrifuged at 14,000 *xg* for 10 minutes, however this step seemed to be excessive since the Mitra™ tips were intact after the extraction procedure, yielding a clear extract of the analytes. A 100 µL aliquot of the supernatant was pipetted into tapered glass autosampler vial inserts in 2 mL amber glass autosampler vials and diluted with 100 µL of aqueous 10 mM ammonium formate solution containing IS just prior to the LC-MS/MS analysis. Matrix effects were assessed similarly by extracting a blank whole blood sample collected onto Mitra™ tips and adding 100 µL of the extract supernatant to 100 µL of aqueous 10 mM ammonium formate IS solution spiked at the three different concentration levels across the calibration range for a combination of all the analytes.

5.2.4 LC-MS/MS method validation in human plasma and dried blood spots collected with the new Mitra™ device

The analytical method for quantitation was validated according to ICH guidelines for matrix effects, recovery, linearity, LOD, LOQ, carry-over, inter and intraday precision and accuracy and analyte stability in plasma and whole blood matrixes.

5.2.4.1 Evaluation of matrix effects

Sources of interference from endogenous components present in plasma and dried blood spot matrixes were assessed by three methods. The influence of the co-eluting matrix on ionisation enhancement or suppression was first assessed by comparing the slopes of the matrix matched calibration curves, the equivalent matrix-based curves spiked after extraction, and equivalent solvent only based calibration curves. Subsequently, relative matrix effects were evaluated from three batches on different days at three different concentration levels (Table 5.2), covering the expected concentration range that would result from poor metabolism phenotype after drug cocktail dosing, taking into account the dilution factors during sample preparation. The signal response of each individual analyte in post extracted matrix was compared to pure solvent spiked at the same concentration level and the matrix effect determined using Equation 7[226].

$$\textbf{\textit{Matrix effect}}\ (\%) = \frac{\textbf{\textit{Matrix matched std spiked post extraction}}}{\textbf{\textit{Spiked solvent standard solution}}} \times \mathbf{100} \qquad \text{Equation 7}$$

Matrix effects were also assessed by direct infusion at a constant flow rate of 20 µL.min^{-1} (using a Harvard syringe pump) of a solvent mixture (1:1 MeOH: H_2O 10 mM NH_4COOH) containing all the analytes into the post column eluent via a "T" piece connector just before the ESI source, whilst running a 10 µL injection of an extracted blank whole blood matrix with the optimised chromatographic method with scheduled MRM detection. Sources of interference present in the sample matrix and eluting from the column during the chromatographic run were visualised as an enhancement or suppression of all the individual analyte signals at specific retention times in the resulting chromatogram. The concentration of the analytes in the mixture matched the second lowest concentration level of the calibration curve given in Table 5.1.

5.2.4.2 Analyte recovery from plasma and dried blood spots

Plasma, dried blood spot and solvent samples were spiked at three concentrations levels corresponding to low, medium and high concentrations given in Table 5.1 and extracted

according to the methods described in Sections 5.2.3.3 and 5.2.3.4, followed by LC-MS/MS analysis with the optimised chromatographic method. Each sample was injected in triplicate and the mean analyte responses calculated for all analytes measured in DBS and plasma compared to the mean analyte responses obtained in solvent, at the same spiked concentration levels, subjected to the same extraction procedures. Mean analyte responses obtained after extraction from the two matrices were compared to the mean analyte responses obtained from solvent and expressed as analyte recovery percentage ± the standard deviation (SD). Analyte recovery assessments were repeated on three separate days during validation. The average recovery over three days was assessed further as the percentage ratio of the experimentally determined calibration slopes in both matrixes and their corresponding solvents, spiked before extraction, and the precision calculated (% CV).

5.2.4.3 Linearity, range, limit of detection (LOD), limit of quantitation (LLOQ) and carry-over

The ability of the analytical method to deliver quantitative results that are directly proportional to the amount of analyte present in a sample is imperative when implementing a method for routine phenotyping. Thus, the linearity of the method, over the expected concentration ranges (for all the analytes in biological matrixes (plasma and DBS) after oral phenotype dosing), was determined using the quantitation function in Analyst® 1.5.2 software. Post-extracted spiked plasma and DBS samples were injected in triplicate for LC-MS/MS analysis and repeated on three separate days. Matrix matched calibration curves were plotted as the ratio of each analyte peak area versus the internal standard peak area as a function of the analyte concentration using a weighted linear regression function (of 1/x) to account for the heteroscedasticity of the data in the lower concentration range. The linear calibration equations of ($y = mx+c$) and the coefficient of determination (r^2) for each individual analyte were determined mathematically.

The average signal to noise ratios, at the lowest concentration levels for all analytes, were determined with Analyst ® 1.5.2 software and the LOD estimated at three times the average signal to baseline noise ratio at the retention time and LOQ at 10 times the average signal to baseline noise ratio at the retention time measured when injecting a blank matrix extracted sample.

Carry-over was assessed by injecting two blank samples after the highest concentration of standard in matrix or solvent and determining the average analyte peak areas of each analyte

present in the second blank sample. To limit analyte accumulation and carry-over, the column was washed with high organic after each day of the analysis and injecting two blank samples between each calibration curve acquisition.

5.2.4.4 Intra- and inter-day precision and accuracy

The accuracy and precision, over the entire calibration range, was calculated with Analyst® 1.5.2 software from matrix matched (plasma and dried blood spot) calibration curves. It is an estimation of the total measured bias and constitutes both systematic error (from analytical instruments, i.e. variation in injection volume) and random error (from preparation of working standards or during extraction). The calibration curves were freshly prepared on each day of the analysis.

Precision measures the agreement or repeatability between a series of quantitative measurements (measured analyte peak area versus internal standard peak area) from triplicate LC-MS/MS injections on the same day (intraday precision) and repeatability on separate days (inter-day precision). It is expressed as the relative standard deviation (also called the coefficient of variation (CV)) from the mean in each instance and is acceptable when the value falls within 15%.

Accuracy, on the other hand, measures agreement between quantitative measurements (measured concentration from triplicate LC-MS/MS injections) against the true expected value and was measured over the entire calibration range on each of the three days of the analysis. Accuracy is expressed as a relative percentage and was deemed acceptable if the measured concentration fell within 15% of the true value. The use of QC standards was included to ensure the slope and intercept fell within the expected limitation of 15%.

5.2.4.5 Analyte stability

Analyte stability was assessed during initial method development under different storage and sample preparation conditions. Due to financial considerations only limited quantities of the following analytes could be obtained: paraxanthine, hydroxy-bupropion, omeprazole, hydroxy-midazolam (one ampoule containing 1 mL each at 1 $mg.mL^{-1}$), as well hydroxy-flurbiprofen (one vial containing 1 mg). After initial preparation of standard stock solutions in methanol at 1 $mg.mL^{-1}$, aliquots of 100 µL in 2 mL Eppendorf vials were stored at -80° C. Each individual vial was covered with foil to protect the analytes from light and used a maximum of two freeze-

thaw cycles during preparation of the working standard solution mixtures, as described in Section 5.2.3.1. Four different solvent conditions were chosen; An analyte mixture spiked in methanol alone, a second in 1:1 methanol: water acidified with 0.1% formic acid, a third in 1:1 methanol: water acidified with 0.01% acetic acid and a fourth in 1:1 methanol: water containing 0.025% ammonium formate. The final concentrations of the analytes in the mixtures were 500 $ng.mL^{-1}$ for caffeine, paraxanthine and flurbiprofen and 100 $ng.mL^{-1}$ for all the other analytes. Short term analyte stability at room temperature in the sample vial was investigated by triplicate injections over a 6-hour run-time and variation in analyte peak intensities recorded. These analyte mixtures were stored at -20°C for one month, after which they were thawed at room temperature and injected (in triplicate) to investigate the stability of the analytes within the analyte mixtures after one freeze thaw cycle under the same chromatographic conditions using a Kinetex™ biphenyl column (100 mm × 2.1 mm, 2.6 µm particle size) with the column temperature set at 40°C ± 3°C and flow rate 300 $\mu L.min^{-1}$. Stability of omeprazole and hydroxy-omeprazole under acidified conditions prompted a further experiment in human plasma described in Chapter 3. Spiked plasma and DBS samples, at three QC concentrations, were also assessed after 3 freeze thaw cycles, over the method validation period, to confirm analyte stability. The accuracy and precision at low, medium and high QC concentrations, were measured against freshly made spiked matrix-matched calibration curves.

5.2.5 Statistical analysis comparing plasma and DBS sampling

The results from the validation experiment was used to assess *in vitro* agreement between plasma and DBS sampling for all analytes, at the same known spiked concentration levels, under altered DBS extraction conditions. Blood samples were collected from healthy caffeine-shy volunteers, pooled and used in all experiments to eliminate, as far as possible, any haematocrit bias possible in the dried blood spots. Whole blood calibration curves, from low volume dried blood spots, were used to predict the analyte concentration in plasma, using the linear regression equation, at the same expected (spiked) concentration levels. The percentage difference between the predicted values and the true average observed values were calculated with Excel and plotted against the true average values observed for the spiked plasma samples, using GraphPad prism version 8.0.2 statistical software for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Variation within 20% of the true average observed values indicated agreement between the two sampling methods and possibly linear pharmacokinetics between the analyte concentrations obtained from the Mitra™ sampling device and plasma. A variation of more than 20% would indicate that a non-linear correlation

exist between the concentration of the analyte in plasma and DBS.[208] The altered extraction times and temperatures, used for analyte recovery from the Mitra™ tip, together with analyte stability and properties were also considered as contributing factors to the variation across the three days of validation.

5.2.6 *In vivo* pilot pharmacokinetic study

As an initial proof of concept, the "home-made" phenotyping cocktail (Chapter 4) was given to a healthy volunteer followed by an *in vivo* pharmacokinetic study with sparse plasma and DBS sampling using the Mitra™ device for whole blood collection. Simultaneous venous (using EDTA vacutainers) and capillary sampling was done at baseline and at 1 hour, 2.5 hours, 3 hours and 3.5 hours after oral administration of the 6-drug "home-made" phenotyping cocktail and a commercially bought Omez™ capsule, containing 10 mg omeprazole. Plasma was obtained by centrifugation (1500 *xg* for 15 minutes) of venous blood at room temperature within one hour after collection. The same sample storage, preparation and extraction procedures as described above were followed before LC-MS/MS analysis using the validated method.

5.3 RESULTS AND DISCUSSION

5.3.1 Optimised chromatographic separation method

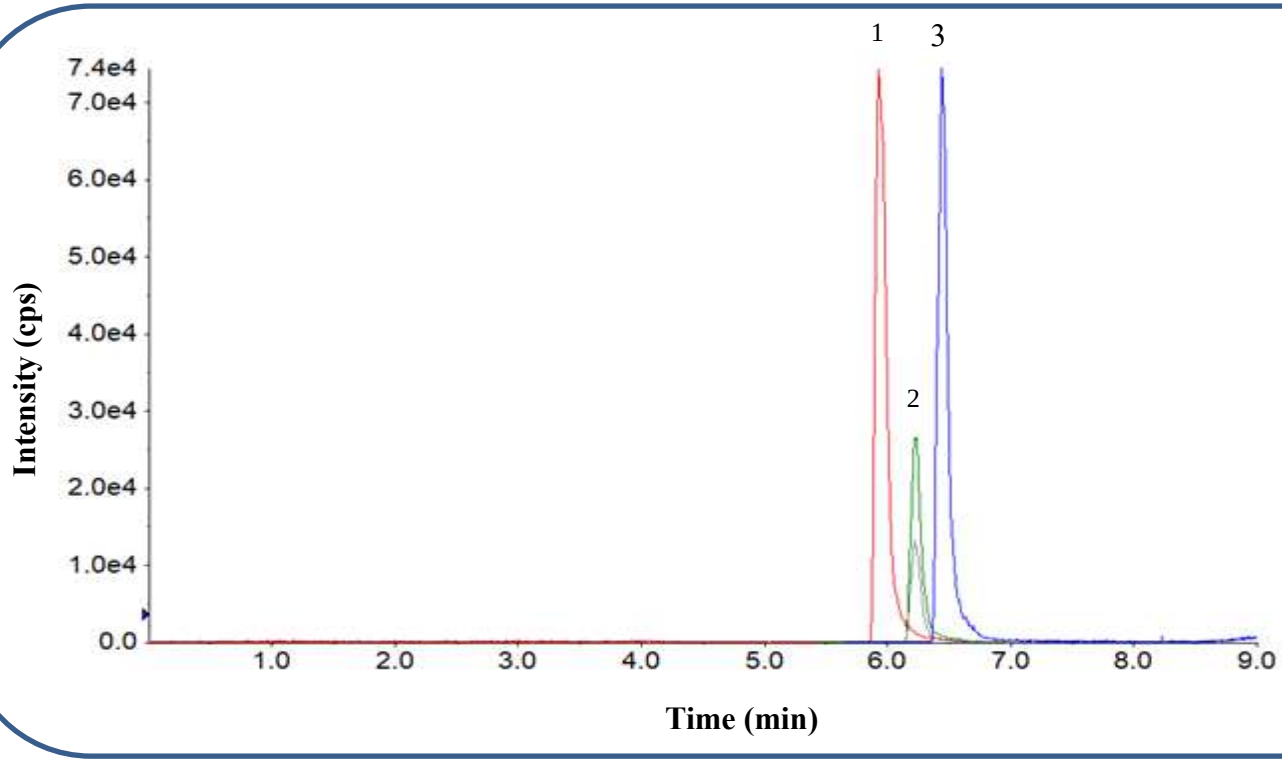
An optimised gradient elution method using a biphenyl phase column and increasing percentage of organic eluent consisting of 80:20 methanol: acetonitrile with 0.1% formic acid, was chosen as the final method to use and validated according to the ICH guidelines. Acetonitrile was used in the solvent mixture to reduce the high backpressures experienced when using 100% methanol. Chromatographic separation was optimised for all thirteen analytes including imipramine and probenecid as the internal standards for positive and negative mode ionisation respectively. It was important to ensure that the internal standards did not co-elute with any of the other analytes, due to the possibility of ionisation suppression of these analytes. Another requirement was that the method be suitable for high throughput limiting the runtime while maintaining optimal resolution of all analytes.

The sample extraction for both plasma and DBS should not introduce bias as the solvents contained no additives that might favour extraction of some analytes above others (due to their physicochemical properties) and avoided acidic degradation of omeprazole and hydroxy-omeprazole. After extraction aliquots were diluted (50:50) with 10 mM ammonium formate to

improve peak shape (together with the mobile phase containing 0.1% formic acid) and to ensure analyte stability in the sample vial. Ammonium formate also assists ionisation efficiency in negative ESI mode.

Examples of typical chromatograms in negative and positive ESI mode with optimised elution gradients are shown in Figures 5.1 and 5.2 respectively. The Turbo V™ (ESI) ionisation source parameters were optimised with a curtain gas pressure of 23 units, nebuliser gas (GS1) pressure of 35 units and auxiliary gas (GS2) pressure of 30 units. The ESI capillary potential was operated at -4500 V for negative mode and +5000 V for positive mode acquisitions respectively. The source temperature was 450ºC with an entrance potential ± 10 V. Scheduled MRM acquisition methods were set up in positive and negative ESI mode, with a 90 second detection window for each transition and target scan time of 100 msec for increased quantitative reproducibility, optimal sensitivity and better signal to noise ratio's (S/N). All other mass spectrometer parameters were tuned and optimised for each individual analyte during infusion of a 500 $ng.mL^{-1}$ solution of individual analytes as discussed in Chapter 3. Retention times for all analytes were stable across all validation assays. Elution order with average retention times and precision (% CV) are also given in Figures 5.1 and 5.2. All peaks in negative ESI mode acquisition were baseline separated with an elution gradient starting at 5% organic and increased to 85% over 3 minutes. The high organic percentage was maintained for 2 more minutes to ensure wash-out of lipophilic matrix components before a re-equilibration step of 3 minutes resulting in a total run time of 9 minutes. In positive mode acquisition the retention behaviour of the analytes changed slightly from the chromatogram shown in Figure 3.5 due to the addition of 20% acetonitrile that was necessary to reduce system backpressure. With the 80:20 methanol: acetonitrile solvent mixture, the gradient ensured baseline separation of the IS, imipramine, from all other analytes.

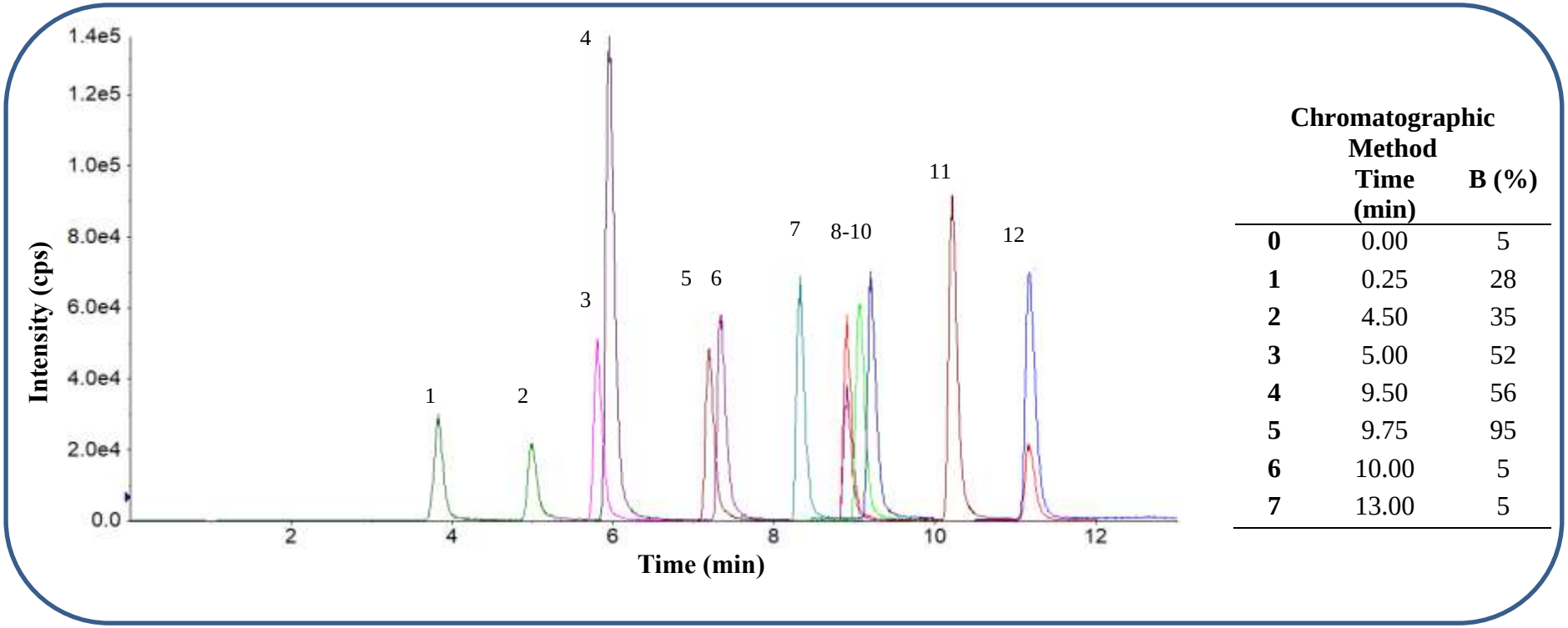
Ideally, deuterium labelled isotope internal standards for each analyte would be preferable to compensate for analyte recovery and matrix interferences, but was not economically viable in the resource constrained environment of a developing country. Targeted MRM analysis allowed accurate identification and quantification, even of the partially co-eluting analytes such as hydroxy-bupropion (5.79 ± 0.01 min.) and dextrorphan (5.93 ± 0.02 min.), bupropion (7.16 ± 0.02 min.) and hydroxy-omeprazole (7.32 ± 0.02 min.) and dextromethorphan (8.88 ± 0.02 min.), midazolam (9.04 ± 0.02 min.) and hydroxymidazolam (9.18 ± 0.01 min.).



Chromatographic Method

	Time (min)	B (%)
0	0.00	5
1	0.50	5
2	3.50	85
3	5.50	85
4	6.00	5
5	9.00	5

Figure 5.1: Chromatogram obtained with scheduled MRM acquisition method for analytes in negative ESI mode. Separation was achieved on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm) with a flow rate of 300 $\mu L.min^{-1}$ and mobile phase composition A: H_2O 0.1% formic acid and B: 80:20 MeOH: ACN 0.1% formic acid with 10 mM ammonium formate in the sample vial. The elution order, with average retention times and % CV was: 1 – hydroxyflurbiprofen, 5.94 min. (0.21%); 2 – internal standard probenecid, 6.23 min. (0.15%) monitoring two MRM transitions; 3 – flurbiprofen, 6.45 min. (0.15%).



Chromatographic Method		
	Time (min)	**B (%)**
0	0.00	5
1	0.25	28
2	4.50	35
3	5.00	52
4	9.50	56
5	9.75	95
6	10.00	5
7	13.00	5

Figure 5.2: Chromatogram obtained with scheduled MRM acquisition method for analytes in positive ESI mode at optimised elution gradient. Separation was achieved on a Kinetex™ Biphenyl column (100 mm × 2.1 mm, 2.6 µm) with a flow rate of 300 µL.min^{-1} and mobile phase composition A: H_2O 0.1% formic acid and B: 80:20 MeOH: ACN 0.1% formic acid with 10 mM ammonium formate in the sample vial. The elution order with average retention times and % CV was: 1 – paraxanthine 3.83 min. (0.45%), 2 – caffeine 4.99 min. (0.35%), 3 – hydroxy-bupropion 5.79 min. (0.25%), 4 – dextrorphan 5.93 min. (0.36%), 5 – bupropion 7.16 min. (0.30%), 6 – hydroxy-omeprazole 7.32 min. (0.21%), 7 – omeprazole 8.30 min. (0.18%), 8 – dextromethorphan (two MRM transitions) 8.88 min. (0.19%), 9 – midazolam 9.04 min. (0.19%), 10 – hydroxymidazolam 9.18 min. (0.15%), 11 – IS imipramine 10.18 min. (0.24%), 12 – fexofenadine (two MRM transitions) 11.13 min. (0.29%).

The total run time of the final acquisition method was 13 minutes including a 3-minute re-equilibration step. A shallow gradient from 28 – 35% phase B was maintained from 0.25 and 4.50 minutes to separate the early eluting analytes. A 0.50 minute increase to 52% with another shallow gradient from 52 – 56% gave acceptable resolution of the lipophilic analytes before increasing the organic to 95% to elute possible lipophilic matrix components that could be retained. The chromatographic separation method is given in Figure 5.2.

5.3.2 LC-MS/MS method validation in human plasma and dried blood spots collected with the new Mitra™ device

5.3.2.1 Evaluation of matrix effects

Initial absolute matrix effects, evaluated by comparing the ratio of the slopes from calibration curves in plasma and solvent (spiked post extraction), resulted in overall matrix effects showing the following average quantitation relative to the solvent based calibration slopes; 102.48% for caffeine, 99.08% for paraxanthine, 98.45% for bupropion, 97.37% for hydroxy-bupropion, 90.45% for flurbiprofen, 106.25% for hydroxy-flurbiprofen, 90.18% for hydroxy-omeprazole, 95.26% for dextromethorphan, 96.86% for dextrorphan, 91.58% for midazolam, 90.99% for hydroxymidazolam and 93.31% for fexofenadine. The matrix effect for omeprazole could not be calculated as these run times for the batch were 42-hours and omeprazole degraded completely during this time.

Subsequently, relative matrix assessments in plasma and DBS were performed by comparing analyte responses, (peak area in counts per second), in post extracted matrix compared to the response in post extracted solvent. Results calculated from triplicate injections repeated on 3 separate days from different batches at three different concentration levels are shown in Table 5.3. Ionisation suppression or enhancement were analyte specific with relative matrix effects between 90 and 110% for most analytes in post extracted plasma and DBS matrixes, except for bupropion and hydroxy-bupropion where the ionisation suppression was more than 10% in both plasma and dried blood spots with precision between 14.01 and 23.66%, with the latter variance observed at the lowest concentration level in the DBS matrix. Flurbiprofen and hydroxyflurbiprofen also showed ionisation suppression with matrix effects suppressing the peak areas to 64.32 and 71.12% respectively at the lowest spiked concentration, thereby affecting the precision of the method at low concentrations more than medium and higher concentrations, which confirms findings reported by Matuszewski *et al.*[243]

Table 5.3: Relative mean matrix effects (%) in plasma and DBS (n=9) with precision (% CV).

Analyte	Plasma			DBS		
	Conc. ($ng.mL^{-1}$)	Mean matrix effect (%)	CV (%)	Conc. ($ng.mL^{-1}$)	Mean matrix effect (%)	CV (%)
CAF	37.50	100.79	6.98	15.00	92.01	2.74
	187.50	95.95	3.91	75.00	104.55	10.68
	375.00	107.70	8.02	150.00	102.60	12.88
PAR	18.75	98.58	6.14	7.50	106.00	11.96
	93.75	96.44	0.43	37.50	102.22	5.67
	187.50	108.45	10.35	75.00	103.67	13.82
BUP	18.75	89.46	14.01	7.50	77.11	23.66
	93.75	88.10	14.34	37.50	92.61	17.25
	187.50	96.46	17.78	75.00	98.90	11.08
OH-BUP	18.75	102.56	9.48	7.50	81.53	10.84
	93.75	98.01	7.62	37.50	96.81	3.25
	187.50	105.20	16.97	75.00	99.72	10.55
FLB	18.75	64.32	23.75	7.50	101.36	0.89
	93.75	82.54	12.66	37.50	101.66	0.88
	187.50	98.41	11.09	75.00	96.62	9.24
OH-FLB	18.75	71.12	14.24	7.50	99.51	14.85
	93.75	90.98	2.83	37.50	103.28	2.81
	187.50	98.94	5.97	75.00	96.96	9.48
OPZ	18.75	105.29	13.07	7.50	98.83	16.23
	93.75	98.17	3.07	37.50	96.36	1.02
	187.50	109.90	12.90	75.00	99.67	10.12
OH-OPZ	18.75	99.47	4.12	7.50	100.20	16.90
	93.75	97.84	2.20	37.50	98.73	1.74
	187.50	107.42	11.72	75.00	97.23	10.00
DEX	18.75	101.11	10.98	7.50	100.06	18.36
	93.75	99.49	3.02	37.50	93.53	2.98
	187.50	105.92	17.47	75.00	103.90	12.23
DTP	18.75	98.34	6.28	7.50	95.63	18.31
	93.75	100.47	4.48	37.50	98.33	2.19
	187.50	108.05	17.06	75.00	101.97	9.33
MDZ	7.50	105.00	14.50	3.00	108.47	15.60
	37.50	95.57	5.72	15.00	103.67	19.93
	75.00	104.66	14.40	30.00	101.33	12.93
OH-MDZ	7.50	107.66	12.95	3.00	104.91	16.26
	37.50	97.58	2.10	15.00	95.32	4.52
	75.00	104.99	14.38	30.00	97.89	9.47
FEX	18.75	98.17	9.81	7.50	106.34	15.37
	93.75	98.68	1.97	37.50	98.84	3.50
	187.50	108.35	14.42	75.00	102.03	9.04

Although ionisation suppression was more pronounced at the lowest concentration for these analytes, the expected plasma or DBS concentration levels *in vivo* correspond to the higher spiked levels, for which acceptable matrix effects were observed. Use of matrix matched calibration curves, from pooled population samples, were used to compensate for the problems associated with matrix effects during the method development and assessment of inter-method agreement of sampling methods.

The third method used to assess the matrix effects was the post column infusion method, where the overall matrix effects at each analyte retention time could be observed by directly infusion a constant stream of the analyte mix into the column eluant before the ionisation source whilst injecting an extract of a blank sample. TIC chromatograms are shown in Figure 5.3 and 5.4 for positive and negative acquisition methods respectively. Numeric values indicated by green arrows in Figure 5.3 and 5.4 represent the elution times for the analytes in positive and negative mode and correspond to Figures 5.1 and 5.2. The concentrations of the analytes in the mix were 150 ng.mL^{-1} for caffeine and flurbiprofen, 75 ng.mL^{-1} for paraxanthine, bupropion, hydroxy-bupropion, hydroxyflurbiprofen, omeprazole, hydroxy-omeprazole, dextromethorphan, dextrorphan and fexofenadine and 30 ng.mL^{-1} for midazolam and hydroxymidazolam.

5.3.2.2 Analyte recovery from plasma and dried blood spots

The measured concentrations (mean ± SD in ng.mL^{-1} from triplicate injections) and calculated precision (% CV), derived from matrix matched calibration curves in plasma and DBS on three separate days, at three spiked concentration levels are summarised in Table 5.4. Relative recoveries fell within the 15% bias with good precision between 93.50 to 107.38% in human plasma and 94.27 to 108.44% in DBS except for a single timepoint for flurbiprofen with a recovery of 84.77%. Optimised recoveries from plasma resulted from the two-step addition of acetonitrile for protein precipitation and extraction with ultrasonication between the addition of each acetonitrile addition. This sequence liberated protein bound drug after an initial partial denaturing of plasma proteins during the first acetonitrile addition, which confirmed earlier findings in our laboratory [(244)].

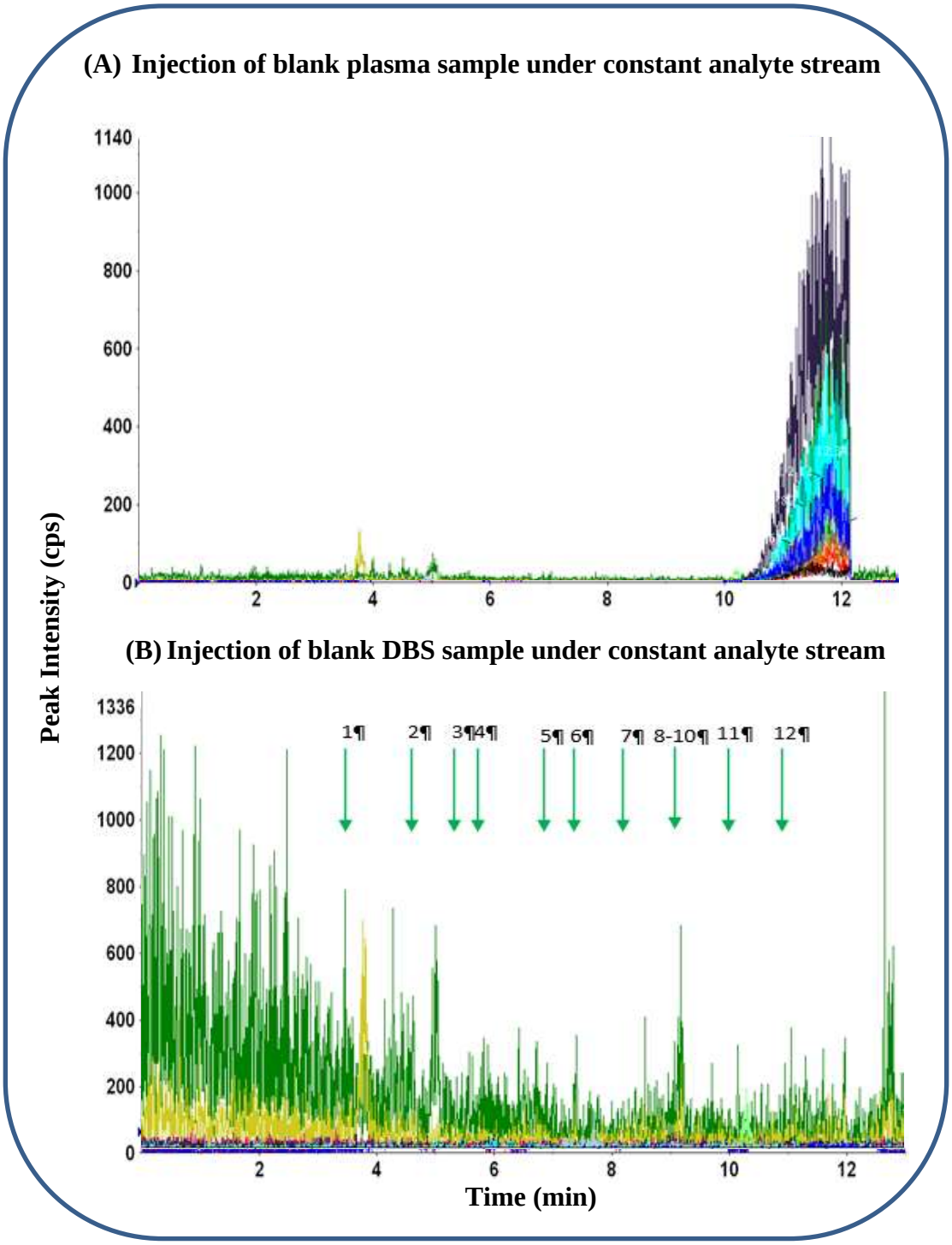


Figure 5.3: TIC chromatograms obtained in ESI+ mode under constant analyte stream with post column infusion method (20 µL.min^{-1}). Total analyte signal obtained from a constant post column infusion with injection of a blank plasma (A) or DBS (B) sample.

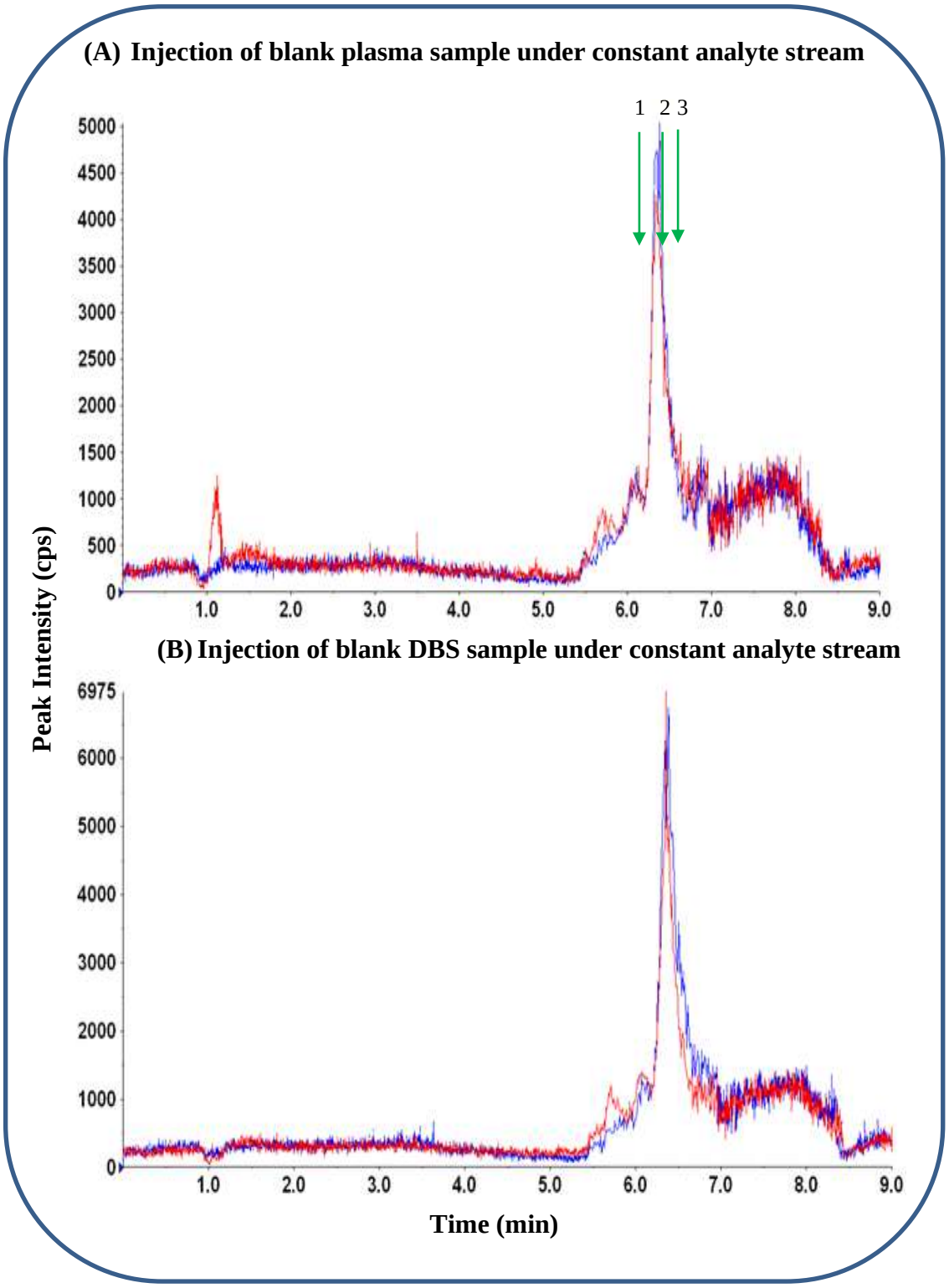


Figure 5.4: TIC chromatograms obtained in ESI- mode under constant analyte stream with post column infusion method (20 µL.min^{-1}). The total analyte signal obtained from a constant post column infusion with injection of a blank plasma (A) or DBS (B) sample.

Table 5.4: Average percentage relative recovery with precision (% CV) from triplicate injections in human plasma and DBS at different spiked concentrations on separate days (n=9)

Analyte	Human plasma			Dried Blood Spots (Mitra™)		
	Spiked conc. ($ng.mL^{-1}$)	Recovery (%)	CV (%)	Spiked conc. ($ng.mL^{-1}$)	Recovery (%)	CV (%)
FEX	150	103.88	9.58	150	108.58	16.44
	1500	98.15	0.60	1500	102.74	2.12
	4500	99.11	2.16	4500	96.25	3.08
CAF	150	94.89	2.13	150	98.38	11.57
	3000	102.69	6.74	3000	102.89	3.07
	9000	95.01	3.61	9000	98.16	3.06
PAR	150	93.50	5.18	150	100.61	5.42
	1500	101.21	1.25	1500	103.02	6.41
	4500	97.20	3.94	4500	96.09	4.35
BUP	150	107.38	10.38	150	106.96	9.89
	1500	102.78	1.72	1500	97.89	2.11
	4500	97.07	3.59	4500	97.89	5.61
OH-BUP	150	103.36	5.73	150	99.30	3.52
	1500	97.62	1.13	1500	98.41	3.62
	4500	99.92	2.74	4500	97.85	3.09
FLB	150	100.11	7.57	150	108.44	10.04
	1500	94.31	4.66	1500	84.77	10.93
	9000	101.88	1.66	9000	105.34	8.38
OH-FLB	150	100.04	8.49	150	104.47	3.11
	1500	98.97	2.05	1500	99.69	6.18
	4500	99.88	0.42	4500	99.69	2.72
OPZ	150	104.31	9.12	75	94.27	4.90
	1500	98.54	5.35	750	102.39	6.68
	4500	99.77	0.40	1500	99.70	4.48
OH-OPZ	150	102.45	6.77	150	98.25	2.27
	1500	97.43	3.61	1500	99.10	3.79
	4500	95.02	7.08	3000	100.49	2.92
DEX	150	98.94	2.25	150	103.82	4.55
	1500	101.98	4.14	1500	101.79	1.35
	4500	96.63	5.43	4500	98.77	0.75
DTP	150	99.42	4.74	150	100.30	4.44
	1500	100.30	6.31	1500	94.90	3.63
	4500	99.11	4.21	4500	101.03	0.92
MDZ	60	98.28	5.75	60	103.79	3.34
	600	98.11	3.07	600	103.66	3.31
	1800	100.73	1.19	1800	97.44	2.03
OH-MDZ	60	99.52	1.55	60	101.36	1.57
	600	100.45	4.86	600	101.23	1.89
	1800	98.62	1.08	1800	98.03	2.73

To optimise the extraction recovery from the polymeric Mitra™ tip, a similar two step extraction with 1:1 acetonitrile: methanol was carried out with sonication and vortex mixing after each addition of extraction solvent. The 50:50 acetonitrile: methanol extraction solvent fixed plasma proteins and erythrocytes on the polymeric tip, resulting in a clear supernatant despite the presence of red blood cells, a factor that could be highly beneficial for automation of extraction before LC-MS/MS analysis for high throughput analysis. Recoveries were assessed by comparing the average of the slopes of the matrix matched calibration curves to that of the equivalent solvent curves subjected to the same extraction methods on all three days of the validation (Table 5.5).

Table 5.5: Percentage absolute recovery and precision (% CV) calculated from the ratio between slopes of matrix matched calibration curves and solvent calibration curves.

Analyte	Human plasma		Dried Blood Spots (Mitra™)	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Fexofenadine	106.99	6.76	100.16	13.88
Caffeine	97.35	11.46	118.23	26.38
Paraxanthine	102.23	11.44	116.30	30.11
Bupropion	98.86	7.20	32.01*	0.15*
Hydroxybupropion	108.18	2.74	107.64	15.41
Flurbiprofen	99.43	4.21	107.98	15.09
Hydroxyflurbiprofen	100.52	5.12	93.25	8.77
Omeprazole	113.73	12.46	89.88	10.42
Hydroxy-omeprazole	108.24	8.11	94.29	4.28
Dextromethorphan	110.60	10.74	94.23	17.90
Dextrorphan	109.28	14.16	97.34	18.68
Midazolam	112.68	13.21	107.46	18.60
Hydroxymidazolam	102.87	11.37	106.93	13.27

* *Bupropion average % recovery calculated from results on days two and three. On day one average absolute recovery were 66.63%. Conditions were altered over the three days to test extraction efficiency of all analytes. See text for explanation.*

Absolute recovery of all analytes consistently ranged from 97.35 to 113.73% in plasma with precision values of ≤ 14.16%. while mean recoveries from DBS across the three days were more inconsistent (precision 26.38% for caffeine. 30.11% for paraxanthine 17.90% for dextromethorphan. 18.68% for dextrorphan and 18.60% for midazolam). A positive recovery bias seen for caffeine and paraxanthine was consistent with results found in a study by De Kesel *et al.* [245]. They found that volumetric absorptive microsampling (VAMS) technology overestimated whole blood concentrations of caffeine and paraxanthine as a result of differences in spiked samples against patient samples at different haematocrit values and higher

recovery at lower haematocrit. It was found that elevated erythrocytes concentrations at higher haematocrit inversely influenced desorption of analytes from the hydrophilic polymeric tip. They further reported that sonication and increased temperatures during the extraction could be employed to overcome this. On the other hand, these conditions affected the average absolute recovery of bupropion, i.e. 32% at higher temperatures and longer sonication step compared to day one of the validation with 70% recovery where spiked DBS samples were sonicated for 30 minutes at room temperature (±19ºC). Bupropion stability at higher temperatures and with extraction pH higher than 5 probably affected the extraction recovery.[212] This reiterates the challenge faced for analysing samples containing multiple analytes such as when administering a drug cocktail, as the analytes have different physicochemical properties. Bosilkovska *et al.* reported the instability of bupropion on DBS filter cards and recommended that cards containing bupropion be stored at -20ºC.[37] Flurbiprofen, hydroxyflurbiprofen, omeprazole and hydroxy-omeprazole recoveries increased with increased temperature and longer sonication. Similar results were seen with immunosuppressant drugs where recovery at different haematocrit levels could be improved by heated desorption.[246]

Recovery of the more hydrophobic analytes midazolam, hydroxymidazolam, dextromethorphan, dextrorphan and fexofenadine were lower with longer sonication and at higher temperatures. This might be due to the fact that recoveries of lipophilic interfering matrix components were also higher under these conditions that probably caused ionisation suppression.[59] Further optimisation of the extraction conditions may improve the recovery from Mitra™ devices.

5.3.2.3 Linearity, limit of detection (LOD). lowest limit of quantitation (LLOQ) and carry-over

Method linearity was established with analyte specific concentration ranges that fell within the expected *in vivo* concentrations for each analyte following the administered phenotyping cocktail dosages. Expected *in vivo* concentrations were estimated for an average person of 70 kg with a volume of distribution (V_d) of 5 L and the assumption that approximately 60% of unbound drug would be detected in the systemic circulation. At these concentrations all analytes gave sufficient responses in the LC-MS/MS system. Signal to noise ratios at the lowest spiked concentrations for plasma and DBS with estimated LOD and LLOQ are given in Table 5.6. These lowest spiked concentrations are 10-fold (for midazolam and hydroxymidazolam) to 100-

fold (for fexofenadine) lower than concentrations expected to be found in whole blood and plasma after dosing with the phenotyping cocktail.

The calibration equations with coefficient of determination (r^2) and slopes are shown for each analyte in Table 5.7. Matrix matched calibration curves were plotted using a weighted linear regression function (of 1/x) to establish the best-fit line of heteroscedastic data covering the expected *in vivo* concentration ranges after extraction. The coefficient of determination exceeded 0.9936 for human plasma and 0.9929 for DBS with the slopes of the calibration equation between 0.0613 and 0.4300 for plasma and 0.0583 and 0.4550 for DBS. These slopes were calculated from the ratio of analyte peak area against the peak area of internal standards at different spiked concentrations.

Table 5.6: Signal to noise ratio at lowest spiked concentrations in DBS and plasma with estimated LOD and LLOQ.

Analyte	Lowest spiked concentration ($ng.mL^{-1}$)	DBS (dilution factor 1:20)			Plasma (dilution factor 1:8)		
		S/N	LOD	LLOQ	S/N	LOD	LLOQ
FEX	30.00	3.70	1.02	3.41	13.10	0.86	2.86
CAF	150.00	2.60	8.65	28.85	5.90	9.53	31.78
PAR	30.00	3.10	1.45	4.84	6.40	1.76	5.86
BUP	30.00	4.80	0.94	3.13	18.10	0.62	2.07
OH-BUP	30.00	6.40	0.70	2.34	16.60	0.68	2.26
FLB	150.00	7.00	3.69	12.30	5.40	10.42	34.72
OH-FLB	75.00	4.90	2.30	7.65	16.10	1.75	5.82
OPZ	30.00	9.20	0.49	1.63	52.30	0.22	0.72
OH-FLB	30.00	5.50	0.82	2.73	19.10	0.50	1.67
DEX	30.00	6.10	0.74	2.46	16.80	0.67	2.23
DTP	30.00	13.20	0.34	1.14	42.00	0.29	0.96
MDZ	12.00	9.90	0.18	0.61	17.30	0.27	0.90
OH-MDZ	12.00	3.80	0.47	1.58	6.30	0.71	2.38

Both internal standards, imipramine and probenecid showed high ionisation efficiency in positive and negative modes respectively at concentrations of 25 $ng.mL^{-1}$. Better extraction efficiency and liberation of highly protein bound analytes, such as imipramine, could account for the high peak intensities of these highly ionisable analytes that influenced analyte/ IS concentration ratio's and influenced the slopes of the regression lines. Calibration curves were

plotted as the Analyte peak area/ Internal Std peak area against Analyte concentration/ IS concentration.

Table 5.7: Linearity (coefficient of determination r^2) and calibration equation of all analytes in DBS and plasma.

Analyte	Human plasma Calibration curve parameters (y = mx + c)			Dried Blood Spots (Mitra™) Calibration curve parameters (y = mx + c)		
	Linearity (r^2)	**m**	**c**	**Linearity (r^2)**	**m**	**c**
FEX	0.9997	0.4075	0.0883	0.9994	0.3975	-0.0032
CAF	0.9958	0.0613	0.0110	0.9971	0.0583	-0.0002
PAR	0.9973	0.1398	0.0164	0.9929	0.1285	0.0083
BUP	0.9990	0.1588	0.0316	0.9983	0.0613	-0.0051
OH-BUP	0.9997	0.0943	0.0139	0.9995	0.1053	-0.0021
FLB	0.9936	0.2193	0.0485	0.9981	0.1995	0.0172
OH-FLB	0.9939	0.3000	0.0079	0.9983	0.3025	-0.0042
OPZ	0.9968	0.3825	0.1970	0.9986	0.4400	0.0112
OH-FLB	0.9985	0.3350	0.0271	0.9979	0.2950	0.0078
DEX	0.9958	0.1865	-0.0001	0.9997	0.1738	-0.0017
DTP	0.9999	0.2258	0.0283	0.9996	0.2215	-0.0057
MDZ	0.9976	0.4300	0.0537	0.9987	0.4550	0.0339
OH-MDZ	0.9978	0.2650	0.0189	0.9988	0.2975	0.0018

Carry over was assessed by injection of a double blank matrix matched sample after the highest concentration calibration standard. Figure 5.5 (A) shows an extracted ion chromatogram of all analytes acquired with scheduled MRM method in ESI+ mode. Signal to noise ratio's (measured as the peak intensity around the retention time of each analyte and the noise area within a 30 second time window after the retention time) are shown for paraxanthine, omeprazole, dextromethorphan, midazolam, hydroxy-midazolam and fexofenadine with S/N greater than the LOQ for four of these analytes. The carry-over was largely reduced by injection of a second double blank sample and an organic wash step of the sample needle between each sample injection, as shown in Figure 5.5 (B). For fexofenadine and dextrorphan S/N are above the LOD but below the LOQ after the introduction of this step with S/N 4.3 for fexofenadine and 4.9 for dextrorphan. In accordance with international guidelines the carry-over did not exceed 20% of the lower limit of quantification when assessing the % area of blank injection to that of the LLOQ after highest concentration sample injection.

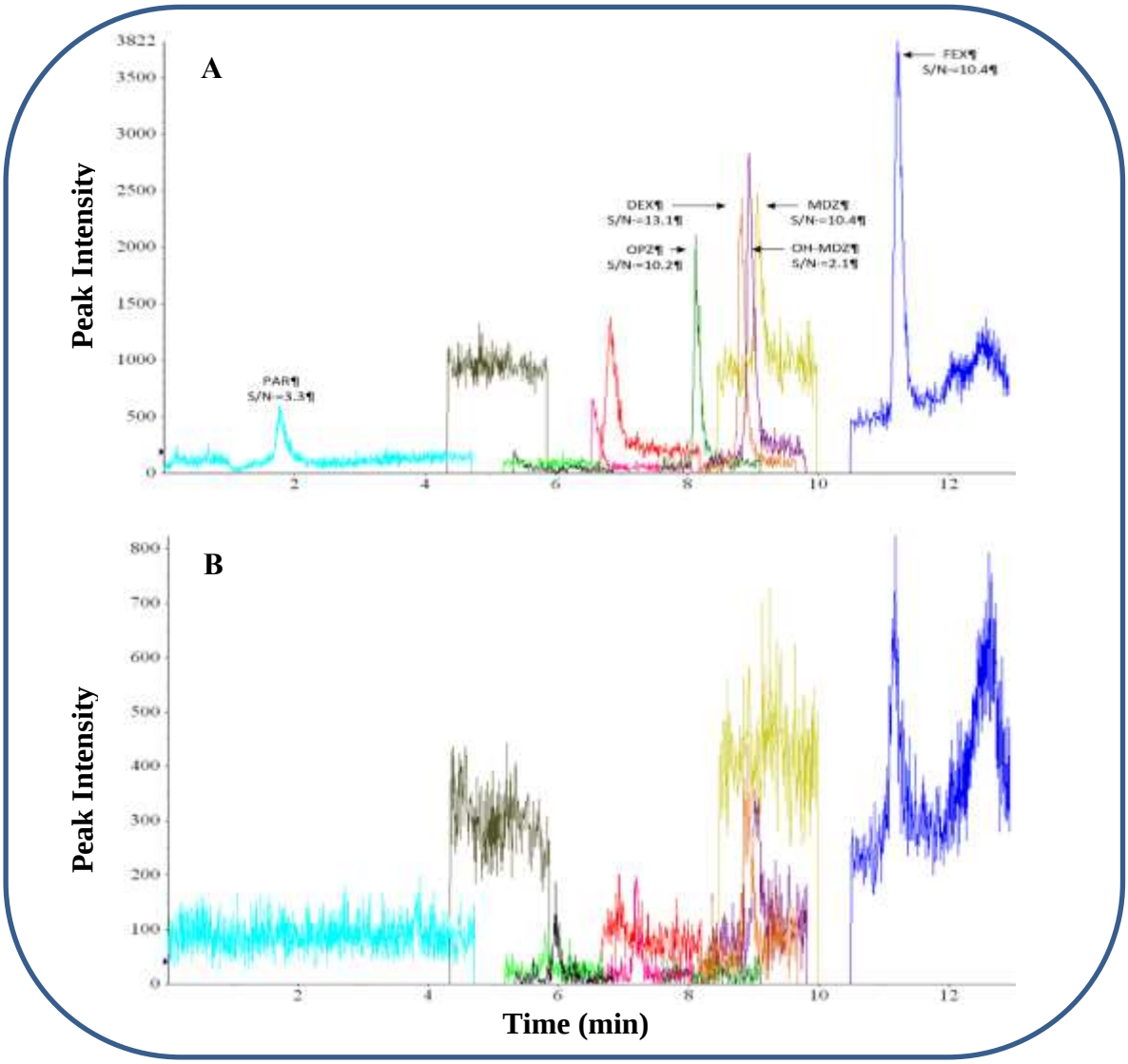


Figure 5.5: Carry over of analytes acquired in ESI+ mode after injection of the first blank after the highest calibrant (A) and after second double blank injection (B).

5.3.2.4 Intra- and inter-day precision and accuracy

Intra- and inter-day precision and accuracy were calculated from triplicate injections on three separate days of the validation and are expressed as percentages of the mean concentration found against the known spiked values in plasma and DBS. The results of intra-day precision and accuracy found in plasma are summarised in Table 5.8 and results found in DBS in Table 5.9. Inter-day precision in plasma and DBS are given in Table 5.10. All tables report the variance in accuracy and precision for each analyte and were within the 15% acceptable bias above the LLOQ and below 20% near or below the LLOQ for plasma and DBS.

Table 5.8: Intra-day accuracy (%) and precision (%) in human plasma (n=3) spiked at 5 different concentration on three separate days from separately made up working standard solutions.

Analyte in plasma	Spiked conc. (ng.mL^{-1})	Intra-day (n=3) Day 1			Intra-day (n=3) Day 2			Intra-day (n=3) Day 3		
		Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)	Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)	Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)
FEX	75.00	74.87 ± 2.14	99.77	2.86	73.82 ± 5.48	98.42	7.43	70.57 ± 3.30	94.09	4.68
	150.00	158.95 ± 2.25	105.68	1.42	147.30 ± 2.16	98.20	1.47	154.49 ± 4.49	102.99	2.90
	750.00	781.52 ± 68.87	104.15	8.81	782.45 ± 37.52	104.33	4.79	791.94 ± 10.57	105.59	1.33
	1500.00	1523.63 ± 168.28	101.30	11.04	1504.45 ± 25.06	100.30	1.67	1539.07 ± 9.19	102.60	0.60
	4500.00	4433.17 ± 492.42	97.56	11.11	4475.81 ± 44.30	99.46	0.99	4430.01 ± 158.55	98.44	3.58
CAF	150.00	160.62 ± 17.84	106.79	11.11	153.62 ± 8.96	102.41	5.83	137.77 ± 27.55	91.85	19.99
	300.00	294.22 ± 36.58	98.07	12.43	295.77 ± 13.80	98.59	4.67	308.98 ± 45.31	102.99	14.67
	1500.00	1558.30 ± 58.00	103.61	3.72	1500.07 ± 4.25	100.00	0.28	1587.29 ± 107.18	105.82	6.75
	3000.00	2857.16 ± 147.98	95.24	5.18	3007.06 ± 97.55	100.24	3.24	3076.72 ± 271.13	102.56	8.81
	9000.00	8745.73 ± 702.36	96.74	8.03	9020.00 ± 143.49	100.22	1.59	8886.63 ± 911.67	98.74	10.26
PAR	75.00	79.47 ± 14.69	105.90	18.48	76.98 ± 4.58	102.64	5.95	69.62 ± 8.40	92.83	12.06
	150.00	138.04 ± 26.47	91.78	19.18	147.53 ± 6.70	98.35	4.54	147.85 ± 18.62	98.57	12.60
	750.00	804.85 ± 17.27	107.26	2.15	745.49 ± 4.47	99.40	0.60	803.85 ± 57.46	107.18	7.15
	1500.00	1502.39 ± 26.65	99.89	1.77	1494.28 ± 54.49	99.62	3.65	1554.91 ± 106.41	103.66	6.84
	4500.00	4386.53 ± 328.73	96.53	7.49	4515.13 ± 25.90	100.34	0.57	4406.75 ± 315.31	97.93	7.16
BUP	75.00	78.27 ± 0.90	104.31	1.16	80.71 ± 1.15	107.61	1.42	- ± -	-	-
	150.00	141.06 ± 9.88	93.79	7.01	140.75 ± 9.49	93.83	6.74	131.80 ± 14.99	87.87	11.38
	750.00	831.70 ± 65.10	110.83	7.83	759.86 ± 21.60	101.31	2.84	789.75 ± 5.38	105.30	0.68
	1500.00	1546.17 ± 132.17	102.80	8.55	1507.04 ± 81.40	100.47	5.40	1575.00 ± 30.21	105.00	1.92
	4500.00	4309.07 ± 261.34	94.83	6.07	4496.04 ± 162.88	99.91	3.62	4410.91 ± 85.12	98.02	1.93

Analyte in plasma	Spiked conc. ($ng.mL^{-1}$)	Intra-day (n=3) Day 1			Intra-day (n=3) Day 2			Intra-day (n=3) Day 3		
		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)
OH-BUP	30.00	26.32 ± 2.04	87.74	7.77	31.01 ± 1.45	103.37	4.69	33.53 ± 4.08	111.76	12.16
	150.00	- ± -	-	-	147.61 ± 11.90	98.41	8.06	136.75 ± 5.19	91.16	3.79
	750.00	790.61 ± 1.75	105.41	0.22	749.04 ± 13.20	99.87	1.76	759.04 ± 23.03	101.21	3.03
	1500.00	1558.12 ± 21.39	103.87	1.37	1471.02 ± 44.54	98.07	3.03	1504.62 ± 70.45	100.31	4.68
	4500.00	4332.48 ± 349.79	96.28	8.07	4538.78 ± 59.65	100.86	1.31	4506.04 ± 108.02	100.13	2.40
FLB	150.00	145.65 ± 15.85	97.10	10.88	146.49 ± 26.36	97.66	17.99	135.15 ± 0.09	90.10	0.06
	300.00	295.31 ± 32.59	98.44	11.04	304.08 ± 39.91	101.36	13.13	312.15 ± 22.81	104.05	7.31
	1500.00	1525.12 ± 16.76	101.67	1.10	1560.32 ± 190.49	104.02	12.21	1548.10 ± 37.39	103.21	2.42
	3000.00	3212.95 ± 83.98	107.10	2.61	2910.61 ± 228.60	97.02	7.85	3049.52 ± 38.14	101.65	1.25
	9000.00	8817.35 ± 416.46	97.97	4.72	9072.70 ± 398.99	100.81	4.40	8918.65 ± 365.28	99.10	4.10
OH-FLB	75.00	70.83 ± 5.07	94.43	7.15	74.01 ± 4.36	98.69	5.89	69.84 ± 2.61	93.11	3.74
	150.00	152.88 ± 7.72	101.92	5.05	146.52 ± 33.46	97.68	22.83	149.93 ± 5.70	99.95	3.80
	750.00	761.24 ± 5.12	101.50	0.67	801.82 ± 189.54	106.91	23.64	788.61 ± 12.57	105.15	1.59
	1500.00	1529.83 ± 19.88	101.99	1.30	1461.66 ± 258.42	97.44	17.68	1569.02 ± 46.12	104.60	2.94
	4500.00	4468.48 ± 113.83	99.30	2.55	4499.82 ± 389.41	100.00	8.65	4406.45 ± 126.30	97.92	2.87
OPZ	30.00	26.80 ± 11.97	89.34	44.67	- ± -	-	-	28.63 ± 1.29	95.44	4.52
	150.00	167.53 ± 11.50	111.69	6.87	150.73 ± 11.02	100.49	7.31	148.68 ± 10.78	99.12	7.25
	750.00	791.43 ± 16.87	105.52	2.13	786.89 ± 40.69	104.92	5.17	778.23 ± 10.34	103.76	1.33
	1500.00	1575.00 ± 73.61	105.00	4.67	1535.00 ± 35.08	102.33	2.29	1584.62 ± 120.29	105.64	7.59
	4500.00	- ± -	-	-	4439.20 ± 190.45	98.65	4.29	4407.91 ± 412.69	97.95	9.36
OH-OPZ	75.00	76.83 ± 18.40	102.44	23.94	75.60 ± 3.03	100.80	4.01	71.35 ± 3.01	95.14	4.22
	150.00	177.20 ± 9.45	118.13	5.33	150.05 ± 11.63	100.03	7.75	157.11 ± 15.88	104.74	10.11
	750.00	849.78 ± 68.09	113.30	8.01	743.90 ± 20.67	99.19	2.78	760.22 ± 22.70	101.36	2.99
	1500.00	1519.02 ± 160.22	101.27	10.55	1507.06 ± 59.78	100.47	3.97	1491.16 ± 78.53	99.41	5.27
	4500.00	3875.23 ± 97.93	86.12	2.53	4505.89 ± 24.25	100.13	0.54	3914.82 ± 241.70	87.00	6.17

Analyte in plasma	Spiked conc. (ng.mL^{-1})	Intra-day (n=3) Day 1			Intra-day (n=3) Day 2			Intra-day (n=3) Day 3		
		Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)	Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)	Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)
DEX	75.00	71.65 ± 3.91	95.53	5.46	77.74 ± 2.05	103.65	2.63	68.73 ± 3.51	91.64	5.10
	150.00	142.78 ± 16.65	95.19	11.66	144.85 ± 4.22	96.57	2.91	154.13 ± 9.61	102.75	6.24
	750.00	842.97 ± 28.20	112.40	3.35	763.20 ± 11.11	101.76	1.46	769.19 ± 15.21	102.56	1.98
	1500.00	1602.97 ± 21.01	106.86	1.31	1470.19 ± 16.72	98.01	1.14	1589.48 ± 122.16	105.97	7.69
	4500.00	4162.87 ± 140.75	92.51	3.38	4519.51 ± 29.48	100.43	0.65	4402.31 ± 147.39	97.83	3.35
DTP	75.00	70.89 ± 3.14	94.52	4.42	73.60 ± 3.24	98.13	4.40	70.59 ± 4.32	94.12	6.11
	150.00	155.12 ± 5.59	103.42	3.60	151.27 ± 5.40	100.85	3.57	148.65 ± 7.95	99.10	5.35
	750.00	- ± -	-	-	764.85 ± 21.29	101.98	2.78	809.27 ± 40.77	107.90	5.04
	1500.00	1637.47 ± 57.91	109.16	3.54	1496.05 ± 12.66	99.74	0.85	1513.34 ± 39.69	100.89	2.62
	4500.00	4270.30 ± 261.71	94.90	6.13	4496.72 ± 6.94	99.93	0.15	4441.98 ± 119.50	98.71	2.69
MDZ	30.00	28.81 ± 3.98	96.03	13.80	31.66 ± 5.83	105.54	18.41	27.79 ± 1.31	92.64	4.72
	60.00	59.25 ± 3.96	98.75	6.69	56.44 ± 8.05	94.07	14.26	63.02 ± 10.03	105.03	15.92
	300.00	310.29 ± 13.63	103.43	4.39	300.36 ± 5.08	100.12	1.69	308.17 ± 38.74	102.72	12.57
	600.00	596.71 ± 12.39	99.45	2.08	600.84 ± 4.97	100.14	0.83	600.99 ± 48.47	100.16	8.07
	1800.00	- ± -	-	-	1800.06 ± 38.40	100.00	2.13	1790.03 ± 100.03	99.45	5.59
OH-MDZ	30.00	28.62 ± 2.26	95.38	7.90	29.22 ± 2.23	97.41	7.62	28.78 ± 1.54	95.95	5.35
	60.00	58.85 ± 3.11	98.08	5.29	60.04 ± 2.70	100.06	4.50	59.31 ± 3.88	98.86	6.54
	300.00	312.00 ± 2.44	104.00	0.78	308.71 ± 17.60	102.90	5.70	312.04 ± 12.08	104.01	3.87
	600.00	602.81 ± 12.40	100.47	2.06	600.61 ± 14.90	100.10	2.48	625.74 ± 62.00	104.29	9.91
	1800.00	1788.10 ± 54.20	99.34	3.03	1791.42 ± 62.20	99.52	3.47	1768.14 ± 95.48	98.23	5.40

FEX – fexofenadine; CAF – caffeine; PAR – paraxanthine; BUP – bupropion; OH-BUP – hydroxy-bupropion; FLB – flurbiprofen; OH-FLB – hydroxyflurbiprofen; OPZ – omeprazole; OH-OPZ – hydroxy-omeprazole; DEX – dextromethorphan; DTP – dextrorphan; MDZ – midazolam; OH-MDZ – hydroxymidazolam.

Table 5.9: Intra-day accuracy (%) and precision (%) in human DBS (n=3) using a Mitra™ device, spiked at 5 different concentration on three separate days from separately made up working standard solutions.

Analyte in plasma	Spiked conc. (ng.mL^{-1})	Intra-day (n=3) Day 1			Intra-day (n=3) Day 2			Intra-day (n=3) Day 3		
		Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)	Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)	Found (mean ± SD in ng.mL^{-1})	Accuracy (%)	Precision (%)
FEX	75.00	70.83 ± 5.18	94.44	7.32	73.25 ± 1.81	97.67	2.48	65.52 ± 2.15	87.36	3.29
	150.00	146.17 ± 8.77	97.45	6.00	148.77 ± 4.26	99.18	2.86	151.43 ± 11.74	100.95	7.75
	750.00	633.29 ± 45.58	84.44	7.20	765.52 ± 26.56	102.07	3.47	820.87 ± 26.03	109.45	3.17
	1500.00	1558.49 ± 156.33	103.90	10.03	1530.68 ± 84.79	102.05	5.54	1581.73 ± 92.99	105.45	5.88
	4500.00	4402.68 ± 538.37	97.84	12.23	4456.79 ± 343.59	99.04	7.71	4355.45 ± 294.41	96.79	6.76
CAF	150.00	138.25 ± 7.47	92.17	5.40	152.62 ± 9.76	101.75	6.40	140.47 ± 7.46	93.65	5.31
	300.00	281.83 ± 20.90	93.94	7.41	294.20 ± 18.93	98.07	6.44	305.98 ± 3.02	101.99	0.99
	1500.00	1275.15 ± 182.62	85.01	14.32	1507.50 ± 112.98	100.50	7.49	1572.50 ± 63.89	104.83	4.06
	3000.00	2971.97 ± 175.08	99.07	5.89	2991.45 ± 182.10	99.71	6.09	3073.58 ± 332.58	102.45	10.82
	9000.00	8907.00 ± 113.43	98.97	1.27	9005.82 ± 290.27	100.06	3.22	8881.63 ± 583.57	98.68	6.57
PAR	75.00	76.68 ± 8.02	102.24	10.46	75.08 ± 3.97	100.11	5.29	70.12 ± 4.12	93.49	5.88
	150.00	150.41 ± 20.40	100.27	13.57	154.55 ± 2.05	103.03	1.32	141.15 ± 13.24	94.10	9.38
	750.00	747.36 ± 154.13	99.65	20.62	742.63 ± 51.68	99.02	6.96	827.02 ± 56.15	110.27	6.79
	1500.00	1485.35 ± 124.97	99.02	8.41	1456.69 ± 115.78	97.11	7.95	1619.78 ± 52.62	107.99	3.25
	4500.00	3115.53 ± 448.54	103.85	14.40	4549.65 ± 177.64	101.10	3.90	4356.86 ± 438.60	96.82	10.07
BUP	75.00	84.24 ± 1.22	112.32	1.44	77.79 ± 4.55	103.72	5.85	78.09 ± 2.68	104.12	3.43
	750.00	623.89 ± 38.85	83.18	6.23	144.20 ± 7.52	96.14	5.21	147.83 ± 3.05	98.56	2.07
	1500.00	1444.15 ± 56.08	96.28	3.88	768.83 ± 83.38	102.51	10.84	742.20 ± 44.86	98.96	6.04
	3000.00	3394.14 ± 35.33	113.14	1.04	1454.60 ± 84.53	96.97	5.81	1459.86 ± 20.61	97.32	1.41
	4500.00	4278.59 ± 155.79	95.08	3.64	4544.36 ± 228.39	100.99	5.03	4547.02 ± 332.82	101.04	7.32

Analyte	Spiked conc. ($ng.mL^{-1}$)	Intra-day (n=3) Day 1			Intra-day (n=3) Day 2			Intra-day (n=3) Day 3		
		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)
OH-BUP	75.00	72.22 ± 6.21	96.30	8.60	73.95 ± 2.91	98.60	3.94	70.56 ± 2.01	94.08	2.84
	150.00	137.82 ± 9.16	91.88	6.65	151.41 ± 4.53	100.94	2.99	148.99 ± 6.27	99.32	4.21
	750.00	734.15 ± 47.46	97.89	6.46	754.65 ± 15.45	100.62	2.05	800.49 ± 37.53	106.73	4.69
	1500.00	1524.50 ± 203.46	101.63	13.35	1505.65 ± 63.05	100.38	4.19	1519.54 ± 108.63	101.30	7.15
	4500.00	4278.27 ± 370.45	95.07	8.66	4489.81 ± 159.39	99.77	3.55	4435.43 ± 48.05	98.57	1.08
FLB	150.00	147.24 ± 6.75	98.16	4.58	159.27 ± 27.00	106.18	16.95	148.54 ± 11.50	99.03	7.74
	300.00	301.20 ± 19.35	100.40	6.43	333.42 ± 23.53	111.14	7.06	320.01 ± 20.94	106.67	6.54
	1500.00	1456.76 ± 143.07	97.12	9.82	1287.19 ± 90.02	85.81	6.99	1428.22 ± 123.83	95.21	8.67
	3000.00	3163.37 ± 202.27	105.45	6.39	2849.32 ± 271.67	94.98	9.53	2969.15 ± 81.36	98.97	2.74
	9000.00	8761.66 ± -	97.35	-	9792.27 ± -	108.80	-	9066.82 ± 183.28	100.74	2.02
OH-FLB	75.00	77.48 ± 5.08	103.30	6.56	71.91 ± 10.07	95.88	14.00	74.47 ± 1.98	99.29	2.66
	150.00	144.11 ± 12.97	96.07	9.00	147.81 ± 7.79	98.54	5.27	156.49 ± 1.55	104.33	0.99
	750.00	747.60 ± 115.90	99.68	15.50	742.21 ± 26.55	98.96	3.58	704.52 ± 14.84	93.94	2.11
	1500.00	1540.75 ± 177.62	102.72	11.53	1611.94 ± 64.15	107.46	3.98	1497.35 ± 48.44	99.82	3.24
	4500.00	4395.27 ± -	97.67	-	4350.15 ± 24.09	96.67	0.55	4527.01 ± 129.87	100.60	2.87
OPZ	75.00	63.83 ± 3.99	85.11	6.25	73.80 ± 7.41	98.39	10.04	67.67 ± 4.44	90.23	6.55
	150.00	- ± -	-	-	145.60 ± 3.64	97.07	2.50	153.92 ± 1.54	102.62	1.00
	750.00	812.26 ± 88.48	108.30	10.89	754.74 ± 4.83	100.63	0.64	805.24 ± 54.95	107.37	6.82
	1500.00	1544.86 ± 115.67	102.99	7.49	1566.20 ± 7.75	104.41	0.49	1567.29 ± 79.95	104.49	5.10
	3000.00	2890.15 ± 122.86	96.34	4.25	4433.20 ± 211.01	98.52	4.76	4399.29 ± 151.33	97.76	3.44
OH-OPZ	30.00	33.67 ± 0.40	112.23	1.18	71.34 ± 0.82	95.12	1.16	68.98 ± 0.96	91.97	1.39
	75.00	67.39 ± 2.02	89.86	3.00	150.55 ± 1.12	100.36	0.74	149.50 ± 7.08	99.66	4.74
	750.00	714.72 ± 99.91	95.30	13.98	761.73 ± 40.30	101.56	5.29	855.00 ± 3.50	114.00	0.41
	1500.00	1487.99 ± 209.87	99.20	14.10	1537.36 ± 120.55	102.49	7.84	1509.89 ± 40.00	100.66	2.65
	3000.00	3043.39 ± 197.68	101.45	6.50	4452.99 ± 285.04	98.96	6.40	4426.63 ± 275.72	98.37	6.23

Analyte	Spiked conc. ($ng.mL^{-1}$)	Intra-day (n=3) Day 1			Intra-day (n=3) Day 2			Intra-day (n=3) Day 3		
		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)
DEX	75.00	71.91 ± 0.94	95.88	1.31	74.93 ± 2.17	99.91	2.90	74.01 ± 3.47	98.68	4.69
	150.00	153.76 ± 10.14	102.50	6.59	149.52 ± 4.90	99.68	3.28	150.25 ± 7.30	100.17	4.86
	750.00	722.96 ± 21.73	96.39	3.01	726.48 ± 17.48	96.86	2.41	757.91 ± 21.20	101.05	2.80
	1500.00	1514.70 ± 30.60	100.98	2.02	1547.41 ± 51.75	103.16	3.34	1505.90 ± 37.80	100.39	2.51
	4500.00	4446.88 ± 66.85	98.82	1.50	4468.79 ± 57.56	99.31	1.29	4486.94 ± 305.60	99.71	6.81
DTP	75.00	71.20 ± 2.74	94.93	3.84	76.83 ± 3.98	102.44	5.18	74.16 ± 4.27	98.88	5.76
	150.00	150.87 ± 8.21	100.58	5.44	147.69 ± 16.24	98.46	11.00	153.17 ± 8.88	102.12	5.80
	750.00	723.18 ± 9.18	96.42	1.27	758.10 ± 16.57	101.08	2.19	746.87 ± 37.49	99.58	5.02
	1500.00	1467.16 ± 163.96	97.81	11.18	1459.16 ± 45.68	97.28	3.13	1486.54 ± 87.07	99.10	5.86
	4500.00	4511.70 ± 258.50	100.26	5.73	4533.21 ± 50.32	100.74	1.11	4514.25 ± 179.92	100.32	3.99
MDZ	30.00	26.13 ± 2.69	87.09	10.31	27.82 ± 0.30	92.75	1.07	27.17 ± 3.54	90.58	13.03
	60.00	60.46 ± 2.40	100.76	3.98	63.87 ± 2.33	106.45	3.65	60.66 ± 2.37	101.11	3.91
	300.00	290.75 ± 25.79	96.92	8.87	303.07 ± 2.77	101.02	0.91	332.83 ± 4.47	110.94	1.34
	600.00	626.87 ± 19.95	104.48	3.18	600.37 ± 13.96	100.06	2.33	619.04 ± 15.61	103.17	2.52
	1800.00	1796.13 ± 70.34	99.78	3.92	1794.87 ± 42.66	99.71	2.38	1761.23 ± 58.78	97.85	3.34
OH-MDZ	30.00	25.98 ± 0.69	86.60	2.64	31.11 ± 2.35	103.71	7.54	29.80 ± 1.53	99.34	5.14
	60.00	61.21 ± 1.19	102.02	1.95	58.48 ± 2.58	97.46	4.42	58.81 ± 1.78	98.02	3.03
	300.00	289.16 ± 38.60	96.39	13.35	295.19 ± 1.49	98.40	0.50	317.45 ± 6.05	105.82	1.91
	600.00	634.54 ± 32.51	105.76	5.12	601.29 ± 9.13	100.21	1.52	594.01 ± 35.43	99.00	5.97
	1800.00	1740.61 ± 64.82	96.70	3.72	1803.93 ± 56.58	100.22	3.14	1795.75 ± 95.79	99.76	5.33

FEX – fexofenadine; CAF – caffeine; PAR – paraxanthine; BUP – bupropion; OH-BUP – hydroxy-bupropion; FLB – flurbiprofen; OH-FLB – hydroxyflurbiprofen; OPZ – omeprazole; OH-OPZ – hydroxy-omeprazole; DEX – dextromethorphan; DTP – dextrorphan; MDZ – midazolam; OH-MDZ – hydroxymidazolam.

Table 5.10: Inter-day accuracy (%) and precision (%) in human plasma and DBS (n=9), using the Mitra™ platform, spiked at 5 different concentration on three separate days from separately made up working standard solutions.

Analyte	Spiked conc. ($ng.mL^{-1}$)	Inter-day plasma (n=9)			Spiked conc. ($ng.mL^{-1}$)	Inter-day DBS (n=9) (Mitra™)		
		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)
FEX	75.00	73.08 ± 2.24	97.45	3.07	75.00	70.45 ± 3.95	93.93	5.61
	150.00	153.58 ± 5.88	102.39	3.83	150.00	149.20 ± 2.63	99.47	1.76
	750.00	785.30 ± 5.76	104.71	0.73	750.00	739.89 ± 96.38	98.65	13.03
	1500.00	1522.38 ± 17.35	101.49	1.14	1500.00	1556.97 ± 25.56	103.80	1.64
	4500.00	4446.33 ± 25.58	98.81	0.58	4500.00	4404.97 ± 50.71	97.89	1.15
CAF	150.00	150.67 ± 11.70	100.45	7.77	150.00	142.91 ± 7.73	95.27	5.41
	300.00	299.66 ± 8.11	99.89	2.71	300.00	295.94 ± 12.07	98.65	4.08
	1500.00	1548.55 ± 44.42	103.24	2.87	1500.00	1451.72 ± 156.33	96.78	10.77
	3000.00	2980.31 ± 112.20	99.34	3.76	3000.00	3012.33 ± 53.93	100.41	1.79
	9000.00	8884.12 ± 137.15	98.71	1.54	9000.00	8931.49 ± 65.62	99.24	0.73
PAR	75.00	75.36 ± 5.12	100.48	6.79	75.00	73.93 ± 3.42	98.58	4.63
	150.00	144.48 ± 5.57	96.32	3.86	150.00	147.19 ± 6.86	98.12	4.66
	750.00	784.73 ± 33.99	104.63	4.33	750.00	774.79 ± 47.41	103.31	6.12
	1500.00	1517.20 ± 32.91	101.15	2.17	1500.00	1535.04 ± 87.08	102.34	5.67
	4500.00	4436.14 ± 69.15	98.58	1.56	4500.00	4428.43 ± 136.32	98.41	3.08
BUP	75.00	79.49 ± 1.72	105.99	2.17	75.00	79.11 ± 3.64	105.48	4.60
	150.00	137.87 ± 5.26	91.91	3.82	750.00	148.92 ± 2.57	99.28	1.72
	750.00	793.77 ± 36.09	105.84	4.55	1500.00	746.10 ± 18.83	99.48	2.52
	1500.00	1542.74 ± 34.11	102.85	2.21	3000.00	1468.00 ± 8.00	97.87	0.54
	4500.00	4405.34 ± 93.61	97.90	2.12	4500.00	4515.67 ± 154.22	100.35	3.42

Analyte	Spiked conc. ($ng.mL^{-1}$)	Inter-day plasma (n=9)			Spiked conc. ($ng.mL^{-1}$)	Inter-day DBS (n=9)		
		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)
OH-BUP	30.00	30.29 ± 3.66	100.96	12.07	75.00	72.59 ± 1.70	96.79	2.34
	150.00	142.18 ± 7.68	94.79	5.40	150.00	145.60 ± 7.25	97.07	4.98
	750.00	766.23 ± 21.70	102.16	2.83	750.00	761.55 ± 33.97	101.54	4.46
	1500.00	1511.25 ± 43.93	100.75	2.91	1500.00	1514.68 ± 9.77	100.98	0.65
	4500.00	4459.10 ± 110.87	99.09	2.49	4500.00	4404.56 ± 109.85	97.88	2.49
FLB	150.00	142.43 ± 6.32	94.95	4.44	150.00	148.60 ± 6.60	99.06	4.44
	300.00	303.85 ± 8.42	101.28	2.77	300.00	307.07 ± 16.19	102.36	5.27
	1500.00	1544.51 ± 17.87	102.97	1.16	1500.00	1461.66 ± 90.79	97.44	6.21
	3000.00	3057.69 ± 151.34	101.92	4.95	3000.00	3044.17 ± 158.49	101.47	5.21
	9000.00	8936.24 ± 128.58	99.29	1.44	9000.00	8942.83 ± 529.40	99.36	5.92
OH-FLB	75.00	71.56 ± 2.18	95.41	3.05	75.00	75.65 ± 2.79	100.87	3.68
	150.00	149.78 ± 3.18	99.85	2.12	150.00	150.20 ± 6.35	100.13	4.23
	750.00	783.89 ± 20.70	104.52	2.64	750.00	734.04 ± 23.47	97.87	3.20
	1500.00	1520.17 ± 54.33	101.34	3.57	1500.00	1512.70 ± 57.85	100.85	3.82
	4500.00	4458.25 ± 47.52	99.07	1.07	4500.00	4474.10 ± 91.90	99.42	2.05
OPZ	30.00	27.72 ± 1.29	92.39	4.67	75.00	71.33 ± 4.33	95.11	6.07
	150.00	155.65 ± 10.34	103.77	6.65	150.00	151.96 ± 5.89	101.31	3.87
	750.00	785.52 ± 6.70	104.74	0.85	750.00	768.41 ± 31.38	102.46	4.08
	1500.00	1564.87 ± 26.32	104.32	1.68	1500.00	1522.43 ± 12.65	101.50	0.83
	4500.00	4423.55 ± 22.13	98.30	0.50	3000.00	4449.65 ± 23.97	98.88	0.54
OH-OPZ	75.00	74.59 ± 2.88	99.46	3.86	30.00	70.46 ± 1.99	93.94	2.82
	150.00	161.45 ± 14.09	107.63	8.73	75.00	149.75 ± 0.74	99.83	0.50
	750.00	784.63 ± 57.01	104.62	7.27	750.00	785.00 ± 71.40	104.67	9.10
	1500.00	1505.75 ± 13.97	100.38	0.93	1500.00	1503.30 ± 24.74	100.22	1.65
	4500.00	4098.65 ± 353.24	91.08	8.62	3000.00	4463.32 ± 18.64	99.18	0.42

Analyte	Spiked conc. ($ng.mL^{-1}$)	Inter-day plasma (n=9)			Spiked conc. ($ng.mL^{-1}$)	Inter-day DBS (n=9)		
		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)		Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)
DEX	75.00	72.71 ± 4.60	96.94	6.32	75.00	73.64 ± 1.55	98.19	2.10
	150.00	147.26 ± 6.04	98.17	4.10	150.00	151.34 ± 2.26	100.89	1.50
	750.00	791.79 ± 44.43	105.57	5.61	750.00	743.62 ± 19.24	99.15	2.59
	1500.00	1554.21 ± 73.08	103.61	4.70	1500.00	1506.87 ± 21.87	100.46	1.45
	4500.00	4361.56 ± 181.78	96.92	4.17	4500.00	4477.94 ± 20.06	99.51	0.45
DTP	75.00	71.69 ± 1.66	95.59	2.31	75.00	73.45 ± 2.82	97.94	3.84
	150.00	151.68 ± 3.26	101.12	2.15	150.00	151.35 ± 2.75	100.90	1.82
	750.00	787.06 ± 31.41	104.94	3.99	750.00	740.02 ± 17.83	98.67	2.41
	1500.00	1548.95 ± 77.14	103.26	4.98	1500.00	1484.57 ± 14.08	98.97	0.95
	4500.00	4403.00 ± 118.14	97.84	2.68	4500.00	4508.65 ± 11.75	100.19	0.26
MDZ	30.00	29.42 ± 2.01	98.07	6.82	30.00	27.77 ± 0.86	92.55	3.09
	60.00	59.57 ± 3.30	99.28	5.54	60.00	60.37 ± 1.91	100.62	3.17
	300.00	306.27 ± 5.23	102.09	1.71	300.00	307.86 ± 21.64	102.62	7.03
	600.00	599.51 ± 2.43	99.92	0.41	600.00	615.30 ± 13.62	102.55	2.21
	1800.00	1795.05 ± 7.09	99.72	0.40	1800.00	1785.79 ± 19.80	99.21	1.11
OH-MDZ	30.00	28.87 ± 0.31	96.25	1.09	30.00	28.59 ± 2.67	95.31	9.33
	60.00	59.40 ± 0.60	99.00	1.01	60.00	60.01 ± 1.49	100.02	2.49
	300.00	310.92 ± 1.91	103.64	0.61	300.00	302.20 ± 14.90	100.73	4.93
	600.00	609.72 ± 13.92	101.62	2.28	600.00	609.52 ± 21.61	101.59	3.55
	1800.00	1782.55 ± 12.59	99.03	0.71	1800.00	1778.79 ± 34.44	98.82	1.94

FEX – fexofenadine; CAF – caffeine; PAR – paraxanthine; BUP – bupropion; OH-BUP – hydroxy-bupropion; FLB – flurbiprofen; OH-FLB – hydroxyflurbiprofen; OPZ – omeprazole; OH-OPZ – hydroxy-omeprazole; DEX – dextromethorphan; DTP – dextrorphan; MDZ – midazolam; OH-MDZ – hydroxymidazolam.

Inter-day variability calculated from separate working standard solutions over three non-consecutive days were acceptable with accuracy between 91.08 – 105.99% for all analytes in plasma matrix with precision less than 12.07% and between 92.55 – 105.48% in DBS with precision less than 13.03% at higher concentration levels.

5.3.2.5 Analyte stability

Analyte stability was assessed under different storage and sample preparation conditions, spiked into methanol alone, 1:1 methanol: water containing 0.1% formic acid (pH ±2.6), 1:1 methanol: water containing 0.01% acetic acid (pH ± 4) and 1:1 methanol: water containing 0.025% ammonium formate (pH ± 8). Short term analyte stability at room temperature in the sample vial showed an average reduction of 86.3% (pH ± 4) and 95.5% (pH ±2.6) in the peak area of omeprazole over a period of 6 hours. A similar decrease in analyte signal were observed for hydroxy-omeprazole, with 59.9% (pH ± 4) to 96.0% (pH ±2.6). Both omeprazole and hydroxy-omeprazole remained stable at higher pH conditions in the sample vial. As expected, the pH of the solution in the sample vial had an influence on the analyte signal intensities corresponding to the degree of ionisation, however this consistently showed a % CV between 0.6 – 6.9% for all analytes except omeprazole and its metabolite.

The same analyte mixtures were stored at -20°C and at ambient temperature (21 ± 3°C) and re-analysed after one month. Variation in the measured analyte peak areas were less than 15% for all analytes kept at -20°C except for omeprazole and hydroxy-omeprazole. The analyte peak area of bupropion, when kept at room temperature for one month, decreased by between 19.8 and 28.3% and confirms findings by Bosilkovska *et al.* This is due to the first-order decay kinetics at basic pH conditions.

Omeprazole and hydroxy-omeprazole showed a decrease of between 72.9 – 94.6% and 64.7 – 99.8% in peak area respectively, when stored in 1:1 methanol: water 0.1% formic acid solution and 1:1 methanol: water 0.01% acetic acid solution, with the characteristic second peak, discussed in Section 3.3.3, visible at an earlier retention time. On the other hand, analyte peak areas and retention times were stable in both the 1:1 methanol: water containing 0.025% ammonium formate (pH corresponding to physiological pH) and pure methanol solutions when kept at -20 ° C. These findings confirmed the instability of omeprazole in acidified media and resulted in use of an unbiased protein precipitation and extraction protocol where the composition of the sample vial solution contained 0.025% ammonium formate, for further

method validation assays. Plasma and DBS samples, spiked at the three QC concentrations, were also assessed after 3 freeze thaw cycles against freshly made matrix-matched calibration curves. Results (given in Table 5.11) indicate that most analytes were stable at higher concentration levels, in both matrixes, with the accuracy, measured against freshly made calibrants, between 85% – 115%. Flurbiprofen concentrations measured in stored plasma samples, showed a positive bias slightly higher than 15% at all QC levels. This could be due to a difference in the extraction recovery or instrument bias when operated in negative ionisation mode. Due to the small sampling volume and higher dilution factor with DBS extraction, the acceptable accuracy at the lowest spiked QC was set at 20%. Again, flurbiprofen did not meet this criterion, with a variance higher than 20% at the lowest spiked QC concentration. Studies have shown that analyte stability might be increased by extraction from the Mitra™ sampler shortly after sample collection and storage of extracted samples at -80°C before further analysis.[59] Analyte stability on the Mitra™ sampler should be studied further, at different storage times and at different temperatures other than the three-freeze-thaw cycles studied here, before final conclusions could be made, since varying results have been recorded in literature.[59]

5.3.3 Assessing inter-method agreement between dried blood spots and plasma sampling

Analyte blood-to plasma ratios are governed by blood cell distribution kinetics, which in turn could be influenced by analyte concentrations, the time it takes to reach the erythrocyte-to-plasma equilibrium and temperature.[201] Acidic analytes are not expected to have high affinity for protein components on or in erythrocytes, whereas the basic pharmaceuticals would be in their ionised state at physiological pH with greater affinity for acidic phospholipids, carbonic anhydrase and intracellular cyclophilin.[248] Variability in the degree of affinity for erythrocyte and plasma protein binding sites and factors affecting the affinity should be estimated *in vitro* from whole blood, collected from a representative study population. This is relevant to any study aimed at using DBS to assess *in vivo* drug exposure, especially in respect to reliability and agreement between DBS and plasma concentrations. Another important consideration is the extent to which the extraction procedure liberates bound drug from either plasma proteins or erythrocytes. Different matrices and different extraction protocols may result in a different amount of drug being liberated from the plasma proteins and can influence the measured concentration.

Table 5.11: Accuracy and precision of spiked QC samples in plasma and DBS after three freeze-thaw cycles at -80 ° C.

	Human plasma				Dried Blood Spots (Mitra™)			
	Spiked conc. ($ng.mL^{-1}$)	**Found (mean ± SD in $ng.mL^{-1}$)**	**Accuracy (%)**	**Precision (%)**	**Spiked conc. ($ng.mL^{-1}$)**	**Found (mean ± SD in $ng.mL^{-1}$)**	**Accuracy (%)**	**Precision (%)**
Fexofenadine	150.00	142.17 ± 15.74	94.53	11.07	150.00	136.97 ± 1.86	91.31	1.36
	600.00	587.21 ± 86.94	97.87	14.81	450.00	410.73 ± 29.24	91.27	7.12
	3000.00	2953.25 ± 349.62	98.44	11.84	3000.00	2529.27 ± 95.76	84.31	3.79
Caffeine	150.00	105.25 ± 17.76	70.16	16.87	150.00	131.88 ± 2.28	87.92	1.73
	600.00	566.56 ± 57.78	94.43	10.20	600.00	515.05 ± 9.32	85.84	1.81
	3000.00	2770.61 ± 378.45	92.35	13.66	3000.00	2642.36 ± 53.60	88.08	2.03
Paraxanthine	75.00	58.02 ± 7.03	77.36	12.12	75.00	63.54 ± 4.04	84.73	6.36
	300.00	285.58 ± 36.22	95.19	12.68	300.00	280.23 ± 17.40	93.41	6.21
	1500.00	1439.21 ± 184.95	95.95	12.85	1500.00	1389.55 ± 72.03	92.64	5.18
Bupropion	150.00	117.58 ± 26.11	78.39	22.20	150.00	138.67 ± 10.00	92.44	7.21
	750.00	671.12 ± 51.89	89.48	7.73	450.00	462.01 ± 36.69	102.67	7.94
	3000.00	3270.21 ± 217.32	109.01	6.65	3000.00	2822.43 ± 220.29	94.08	7.81
Hydroxybupropion	75.00	66.98 ± 3.96	89.31	5.91	75.00	71.09 ± 2.51	94.78	3.54
	300.00	320.10 ± 8.88	106.70	2.77	300.00	278.04 ± 21.46	92.68	7.72
	1500.00	1635.18 ± 12.05	109.01	0.74	1500.00	1576.19 ± 31.09	105.08	1.97
Flurbiprofen	150.00	169.06 ± 18.58	112.71	10.99	150.00	193.96 ± 2.54	129.30	1.31
	600.00	711.69 ± 54.04	118.61	7.59	600.00	650.30 ± 82.69	108.38	12.72
	3000.00	3497.46 ± 115.23	116.58	3.29	3000.00	3283.55 ± 493.34	109.45	15.02
Hydroxyflurbiprofen	75.00	65.43 ± 0.90	87.24	1.38	150.00	122.48 ± 4.42	81.65	3.61
	300.00	284.31 ± 16.94	94.77	5.96	300.00	299.39 ± 14.40	99.80	4.81
	1500.00	1511.98 ± 88.64	100.80	5.86	1500.00	1481.57 ± 6.22	98.77	0.42

Table 5.11 (continued): Accuracy and precision of spiked QC samples in plasma and DBS after three freeze-thaw cycles at -80 ° C.

Analyte	Human plasma				Dried Blood Spots (Mitra™)			
	Spiked conc. ($ng.mL^{-1}$)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)	Spiked conc. ($ng.mL^{-1}$)	Found (mean ± SD in $ng.mL^{-1}$)	Accuracy (%)	Precision (%)
Omeprazole	150.00	160.38 ± 19.83	106.92	12.37	75.00	54.21 ± 2.61	72.29	4.81
	300.00	251.81 ± 26.33	83.94	10.46	300.00	281.02 ± 5.71	93.67	2.03
	1500.00	1399.75 ± 237.21	93.32	16.95	1500.00	1338.81 ± 55.19	89.25	4.12
Hydroxy-omeprazole	75.00	68.39 ± 1.65	91.19	2.41	75.00	71.15 ± 3.37	94.87	4.74
	300.00	313.34 ± 26.25	104.45	8.38	300.00	294.24 ± 12.17	98.08	4.13
	1500.00	1457.62 ± 76.06	97.17	5.22	1500.00	1474.07 ± 39.84	98.27	2.70
Dextromethorphan	75.00	77.41 ± 6.88	103.21	8.89	75.00	75.36 ± 4.96	100.48	6.58
	300.00	333.85 ± 16.94	111.28	5.07	300.00	274.74 ± 10.84	91.58	3.95
	1500.00	1629.79 ± 97.39	108.65	5.98	1500.00	1543.52 ± 50.39	102.90	3.26
Dextrorphan	150.00	150.04 ± 4.32	100.03	2.88	150.00	151.49 ± 5.92	100.99	3.91
	750.00	790.96 ± 48.57	105.46	6.14	750.00	659.63 ± 17.17	87.95	2.60
	3000.00	3127.85 ± 104.21	104.26	3.33	3000.00	3131.03 ± 141.39	104.37	4.52
Midazolam	30.00	26.14 ± 3.14	87.14	12.01	30.00	24.78 ± 1.97	82.62	7.93
	120.00	137.37 ± 16.05	114.48	11.69	120.00	115.10 ± 2.26	95.91	1.96
	600.00	650.37 ± 55.62	108.40	8.55	600.00	559.28 ± 36.62	93.21	6.55
Hydroxy-midazolam	30.00	34.19 ± 5.72	113.97	16.73	60.00	49.65 ± 1.37	82.74	2.75
	180.00	205.49 ± 12.36	114.16	6.01	180.00	164.88 ± 8.48	91.60	5.15
	1200.00	1090.39 ± 129.74	90.87	11.90	1200.00	979.89 ± 31.29	81.66	3.19

Extrapolation between methods would be difficult as the slopes of the calibration curves will differ. In this study pooled whole blood was spiked with an analyte mix within four hours after collection and allowed to equilibrate at ambient temperature for at least one hour. Inter-method agreement between traditionally harvested plasma from venous blood sampling and DBS sampling, using the Mitra™, which is a volumetrically controlled sampling device, was assessed by plotting the predicted plasma concentrations, derived from the *in vitro* DBS calibration curves, against the true measured average plasma concentration (n=9) at known spiked concentrations. The methodology followed is that recommended by the International Consortium for Innovation and Quality in Pharmaceutical Development MWG, using pooled human whole blood and plasma collected from the study population of interest. The percentage difference between the predicted and the true average y values, at the same spiked concentration, were plotted against the true measured plasma concentrations.

Despite using altered sonication times and increased extraction temperature during DBS sample preparation, differences between predicted and true measurements consistently fell within the 20% range over the three separate validation days for fexofenadine, dextromethorphan, midazolam, hydroxymidazolam, indicating fair agreement between the two sampling methods and that equivalent pharmacokinetic parameter determinations could be calculated from the two matrices. The % CV (given in brackets) for the analytes were: fexofenadine (5.9), dextromethorphan (5.4), midazolam (3.5) and hydroxymidazolam (4.8) with hydroxy-bupropion (12.4) showing the highest variation. These analytes are all lipophilic with log P values between 2.48 and 3.49, eluting last off the reversed-phase chromatographic column and are expected to be highly protein bound. Consistent recovery from plasma and the Mitra™ sampler as well as analyte stability probably contributed to this observation. On the other hand, the percentage difference between the predicted and measured plasma concentration were inconsistent for other analytes, which could be attributed to a number of different factors, including their different physicochemical properties, stability and variance in extraction recovery with the altered sonication times and extraction temperature used during DBS sample preparation. Diagnostic plots (Figure 5.6) are shown for the CYP450 probe drugs and their metabolites and for the P-gp probe drug fexofenadine (Figure 5.7).

Hydrophilic and polar analytes are expected to show poor plasma protein binding and usually do not enter erythrocytes, although caffeine enters erythrocytes but does not bind to the cell constituents implying highly correlated concentrations of the drug measured in both whole blood and plasma. In this study, the Mitra™ technology overestimated plasma concentrations

of caffeine and its metabolite paraxanthine, when DBS were extracted at lower temperatures and shorter sonication times, but increased sonication time and higher temperature during extraction reduced the positive recovery bias.

Under these conditions the caffeine and paraxanthine plots indicated agreement with the percentage variation within the acceptable 20% range. This could also be visualised by overlaying the DBS and plasma calibration curves as well as the solvent based calibration curves matching the two different extraction methods. When the extraction from both DBS and plasma were optimised for caffeine and paraxanthine the calibration equations were similar (Figure 5.8) as expected for hydrophilic analytes that are not highly bound to plasma proteins or erythrocytes. It did, however, depend on an optimised extraction protocol. Previous studies indicated that haematocrit-dependent recovery could influence quantitative accuracy compared to other matrices, regardless of the accurate sampling volume, collected independently of the haematocrit.[245, 249, 250] These studies further elucidated the importance of optimising the extraction procedure, conditions and solvents.[239] Thus, caution should be exercised when predicting plasma concentration using DBS calibration curves, since these are highly dependent on the extraction protocol, where the resultant recovery of different analytes using different conditions can be affected by the matrix.

Longer sonication time and higher extraction temperatures from the Mitra™ sampling tip resulted in lower absolute bupropion recovery (32% on days two and three of the study vs 66% on day one when the shorter extraction protocol was used). Poor bupropion stability might be a possible explanation for the altered recovery, since it is unstable at higher temperature, with 3-chlorobenzoic acid identified as a detectable degradation product.[212] Bupropion further undergoes first order decay over two weeks at physiological pH to form N-methyl bupropion.[212] CYP2B6 activity is important in Sub-Saharan African where high frequencies for the CYP2B6*6 allele, which is associated with adverse events during ARV therapy, are reported.[104] Currently, bupropion is the FDA probe drug of choice for CYP2B6. For DBS sampling using the Mitra™ to be accurate, further optimisation of recovery of this probe drug and its metabolite is necessary. Bupropion should be extracted using lower temperatures, shorter sonication times and preferably a lower pH extraction buffer. It is further recommended that the Mitra™ DBS sampler be stored at -20ºC and not ambient temperatures.

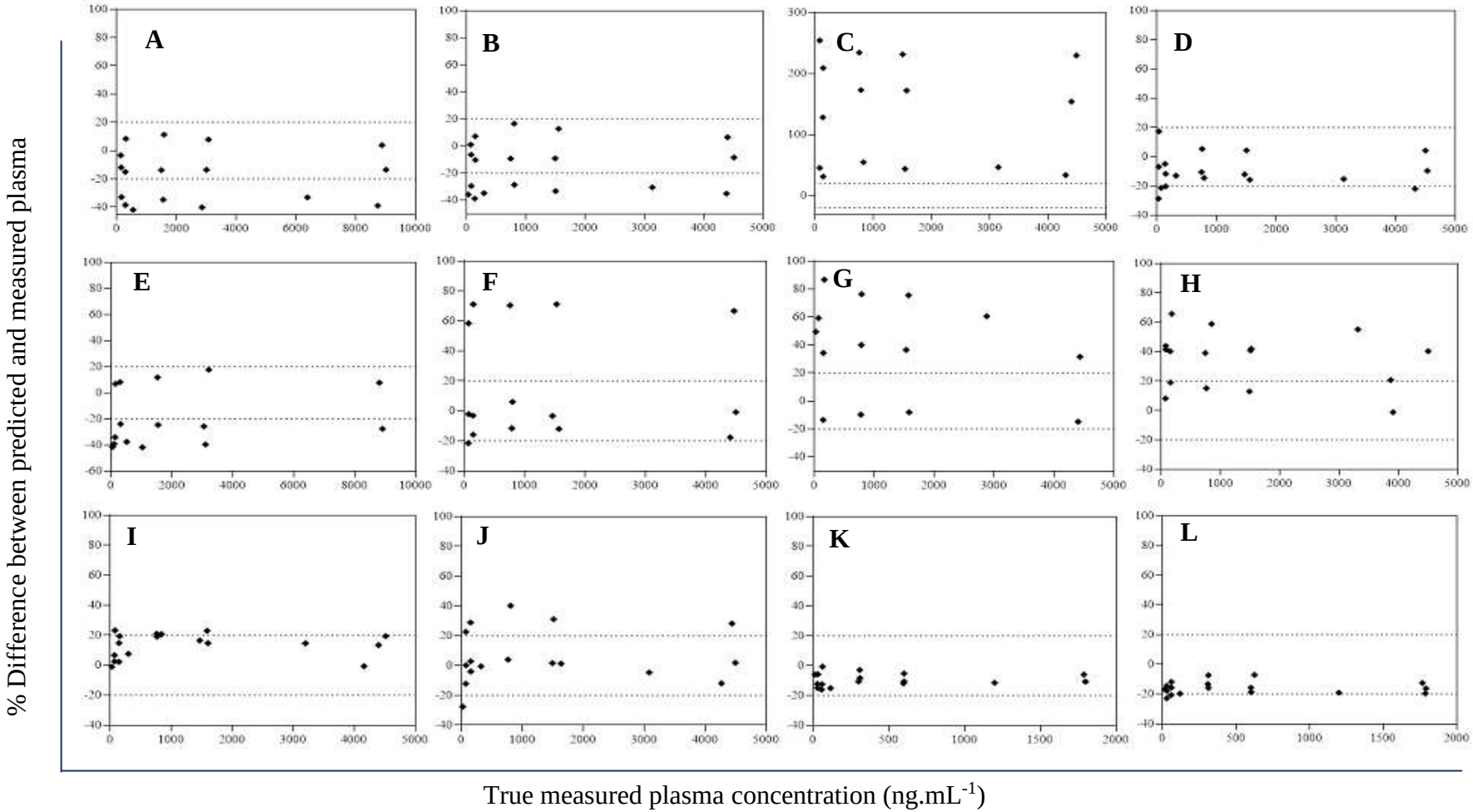


Figure 5.6: Diagnostic plots exploring agreement between predicted and measured plasma concentration of CYP450 probes and metabolites at known spiked concentration levels with A – caffeine, B – paraxanthine, C – bupropion, D – hydroxy-bupropion, E – flurbiprofen, F – hydroxyflurbiprofen, G – omeprazole, H – hydroxy-omeprazole, I – dextromethorphan, J – dextrorphan, K – midazolam, L – hydroxymidazolam.

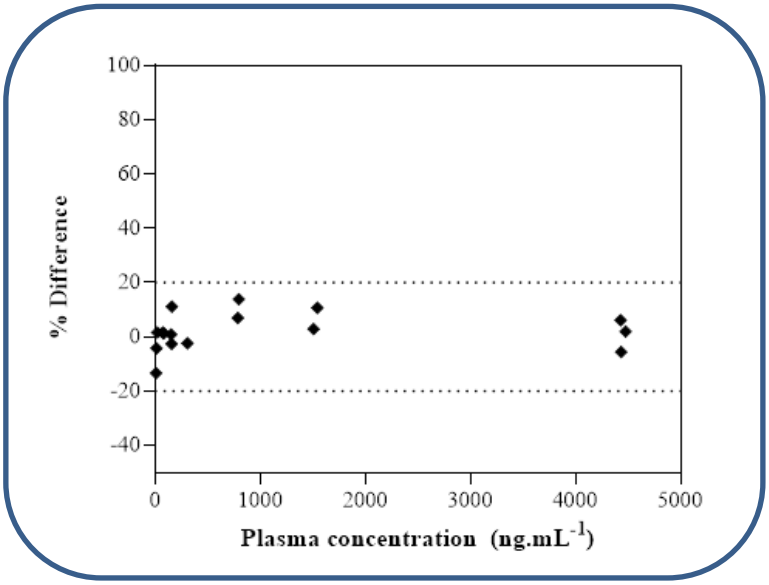


Figure 5.7: Diagnostic plot exploring agreement between predicted and measured plasma concentration of P-gp probe fexofenadine.

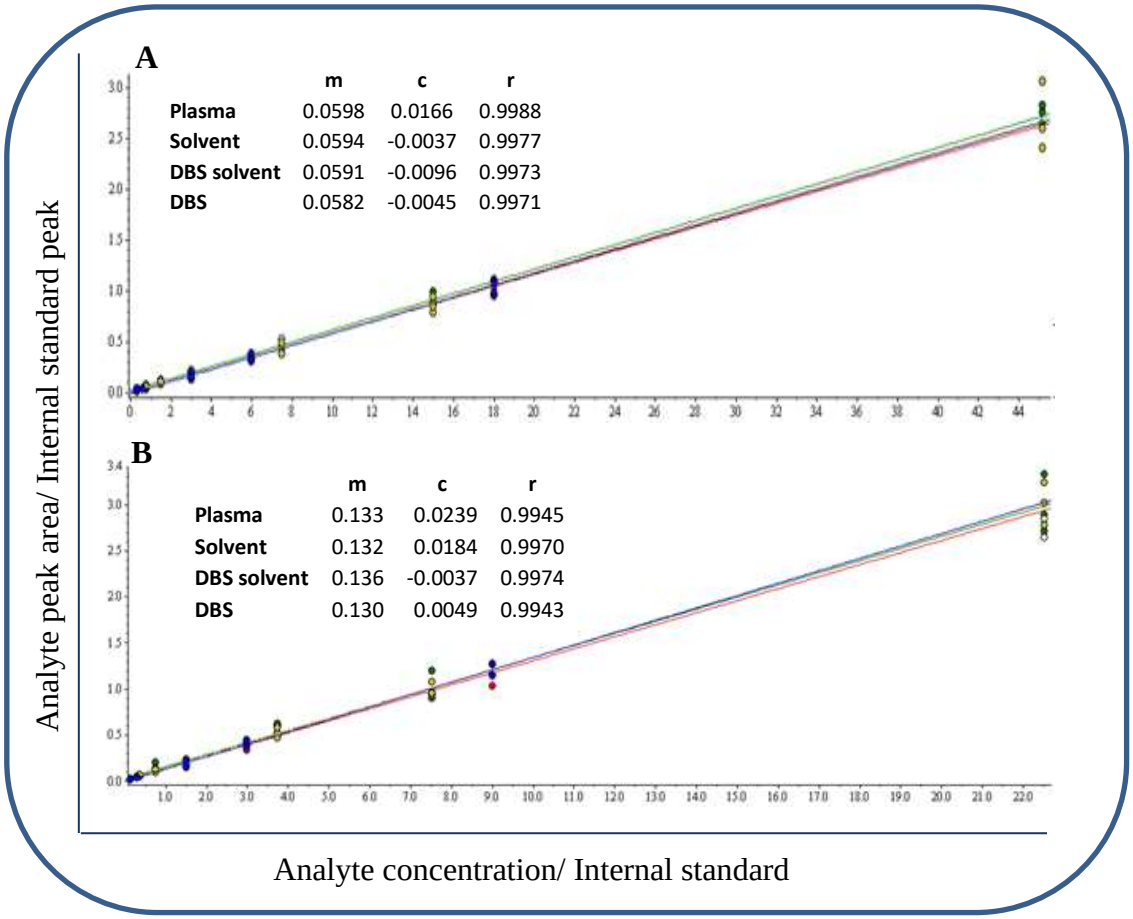


Figure 5.8: Overlaid calibration curves for A – caffeine and B – paraxanthine extracted from Mitra™ DBS sampler and plasma with matching solvent calibration curves.

Flurbiprofen has been used as a probe drug for CYP2C9 activity in the Pittsburg[152] and Geneva[37] phenotyping cocktails. Both flurbiprofen and its hydroxylated metabolite are acidic drugs and thus expected to have little affinity for erythrocytes. As a result, the measured concentrations between plasma and DBS should correlate well. Daali *et al.* investigated the metabolic ratio of flurbiprofen and hydroxyflurbiprofen in DBS as an alternative to plasma sampling. They found higher drug concentrations in plasma that was attributed to the smaller dilution factor due to the lack of erythrocytes and possibility of altered sampling precision using Whatman 903 DBS filter paper. This study found that a DBS calibration curve underestimated the plasma concentration for flurbiprofen at higher extraction temperatures and longer sonication times. It is unclear whether this was due to possible matrix effects or experimental bias and should be further investigated, using fresh stock solutions and QC samples on each day. Higher extraction temperatures and longer sonication times, as used on days two and three of the validation during this study showed the hydroxy-flurbiprofen DBS calibration curve matched predicted plasma concentrations within the accepted 20% variance.

Large variability in predicted and measured plasma concentrations were also evident for omeprazole and hydroxy-omeprazole, which could be attributed to analyte instability (discussed in Section 3.3.3). However, increased extraction temperature and sonication resulted in a lower bias when the DBS curves were used to predict plasma concentrations. Under these conditions more of the erythrocyte-associated drug is expected to be extracted, although better correlation between the concentrations of the analytes measured in both matrixes is still not to be expected. Figure 5.9 shows overlaid analyte calibration curves for DBS and plasma together with their matrix matched solvent curves on day three of the experiment. To overcome issues with analyte stability, it is recommended that the more stable pantoprazole be used as a substitute for the omeprazole. Pantoprazole has proven to be the most stable equivalent probe drug under different pH conditions used during sample preparation, on autosampler stability and LC-MSMS analysis with improve peak shape and better sensitivity due to efficient ionisation.[211] The main metabolic pathway of pantoprazole is also demethylation by CYP2C19 and it has a lower potential of interaction with other drugs compared to omeprazole[251]. In addition to substitution with alternative probe drugs that are more stable, DBS samples could also be extracted shortly after collection with the Mitra™ device and stored at -80ºC until further analysis, to solve challenges with sample stability.[59]

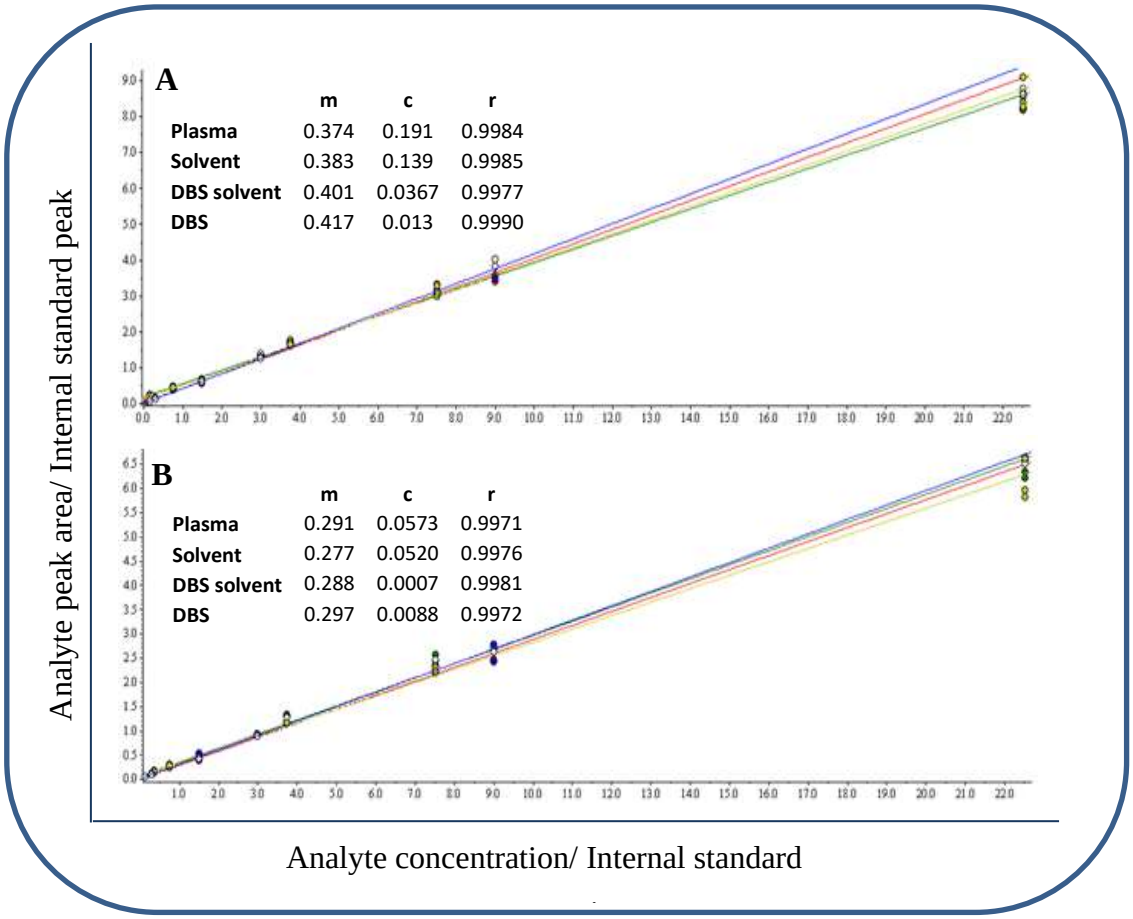


Figure 5.9: Overlaid calibration curves for A – omeprazole and B – hydroxy-omeprazole extracted from the Mitra™ DBS sampler and plasma with matching solvent calibration curves on day three of validation.

Also, conclusive data on the extent to which analytes bind to protein components in or on erythrocytes or to plasma proteins in an *in vitro* environment are lacking and might influence quantitative *in-vitro-in-vivo* correlation. Extensive validation in an *in vivo* population is necessary before implementation of phenotyping in routine practice. Correlation between the measured analyte in DBS and plasma is dependent on the analytical workflow used in this study and the protocols used influenced the analyte recovery. A conclusive confounding factor was the extent to which the extraction procedure liberated bound drug from either plasma proteins or erythrocytes. The different DBS extraction conditions resulted in altered amounts of specific analytes liberated from the Mitra™ sampler and influenced the predicted plasma concentration. Significant effects were seen for the recovery of caffeine, paraxanthine, dextrorphan and bupropion, flurbiprofen, omeprazole and their hydroxylated metabolites.

There are a number of important factors that require careful consideration before agreement between *in vivo* plasma and DBS sampling with the Mitra™ sampler could be assessed. It was evident from the initial *in vitro* assessment of agreement that blood cell distribution kinetics are regulated not only by the blood-to-plasma concentration ratio, but also by the physicochemical properties of the analytes, extraction temperature, total analyte concentration, analyte stability and time dependent equilibrium between different blood compartments. The proposed relationship between analyte pK_a and Log P and blood cell distribution with their hypothesised influence on predicted vs real plasma concentrations based on measurements in DBS matrix are illustrated in Figure 5.10.

In the case of more acidic compounds like flurbiprofen and hydroxy-flurbiprofen, that are not expected to have high affinity for erythrocytes, whole blood would show a lower concentration due to the reduced amount of plasma in the total volume collected and analysed compared to the harvested plasma where erythrocytes are absent. When the recovery from the DBS was optimised, the DBS calibration curve still underestimated the plasma concentration of flurbiprofen in plasma. On the other hand, basic pharmaceuticals are in their ionised state at physiological pH which influences their binding affinity for erythrocytes. Under these circumstances it stands to reason that the measured concentration in plasma would be lower than that in whole blood since whole blood would have analytes bound to the erythrocytes in the sample. A calibration curve developed from DBS would in theory over-estimate the concentration of the drug in plasma, which was observed for dextromethorphan and dextrorphan in this study.

In addition to the differences in erythrocyte affinity seen for acidic versus basic pharmaceuticals, the lipophilicity of the analytes also contributed to the blood cell distribution kinetics *in vitro*. Fexofenadine, midazolam and hydroxy-midazolam are hydrophobic and have higher plasma protein binding than the more hydrophilic analytes caffeine and paraxanthine. This could explain why the harsher DBS extraction methods did not influence predicted vs measured plasma concentration in the case of fexofenadine, midazolam and hydroxy-midazolam since the high proportion of these drugs were bound to plasma proteins and were liberated under the optimised plasma extraction method. These observations are to be confirmed in an *in vivo* environment during a clinical pilot study, which still requires ethical approval from the South African Health Products Regulatory Authority (SAHPRA) which is a legal requirement.

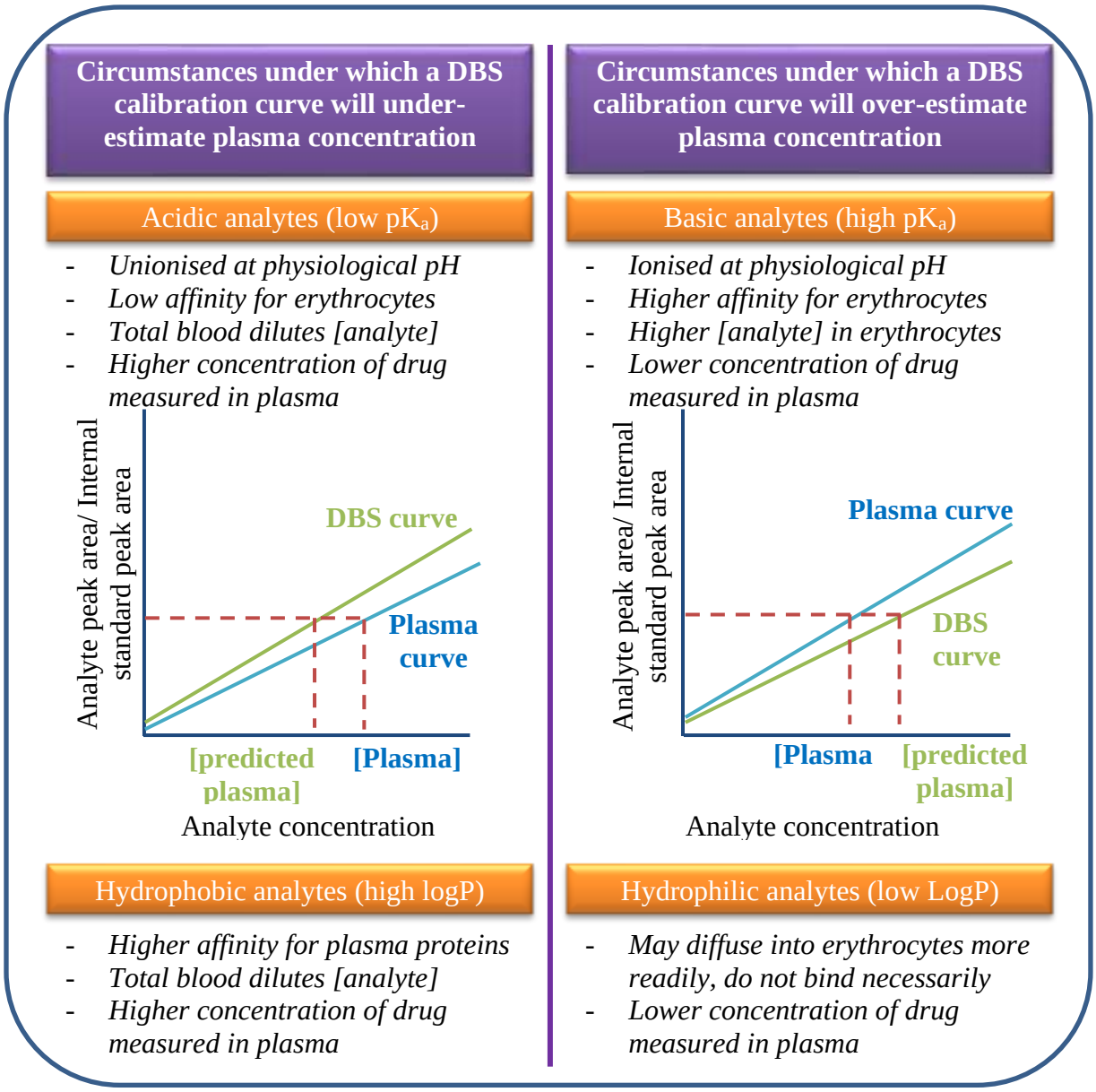


Figure 5.10: Proposed relationship between analyte pK_a and Log P and blood cell distribution with their hypothesised influence on predicted vs real plasma concentrations based on measurements in DBS matrix.

5.3.4 *In vivo* pilot pharmacokinetic study

Differences in measurements from DBS and plasma sampling were also confirmed *in vivo* after oral administration of the phenotyping cocktail to a patient volunteer. XIC chromatograms at different PK sampling times are shown in Figure 5.11. The validated LC-MS/MS method was sensitive enough to be used for phenotypic profiling able to determine inter-individual variability to infer metabolic activity or drug transport activity, from a small volume biological DBS sample, after administration of a low dose "home-made" phenotyping cocktail in combination with optimised analytical extraction protocols.

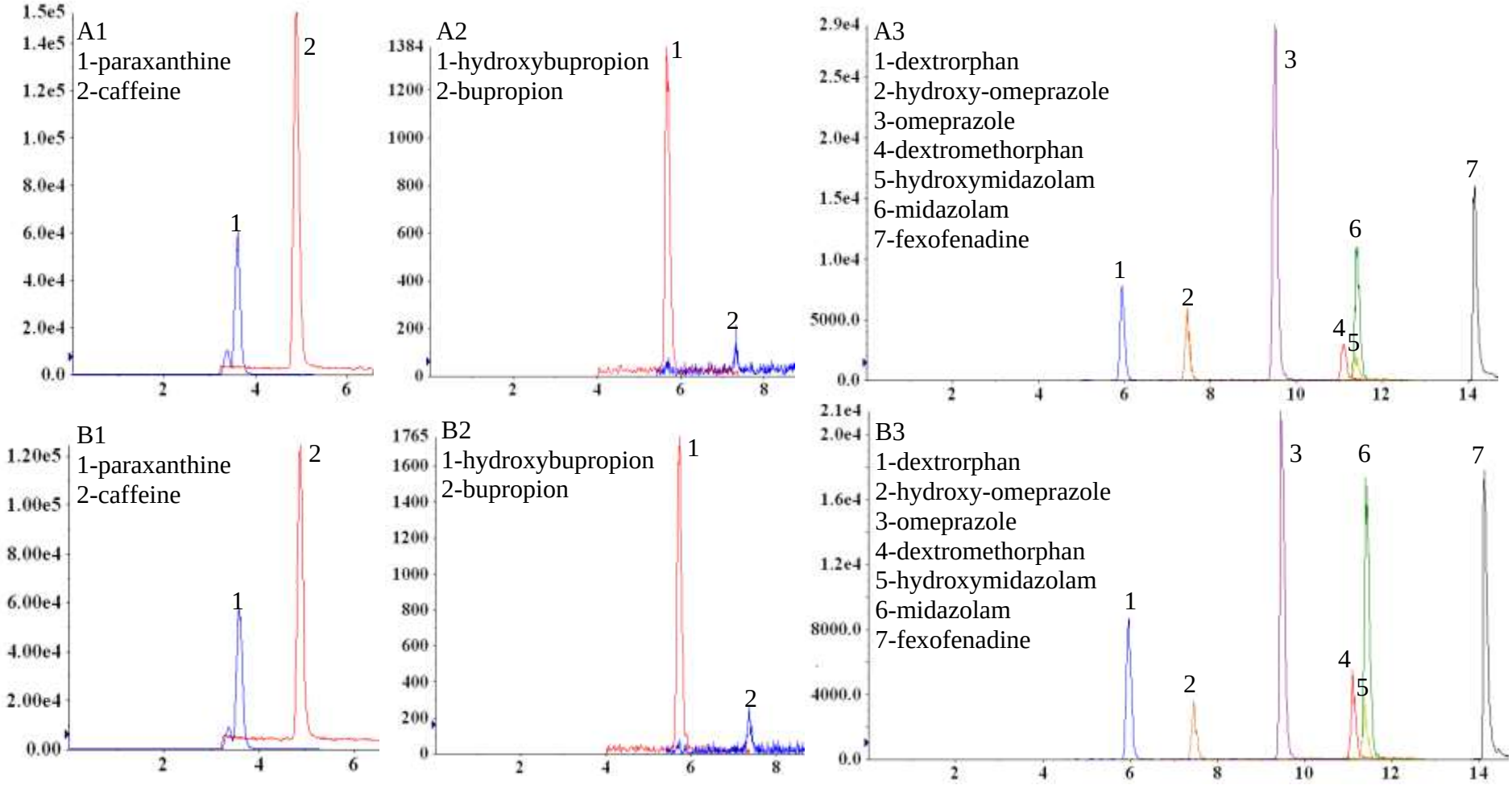


Figure 5.11: Chromatograms obtained with a scheduled MRM acquisition method for analytes in positive ESI mode after oral administration of "home-made" phenotyping cocktail with plasma sampling (A) and DBS sampling (B) using the Mitra microsampling device. A1 and B1 – paraxanthine and caffeine 2.5 hours post dose; A2 and B2 – hydroxy-bupropion and bupropion 3.5 hours post dose; A3 and B3 – dextrorphan, hydroxy-omeprazole, omeprazole, dextromethorphan, hydroxy-midazolam, midazolam and fexofenadine 3 hours post dose.

5.4 CONCLUSION

To our knowledge, this is the first DBS validation study using the Mitra™ device for the purpose of simultaneous phenotyping of the *in vivo* P-gp transport and CYP450 metabolic activity of the CYP1A2, -2B6, -2C9, -2C19, -2D6 and -3A4 enzymes and activity. From the initial *in vitro* assessment of agreement, it was concluded that blood cell distribution kinetics are regulated by the blood-to-plasma concentration ratio and time dependent equilibrium between different blood compartments, the physicochemical properties of the analytes, temperature during extraction, analyte concentration and stability and the extent to which the extraction procedure liberated bound drug from either plasma proteins or erythrocytes.

CHAPTER 6: OVERALL CONCLUSION, STUDY LIMITATIONS AND FUTURE PROSPECTS

Wide inter- and intra-individual pharmacokinetic variability in response to pharmacotherapy has been attributed to variation in drug absorption and metabolism. Assessing *in vivo* drug absorption, via P-gp transport proteins across enterocytes, and metabolism by CYP450 enzymes may contribute to personalised medicine whereby the incidence of adverse drug reactions, drug-drug interaction or therapeutic failure often seen in clinical practice can be reduced. Pharmacokinetic variability is patient specific with several intrinsic and extrinsic factors influencing drug absorption and metabolism. An individualised approach to pharmacotherapy would be beneficial in a South-African population considering the large genetic diversity; influenced by ancestry and ethnicity and geographical location. Genotyping alone is often insufficient to infer metabolic or transport phenotypes due to the complex interplay of many contributing factors that result in altered phenotype. Phenotyping, by measuring the absorption or metabolic conversion rate of specific probe drugs can provide a real-time snapshot of *in vivo* biological drug transport activity and drug metabolism that could be applied in routine clinical practice to guide personalised prescribing, especially for patients with multiple comorbidities on polypharmacy, as well as to monitor therapeutic drug exposure and to assess patient adherence to pharmacotherapy.

For personalised medicine to become a reality in routine practice a number of challenges would have to be addressed. First, clinicians should have a basic knowledge of the factors influencing the activity (induction or inhibition) of individual enzymes, especially considering polypharmacy, high OTC medication use and use of herbal and alternative remedies. More importantly, an economically viable, minimally invasive sampling platform, suitable for home sampling, would improve the feasibility of a routine phenotyping method to measure drug transport and metabolic activities while assessing potential unexpected drug exposure during treatment. Preferably a low dose mixed probe drug combination followed by a single time point sampling by a minimally invasive finger-prick could be used to measure metabolic ratios and drug absorption parameters. Probe drugs should be easily available, safe and tolerable at administered dosages, effective for use in a cocktail for simultaneous assessment and have established CYP450 enzyme and P-gp transport selectivity. To this end the already validated Geneva phenotyping cocktail was used in the current study.

The main question when using capillary blood sampling as an alternative to plasma is whether it provides the same quantitative bioanalytical answer. Considering the potential differences in the composition of capillary whole blood and venous plasma, the dose dependent binding of probe drugs and their metabolites to plasma proteins and erythrocytes as well as the influence of the haematocrit on the viscosity of whole blood and the possibility of contamination of capillary blood with interstitial fluid, differences could be expected. Moreover, the smaller sampling size, when using capillary blood, requires optimal recovery and reproducible sensitive bioanalytical quantitation. Lastly, appropriate statistical methods should be used to prove agreement between quantitative measurements in plasma and DBS. Non-linear statistical models could be used in instances where quantitative drug concentration between the two matrices indicate a non-linear relationship.

Another difficulty, when using multi-drug phenotyping cocktails, are the diverse physicochemical properties of the probe drugs that present quantitative bioanalytical and pharmaceutical challenges. In this regard, the different physicochemical properties of probe drugs require an optimised and validated bioanalytical method with maximal extraction efficiency to separate, detect and quantify the different polycyclic or aromatic pharmaceutical probe drugs and their metabolites in small complex biological samples and must have sufficient analytical sensitivity.

A relatively new development in DBS sampling is the Mitra™ device using VAMS™ technology, that was launched in 2014, to collect an accurate volume of whole blood directly from the sampling site, i.e. the finger prick, to overcome problems with sample homogeneity and influence of haematocrit when using cellulose based dry blood spot cards. More research on individual compounds and method validation is required before the product can be used routinely in clinical practice for simultaneous phenotyping CYP450 metabolism and P-gp absorption.

The aim of this study was to assess inter-method agreement of the measured probe drug and metabolite concentrations, used to determine phenotype, between the DBS using the Mitra™ DBS sampling device and harvested venous plasma, from *in vitro* spiked whole blood samples collected from healthy volunteers. A further aim was to compound and validate the use of the Geneva phenotyping cocktail for future use in a South-African population. To our knowledge, this is the first DBS validation study using the Mitra™ device for the purpose of phenotyping P-gp transport and multiple CYP450 enzyme activity simultaneously. Targeted LC-MS/MS

methods were developed and validated in both negative and positive ESI mode with optimised mass spectrometric detection parameters for each individual analyte then combined into a scheduled MRM transition method with optimised ESI source parameters and LC elution gradient.

As a first phase, the bioanalytical method was investigated using the ICH guidelines, to ensure optimisation of the complete method under conditions most likely to introduce variation in quantitative results. This ensured that repeatable and reliable results would be obtained when analysing real samples. This included exploring alternative column chemistry to improve analyte resolution of the LC-method and the influence of variation in the pH of both the sample vial and mobile phases used for the chromatographic separation. A biphenyl stationary phase with predominantly methanol as the organic eluent provided improved resolution and analyte selectivity of the structurally related aromatic compounds used as probe drugs in the Geneva phenotyping drug cocktail together with their CYP specific metabolites. This improved chromatographic resolution was due to enhanced π-π interactions between the phenyl groups of the stationary phase and the analytes, that were favoured in the presence of methanol. The improved resolution can be advantageous in future *in vivo* PK analysis due to more accurate quantitation of the individual analytes when they are better separated with sharper more symmetrical peaks. The second objective was to study the ionisation efficiency of the individual analytes under different pH conditions in positive ESI mode, to optimise the sensitivity and detection limits of the LC-MS/MS analytical method for simultaneous quantitation of analytes with different physicochemical properties, all present at low concentrations, from a low volume biological sample. Although a mobile phase at a pH of 3.9, containing 10 mM ammonium formate provided statistically significant greater MS sensitivity for most of the analytes, it resulted in longer runtimes with a lower throughput method with unacceptable retention factors. Also, considering the instability of omeprazole and hydroxy-omeprazole at acidic pH, a combination of 10 mM ammonium formate in the LC sample vial paired with a mobile phase containing 0.1% formic acid (pH 2) were chosen for further method validation. With this pairing of sample storage and run conditions, analyte sensitivity was slightly sacrificed for a higher throughput method and improved column efficiency. The complete method was still sensitive enough with sufficient quantitation limits to quantify all analytes from a small volume DBS sample (20 µL) at low phenotyping drug combination dosages.

The third objective was to compound an "in-house" phenotyping cocktail capsule from commercially available dosage forms and analytical standards. This proved to be a challenge due to the difficulty of producing a homogenous powdered mixture, possible interference of different excipients in the commercial dosage forms and low pharmaceutical flowability that complicated hand-filling of the capsules. Regardless of the fact that the compounded cocktail capsules in this study had larger variability in terms of the absolute drug content, their purpose is to measure metabolic conversion ratios within individual patients and not absolute drug concentrations. The ratio of parent drug to metabolite conversion under low concentrations is dependent on the CYP450 enzyme metabolic activity in the gut and the liver, which is not influenced by exact dosage because of the low doses in the phenotyping drug cocktail administered. Similarly, the absorption involving P-gp transporter proteins within an individual patient could still be measured, since the rate of uptake at low phenotyping doses should also not be affected by a 20-30% difference in content.

The quantitative bioanalytical methods, in positive and negative mode, were validated according to ICH guidelines for matrix effects, analyte recovery, linearity, LOD, LOQ, carry-over, inter and intraday precision and accuracy and stability as the third objective of the study. Ionisation suppression or enhancement were analyte specific with relative matrix effects reducing the signal to between 90 and 110% for most analytes in post extracted plasma and DBS matrixes, except for bupropion and hydroxy-bupropion detected in positive ESI mode and for flurbiprofen and hydroxy-flurbiprofen detected in negative ESI mode, where ionisation suppression greater than 10% were observed. This was attributed to the elevated baseline noise observed for these four analytes specific to the MS method and co-elution of matrix components.

Analyte recoveries from plasma and DBS fell within 15% of each other with good precision (< 10%) for all analytes except for one timepoint for flurbiprofen. As expected, the recovery bias was largest at the lowest spiked concentrations. To promote an unbiased extraction, the extraction solvents contained no additives that could favour some analytes above others for both extraction from plasma and DBS. Over the three different days comparing the DBS extraction the extraction temperatures and sonication times were however varied during the validation. This contributed to the more variable absolute recoveries seen for some of the analytes from the Mitra™ sampler. These absolute recoveries from the Mitra™ sampler were analyte specific, with a positive recovery bias at lower extraction temperature and shorter sonication times for the neutral more hydrophilic compounds caffeine and paraxanthine and a

significant decrease in absolute recoveries of bupropion at higher extraction temperatures and longer sonication times. Overall, absolute recoveries of acidic flurbiprofen and hydroxy-flurbiprofen and the less lipophilic omeprazole and hydroxy-omeprazole improved with increased sonication and extraction temperature, whereas recovery of the more lipophilic analytes fexofenadine, dextromethorphan and midazolam and the corresponding hydroxylated metabolites of the latter two decreased with increased sonication and extraction temperature for Mitra™ extraction. Regardless of this finding, a positive recovery bias was still evident for midazolam and hydroxymidazolam. It has been hypothesised that this positive bias seen with VAMS™ technology in relation to plasma might be due to an underestimation of the actual concentration in plasma rather than a positive bias from the DBS sampler. Basic analytes are also ionised at physiological pH with higher affinity for erythrocyte protein binding sites in and on the erythrocytes. Liberation of these analytes from erythrocytes could result in the positive recovery bias, however the different analyte physicochemical properties, possible interaction of analytes with the Mitra™ sampling tip, analyte stability and plasma-to-erythrocyte binding ratios can all contribute. It is uncertain which of these factors contributed most to the variability seen in absolute recoveries from the Mitra™ sampler.

The complete validated method including sample preparation, met the criteria for linearity, LOD, LLOQ and linear dynamic range covering the expected *in vivo* concentrations in both DBS and plasma matrixes. Method linearity was established within the expected *in vivo* concentration ranges corresponding to the specific phenotyping dosages for each analyte, with r^2 values exceeding 0.9936 in human plasma and 0.9929 in DBS. The slopes of the calibration curves for individual analytes were between 0.0613 and 0.4300 for plasma and 0.0583 and 0.4550 for DBS. The LOD and LLOQ for each analyte was calculated at 3 and 10 times the analyte signal-to-noise ratio respectively. The LOD's were between 0.18 and 8.65 $ng.mL^{-1}$ in the DBS matrix and 0.22 and 10.42 $ng.mL^{-1}$ in plasma, with the LLOQ between 0.61 and 28.85 $ng.mL^{-1}$ in DBS and 0.72 – 31.78 $ng.mL^{-1}$ in plasma. The LLOQ in DBS were below 5 $ng.mL^{-1}$ except for caffeine and flurbiprofen and hydroxy-flurbiprofen, where the high baseline noise caused an increase in the LOD. These findings were similar for plasma, but regardless of the estimated LLOQ values, the expected *in vivo* concentrations are between 10 to 100-fold above the lowest spiked concentrations. Carry-over was reduced by introduction of a needle wash step following injection of the highest concentration standard and did not exceed 20% of the LLOQ.

Intra and inter-day precision and accuracy for all analytes over the three non-consecutive days fell within the acceptable 15% bias. Most analytes were stable in plasma over three freeze - thaw cycles when stored at -80°C and at -20°C in spiked solvent for one month. Short-term stability in the LC autosampler was also established for most analytes. Exceptions were omeprazole and hydroxy-omeprazole that degraded rapidly in the sample vial under acidic conditions. Bupropion on the other hand degraded at room temperature, especially under higher pH conditions. Matrix matched spiked DBS and plasma QC's (at physiological pH and freshly made up from stock standards) did comply with the required 85 – 115% accuracy at medium and high concentrations and 20% accuracy at the lowest spiked concentration in DBS. For both plasma and DBS matrixes the measured accuracy for flurbiprofen did not comply with these criteria due to large instrument bias when operated in negative ESI mode, or alternatively as a result of differences in the extraction recovery. It is recommended that DBS be extracted soon after sample collection and the extract stored at -80°C until further analysis, that omeprazole be substituted with pantoprazole and that analyte stability of flurbiprofen in DBS be re-evaluated under the same extraction conditions.

As a final objective this study measured the *in vitro* agreement between the two sampling methods under different DBS extraction conditions. The DBS calibration curves derived from spiked samples were highly dependent on the extraction protocol, evident from the large variability in % CV and the recovery of different analytes under the different conditions. Further extraction protocol optimisation could enable better extrapolation between DBS and plasma concentrations where a DBS calibration curve is used to predict plasma concentration. It was evident that blood cell distribution kinetics are regulated by the whole blood/plasma concentration ratio, the physicochemical properties of the analytes, extraction temperature, analyte concentration, stability and equilibration rate between different blood compartments. A clinical PK experiment for the purpose of measuring *in vivo* agreement within subjects, is currently awaiting ethical approval by SAHPRA.

Limitations of the current study included limited availability of analytical standards and delays in obtaining import licences for selected analytes like hydroxy-midazolam. Only single ampoules containing one mL each at 1 mg. mL^{-1} for paraxanthine, hydroxy-bupropion, omeprazole, hydroxy-midazolam and hydroxy-flurbiprofen could be easily procured. These standard stock solutions in methanol at 1 mg. mL^{-1} were aliquoted and stored at -80° C.

Even though care was taken to protect the analytes from light and working standards were freshly prepared from these aliquots (after no more than two freeze-thaw cycles), there were concerns about the adherence of analytes to the plastic Eppendorf vials as well as the possibility of evaporation of methanol during preparation of working standards.

In conclusion, this study successfully validated the use of DBS, collected with the volumetrically controlled absorptive microsampling device Mitra™, to measure expected probe drug and metabolite concentrations using the "Geneva phenotyping cocktail" that will be used in future to simultaneously quantifying *in vivo* P-gp transport and CYP450 metabolic activity of CYP1A2, -2B6, -2C9, -2C19, -2D6 and -3A4 enzymes. The advantage of using the Mitra™ device in personalised medicine is the possibility of home sampling after self-administration of the phenotyping cocktail. This "home-made" cocktail could be successfully used to assess inter-method agreement of drug transport and metabolic ratios using both plasma and DBS collected with the Mitra™ device for within subject comparisons using the Bland-Altman and the Intraclass Correlation Coefficient statistical tests. The pharmacokinetic outcomes reported for Mitra™ and traditional plasma collection, should be assessed *in vivo* to confirm the initial findings in this study and determine whether these results are reproducible enough to allow the determination of distinct threshold ratios for each of the different pharmacogenetics classifications to be determined. This could contribute towards creating a mathematical model based on analyte properties, their blood cell distribution kinetics, individual patient haematocrit and absolute analytical recovery and matrix effects, to accurately predict plasma concentration from DBS samples.

The validated targeted LC-MS/MS method is sensitive enough to be used for phenotypic profiling able to determine inter-individual variability of probe drug versus metabolite ratios (to infer metabolic activity) or drug absorption rates (to infer drug transport activity), from a small volume biological DBS sample, collected with VAMS technology™, after administration of a low dose "home-made" phenotyping cocktail in combination with optimised analytical extraction protocols. The study has achieved the aims originally established and further highlights the importance of standardised manufacturing of low-dose phenotyping cocktails as well as the need for standardised statistical analysis when assessing statistical agreement between sampling methods in future targeted metabolomics research.

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APPENDIX I: RESEARCH AND ETHICS COMMITTEE APPROVAL LETTER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/29/2022
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020

17 July 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 209/2016
Title: Assessment of drug-based phenotyping metrics between dried blood spots and plasma sampling.

Dear Mrs M Leuschner

The **Annual Renewal** as supported by documents received between 2019-06-18 and 2019-07-17 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-07-17.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-07-17.
- Please remember to use your protocol number (209/2016) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 8.60, Level 8, Tswelopele Building
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

APPENDIX II: PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

Researcher's name: Machel Leuschner
Student Number: 15240054
Department: Pharmacology
Institution: University of Pretoria
Study Title: Assessing inter-method agreement of drug-based phenotyping metrics between dried blood spots and plasma sampling.

Dear Participant,

I am a PhD student in the field of Pharmacology (that is study of how medicine works in your body and how your body work to get rid of medication) at the University of Pretoria. You are invited to volunteer to give blood that will be used in this study to develop and test a new laboratory method that can be used to determine how your body breaks down medication. This is important, because it might help doctors in future to test patients before they are given medication that could either not work for them or have bad effects on them. This would help the doctor prescribe the right medication, at the right times to the right patient.

This letter will give you information to help you decide if you want to take part in this study. Before you agree to participate in the study you should understand everything that will happen to you. This letter will give you information on the process and procedure that we will use to collect your blood. If you feel uncomfortable about the blood collection, or do not understand the process, I will take time to explain them until you are comfortable about everything that will happen to you. You should not agree to take part unless you are completely happy about what we expect of you. You will also receive a copy of this document to keep, if you decide to participate.

Purpose of the research project

We would like to find out if we can use whole blood from a dried blood spot collected onto a special device to find out how the body works to break down medication. We want to compare this method to another method where we use blood plasma, that is the yellowish part of your blood that we separate from the red blood cells. It will help us to find out if the dried blood spot method is sensitive enough to be used by doctors and clinics in the future to help them in giving the right medication to the right person at the right dose.

Taking part is voluntary

Taking part in the study is completely voluntary. You decide if you want to take part or not. You may withdraw your consent to take part at any time. If you decide to withdraw from the study the clinic will still care for you in the same way and nothing will change. If you withdraw, all the blood taken from your arm will be destroyed in the correct way and it will not be used any further. You do not have to decide today and will have enough time to think about whether you want to take part or not.

Approval for the study

The Research and Ethics Committees of the University of Pretoria, Tswelopele Building, Dr. Savage Road, Gezina, of the Faculty of Health Sciences, and telephone number 012 356 3084 / fax 086 651 6047 granted written approval for this study. They make sure that you are protected from harm. All procedures will be done according to national and international guidelines on good clinical practice. This means that your health will be put first and that you will understand all tests that we will do.

The procedure

Blood will be taken from the inside of your arm where it bends using a needle and a syringe. You will be asked to give enough blood to fill five blood collection tubes of 4mL each.

The process

During this study, you will be asked to come to the clinic once to give blood. We will ask you to do the following:

- You will be asked to not eat or drink any product containing caffeine for at least 72 hours before giving blood.
- You will be asked to not eat or drink anything except water from the previous evening at ten o'clock before you come to the clinic.
- You should bring all medication or herbal products that you take on a daily or regular basis with you to the clinic so we can write them down.
- Blood samples from your arm will be taken at a time prearranged with the Clinical Research Unit Staff that suits you.

All blood that we take will be used for this study only and all left over blood will be destroyed. Should you decide to withdraw from the study at any time, all the blood collected up to that point will be destroyed and will not be tested.

Patient Confidentiality

All the information that we collect during this study will be kept strictly confidential. We will put it away and only the researchers will see it. We will also use a number instead of your real name when we use the information to share the results of the research through meetings or in journals so that other people can learn from it as well.

Machel Leuschner

CERTIFICATE OF CONSENT

I have read the patient information given to me, OR it has been read to me in the presence of a witness (please scratch out what is not applicable). I have been given the time to ask questions about it and all the questions have been answered in a way that I understand. I agree to take part in this research study out of my own free will. I also understand the different tests and the process and possible risks involved and that I may withdraw from the study at any time. I will be given a copy of this signed form to keep for my records.

Print Name of Participant _____

Signature of Participant _____ Date _____

Day/month/year

A copy of this ICF has been provided to the participant.

Print Name of Researcher _____

Signature of Researcher _____ Date_____

Day/month/year

Print Name of Witness _____

Signature of Witness _____ Date _____

Day/month/year

APPENDIX III: PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

Researcher's name: Machel Leuschner
Student Number: 15240054
Department: Pharmacology
Institution: University of Pretoria
Study Title: Assessing inter-method agreement of drug-based phenotyping metrics between dried blood spots and plasma sampling.

Dear Participant,

I am a PhD student in the field of Pharmacology (that is study of how medicine works in your body and how your body work to get rid of medication) at the University of Pretoria. You are invited to volunteer to take part in a pilot study to determine if finger prick tests can be used to determine how your body breaks down medication. This is important, because it might help doctors in future to test patients before they are given medication that could either not work for them or have bad effects on them. This would help the doctor prescribe the right medication, at the right times to the right patient. If this could be done by simply pricking a person's finger, it gives the doctors a cheap and easy way to help their patients in getting the correct medication.

This letter will give you information to help you decide if you want to take part in this study. Before you agree you should understand everything that will happen to you. This letter will also give you information on all the different types of tests that will be done on you if you agree to participate in the study. If you feel uncomfortable about any of the tests, or do not understand some of the questions, I will take time to explain them until you are comfortable about everything that will happen to you if you decide to take part. You should not agree to take part unless you are completely happy about what we expect of you. You will also receive a copy of this document to keep, if you decide to participate.

Purpose of the research project

People are different in how their bodies deal with medications that they take. The same medication that works for one person will cause a negative or bad reaction in another. We would like to find out if we can use blood from a finger prick to find out how your body works to break down medication. We want to compare this method to another method where blood is drawn from a vein in your arm. It will help us to find out if the finger prick method is sensitive

enough to be used by doctors and clinics in the future to help them in giving the right medication to the right person at the right dose to limit the amount of people experiencing negative effects to medication.

Tests that will be done

In this one-day study you will be asked to take small doses of medicine that we will give you and then have 5 finger prick tests and 5 blood samples drawn from your arm. The finger prick tests and blood samples will be done at the same time, starting just before you take the medicine and then 1, 2.5, 3 and 3.5 hours after we give you the medication to drink.

Taking part is voluntary

Taking part in the study is completely voluntary. You decide if you want to take part or not. You may withdraw your consent to take part at any time. If you decide to withdraw from the study the clinic will still care for you in the same way and nothing will change. If you withdraw, all the blood taken from your arm and/or your finger will be destroyed in the correct way and it will not be tested any further. You do not have to decide today and will have enough time to think about whether you want to take part or not.

Information on the test medicine we will give you to drink

Your safety and comfort during the study is important to us. You are valued as a partner during this study. You will be asked to take medicine that contains small doses of different ingredients to help us understand how your body works to break them down. This medicine is not enough to treat you for any illness it was made for, because it is given in amounts that are very low (between half and up to 10 times less than the usual amount). It has been safely used before in other similar research, but you should know the things that might happen. Side effects of the medication that have been reported at normal amounts, include the following:

Bupropion;

- Seizure
- High Blood Pressure
- Great excitement linked to mental illness
- Loose contact with reality
- Increased pressure in the front part of the eye
- Hypersensitivity

Caffeine;

- Irritability and/or restlessness
- Increased heart beat
- Upset stomach
- Changes in your blood sugar level (might increase or decrease)
- Increased urination

Dextromethorphan;

- Drowsiness
- Dizziness
- Stomach upset

Fexofenadine;

- Headache
- Dizziness
- Back pain
- Stomach discomfort
- Hypersensitivity

Flurbiprofen;

- Heart problems due to blood clots
- Stomach pain and bleeding of the stomach wall
- Increased blood pressure
- Liver toxicity
- Increased blood pressure
- Heart failure
- Swelling
- Allergic reactions
- Skin rash

Omeprazole;

- Headache (Common; more than 2 in every 100 people reported this effect)
- Stomach pain, nausea, diarrhoea, vomiting (Common)
- Dizziness
- Rash
- Weakness
- Back pain
- Cough

Midazolam;

- Nausea
- Vomiting
- Dizziness
- Rapid or slow or shallow breathing
- Agitation or aggressive behaviour
- Heart beat may change (fast or slow)
- Blurred vision

If you decide to take part we will look after you and write down any negative effect you may experience. You will be allowed to stop with the study at any time due to possible negative effects.

The Procedures

The Research and Ethics Committees of the University of Pretoria, Tswelopele Building, Dr. Savage Road, Gezina, of the Faculty of Health Sciences, and telephone number 012 356 3084 / fax 086 651 6047 granted written approval for this study. They make sure that you are protected from harm. All procedures will be done according to national and international guidelines on good clinical practice. This means that your health will be put first and that you will understand all tests that we will do. You may be asked to undergo some or all of the procedures listed below.

- ***Medicine to be taken***

All participants will be asked to take a low-dose mix of medicine that will help us to find out how their body breaks down medicine.

- ***Blood from your arm***

Five blood samples we will take from your arm with a syringe and needle at 5 different times, at the start and 1,2.5, 3 and 3.5 hours after you take the medicine.

- ***Finger prick testing***

Five finger prick tests will also be done on each of the 5 times as above, where we will take small drops of blood from your fingertips at the same time that we will take the blood from

your arm. We will use this blood to find out if such a small blood drop can be used to test how your body breaks down the medicine.

The process

During this one-day research study, you will be asked to come to the clinic once. We will ask you to do the following:

- You will be asked to not eat or drink anything except water from the previous evening at ten o'clock before you come to the clinic.
- You should bring all medication or herbal products that you take on a daily or regular basis with you to the clinic so we can write them down.
- Blood samples from your arm and finger will be taken just before you take the test medication.
- You will be administered the test agents provided you have no past history of an allergy or a reaction to them.
- You will receive a light meal and a non-caffeinated refreshment.
- We will take 4 more samples of blood from your arm and take 4 more finger prick tests at 1 hour, 2.5 hours, 3 hours and 3.5 hours after you take the test medicine.

All blood that we take will be used for this study only and all left over blood will be destroyed. Should you decide to withdraw from the study at any time, all the blood collected up to that point will be destroyed and will not be tested.

Patient Confidentiality

All the information that we collect during this study will be kept strictly confidential. We will put it away and only the researchers will see it. We will also use a number instead of your real name when we use the information to share the results of the research through meetings or in journals so that other people can learn from it as well.

Machel Leuschner

CERTIFICATE OF CONSENT

I have read the patient information given to me, OR it has been read to me in the presence of a witness (please scratch out what is not applicable). I have been given the time to ask questions about it and all the questions have been answered in a way that I understand. I agree to take part in this research study out of my own free will. I also understand the different tests and the process and possible risks involved and that I may withdraw from the study at any time. I will be given a copy of this signed form to keep for my records.

Print Name of Participant _____

Signature of Participant _____ Date _____

Day/month/year

A copy of this ICF has been provided to the participant.

Print Name of Researcher _____

Signature of Researcher _____ Date_____

Day/month/year

Print Name of Witness _____

Signature of Witness _____ Date _____

Day/month/year

Comparison of different reverse phase columns on the separation of the Geneva phenotyping drug cocktail by high performance LC-MS/MS (September 2017)

Leuschner, Machel; Sheva, Kim; Cromarty, Duncan

Department of Pharmacology, University of Pretoria, Pretoria

INTRODUCTION: Phenotyping cocktails are used to assess metabolic and transport phenotypes in vivo and consist of a number of probe drugs for simultaneous quantitation in different biological matrices. The most commonly used reversed phase column in high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method development are the alkyl C18 phase. Multidrug cocktails often consist of related substituted aromatic and polycyclic compounds that may require more selective separation. The aim of this study was to compare the selectivity of a Kinetex Biphenyl column to a Kinetex C18 column on the separation of the seven probe drug Geneva phenotyping cocktail.

METHODS: After optimisation of source detection parameters, known concentrations of each probe drug, including internal standards, were prepared in 50% methanol. Separation was achieved by HPLC on a triple quadrupole LC-MS/MS system consisting of an Agilent combination 1100 & 1200 series LC system coupled to an AB Sciex 4000 Qtrap mass spectrometer. Isocratic runs with 60:40 methanol: water and 40:60 acetonitrile: water with similar elution strengths were used as mobile phases on both the Kinetex C18 and Kinetex Biphenyl columns with similar silica backbones. Scatter plots were drawn comparing the logarithm of the retention factors (log k') for all compounds on the biphenyl column against their respective log k' values on the C18 column for both mobile phase conditions. The slopes and correlation coefficients were determined from linear regression analysis.

RESULTS: Results showed that the alternative selectivity of the Kinetex Biphenyl column using methanol as co-eluent mostly resolved the separation of similarly structured aromatic compounds. Lower correlation coefficients and slope values gave an indication of the differences in selectivity. Better peak resolution with methanol was attributed to increased non polar interaction of compounds with the biphenyl stationary phase as opposed to acetonitrile that suppressed the π-π interactions of the aromatic compounds with the biphenyl stationary phase by competitive interaction with both solute and stationary phase phenyl groups.

CONCLUSION: In this study the selectivity of the Geneva phenotyping cocktail was altered by pairing a biphenyl stationary phase with methanol taking advantage of the increased non-hydrophobic π-π interactions on the biphenyl column in addition to hydrophobic interactions by using methanol as the mobile eluent. This combination mostly resolved co-elution of similarly structured aromatic compounds.

Bioanalysis of the Geneva phenotyping cocktail in whole blood collected with volumetric absorptive microsampling by LC-MS/MS (September 2019)

Leuschner, Machel; Sheva, Kim; Cromarty, Duncan

Department of Pharmacology, University of Pretoria, Pretoria

BACKGROUND: Poor therapeutic response to medication has been attributed to inter-individual and interethnic variability in cytochrome P450 (CYP450)-dependent metabolism and altered drug retention associated with P-glycoprotein (P-gp). An individualised pharmacotherapeutic approach would benefit South-Africans considering the country's large genetic diversity. A low dose probe drug cocktail followed by a single time point, minimally-invasive capillary sampling, to simultaneously quantify in vivo drug and metabolite concentrations, could enhance the feasibility and cost-effectiveness of routine phenotyping towards personalised medicine. This study aim was to develop a validated, targeted, analytical method to quantify the seven probe drugs and their metabolites in dried blood spots (DBS) when using the Mitra™ volumetric absorptive micro-sampling device for blood collection.

METHODS: An Agilent binary liquid chromatography (LC) system coupled to a Sciex 4000 QTRAP triple quadrupole tandem mass spectrometer (MS) was used for method optimisation and validation. Optimised source conditions and fragmentation parameters were used to monitor the most abundant MRM transitions for all analytes. Targeted LC-MS/MS methods, in negative and positive ESI mode, were validated according to ICH guidelines. Agreement between the in vitro quantitative measurements in DBS and conventional plasma sampling was assessed.

RESULTS: The validated LC-MS/MS method met the required bioanalytical standards for specificity, sensitivity, linearity, accuracy, precision, carry-over and stability. In vitro assessment of agreement between DBS and plasma sampling showed deviations > 20% (measured against predicted plasma concentration). These findings were related to the blood-to-plasma concentration ratio, the physicochemical properties and stability of the analytes as well as the extraction efficiency from the Mitra ™ sampler and degree of liberation of the analytes from plasma proteins and erythrocytes.

CONCLUSION: This study successfully validated the use of DBS, collected with the Mitra™ microsampling device, to measure expected probe drug and metabolite concentrations using the "Geneva phenotyping cocktail" for the purpose of simultaneous phenotyping of the *in vivo* P-gp transport activity and CYP450 metabolic activity of the CYP1A2, -2B6, -2C9, -2C19, -2D6 and -3A4 enzymes, although agreement between the two matrixes were not straightforward.