



**University of
Nottingham**
UK | CHINA | MALAYSIA

An investigation into the drug disposition of the spleen organ as a potential immuno-therapeutic target

By

Patience G. Inuwa

BSc, MSc

**Faculty of Medicine and Health Sciences
School of Veterinary Medicine and Science
University of Nottingham**

Abstract

The development of new therapeutic strategies or treatment options occur, most often, to meet a demand or a need not yet encountered. For the treatment of many diseases, conventional approaches using drugs as therapeutic agents were directed specifically at the indication or in combination with radiotherapy to treat the presumed condition(s). However, severe adverse side effects resulting from these approaches made researchers even more diligent and resilient in striving for solutions to the disease phenomenon. In recent years, immunotherapy has become the emerging new generation strategy to transform the treatment of cancer by using a patients' own immune system to kill cancerous cells. This has since been met with a lot of challenges especially when tumour cells develop the ability to evade the host immune system or where a breakthrough has been made, the success is only apparent on certain types of cancer. The concept of immunotherapy was powerful enough, but it required the right execution; as with cancer, this solution brought many more unanswered questions.

Nonetheless, the revelation and acknowledgment that the immune system is not only fundamental but capable in treating any disease or illness, is a significant step towards changing the direction of how therapeutic strategies are designed. If the immune system is considered as this big switch that can be turned on and off, then research should be focussed on what regulates the immune system. The spleen as a major regulator of the immune system expression, in combination with being a host to a population of immune cells, has catapulted the organ to attention within the clinical scientific community. Stimulating an immune response is the basis of immunotherapy, in which the spleen can be used to demonstrate just that.

Moreover, the clinical significance of drug kinetics and its effects in the liver has shown that drug research in the spleen is limited since interest in the liver is driven by the knowledge as the main site of hepatic drug metabolism. Yet the spleen has potential in establishing itself as a possible therapeutic target even though current research in the spleen has shown no indication that it can metabolise drugs.

To prove the notion that the spleen can process therapeutic drugs and with the use of precision drugs aimed to specifically cause the activation of immune cells that then generates an immense immune response will be a significant event. Therefore, this study aims to examine the drug disposition in the spleen by discovering its drug metabolising features and understanding its interactions with drugs. This will involve investigating the enzymes responsible for breaking down drugs in the body, but it would also be an indicator of the spleen's drug uptake qualities; another confirmation that drug transporters might be at play here and providing insight into the distribution of drugs within the spleen. Cytochrome P450 (CYP450s) and (UDP)-glucuronosyltransferase (UGTs) are known as major contributors to drug metabolism of clinically used drugs, and their activities were investigated to represent phase I and II reactions respectively. The following experimental procedures presented in this study were set to evaluate the expression of these enzymes at genetic, protein and functional level in rat and pig spleens which resulted in delivering some promising data. Hence, the work portrayed here is novel in determining the drug disposition process in the spleen.

In addition, the therapeutic drug substrates known for their major CYP450 or UGT probes were evaluated to monitor the enzyme kinetics in the spleen, and the observations resulting from the metabolite formation profiles in pig spleen suggests that the spleen is capable of metabolising drugs. Consequently, further investigation in assessing the mechanisms of

metabolic enzymes, the identification of the spleen's major CYP450s or UGTs associated to drug metabolism, and extensive kinetic analysis quantitating these enzymes activities are all required to solidify the spleen's status as a potential immunotherapeutic target.

Declaration

I declare that the work presented in this thesis was carried out in accordance with the requirements of the Nottingham University Regulations.

The work is original except where indicated by specific reference in the text and has not been submitted for any other academic award at the University of Nottingham or any other institution.

Name: Patience G. Inuwa

Signed:

Date:

Conferences, Posters and Oral Presentations:

1. The spleen: A potential target in cancer immunotherapy
Summer PGR symposium, University of Nottingham
(Online Poster and Oral presentation) (1st July 2020)
2. Investigating drug distribution and metabolism in the spleen
BBSRC DTP Spring Conference 2021, University of Nottingham
(Online Poster presentation) (14-15th April 2021)
3. Investigating the gene expression of drug metabolising enzymes in the spleen
Summer PGR symposium, University of Nottingham
(Online Poster and Oral presentation) (1st July 2021)
4. Investigating the pharmacokinetics of drugs in the spleen as a potential target in cancer immunotherapy
BBSRC DTP Spring Conference 2022, University Park, University of Nottingham
(Oral Presentation) (27th April 2022)
5. Investigating the pharmacokinetics of drugs in the spleen as a potential target in cancer immunotherapy
DMPK science meeting presentation, Sygnature Discovery, Alderley Park
(Oral Presentation) (24th May 2023)

Dedication

I dedicate this work to my beloved and most cherished parents, Mr. Daniel K Inuwa and Mrs. Grace B Inuwa. You gave the very best of yourselves for my siblings and I to lack absolutely nothing in life. For this, I am eternally grateful and truly honoured.

Mom, Dad, you are the reason of my achievements in life so far. Your life lessons and deep-rooted values you taught us so well, has shaped me into the person I am today; an individual who is resilient, hard-working, diligent, responsible, disciplined, kind, trustworthy, courageous, reliable, pro-active, respectful, not easily-angered, hospitable, someone who perseveres and dares to dream big.

I miss you both terribly and until we meet again, sweet dreams.

Acknowledgements

Firstly, I would like to thank Jesus Christ, my rock and my refuge. In all things, He strengthen my faith in Him and positively encouraged me when I struggled in motivation and the will to carry on. The joy of the Lord has indeed been my strength throughout my life and even more so during and towards the end of my PhD journey. To God be all the glory, all the honour and all the praise.

I would like to thank my supervisors Dr. Stuart Paine, Dr. Cyril Rauch and Dr. Sabine Tötemeyer for all their support, guidance, advice and direction which helped me remain focused along the way. This has truly been a humbling and rewarding experience.

A part of my research study was done at Sygnature Discovery lab, Alderley Park Campus, and the past and current staff – Dr. Timothy Schulz-Utermoehl, Dr. Hayley Butler, Dr. Stuart Best, Dr. Warren Keene, Phi Dinh, Caroline Rook, James Gregg, Molly OConnor-Gill, Oscar Brown and Hannah Neal, have all been in one way or another instrumental in the successful completion of my kinetic analysis. I would like to take this opportunity to give them thanks for all their support.

Most of my trainings in molecular biology techniques and preparation of biological materials were conducted by laboratory technicians, supervisor and fellow PhD colleagues at the Vet School of the University of Nottingham. I would like to thank Ceri Stanley, Scott Hulme, Sophie Galloway, Belinda Wang, Dr. Toshana Foster, Mohammed Aldurdunji and Chloe Whitehouse for their time and patience in training me.

Acknowledgments

A heartfelt and unwavering thanks goes to my siblings and family, who have been a massive support system to me. Thank you for your encouragement, prayers, patience and for your total unified believe in me. May the good Lord bless you in kind.

Last but not the least, I would like to thank the UKRI (BBSRC) and Sygnature Discovery for their financial support. I would also like to express my full appreciation to them for enabling me to participate and complete a DTP PhD programme.

Table of Contents

Abstract	i
Acknowledgements.....	vii
1.0 Introduction.....	1
1.1 Immunotherapeutic Targets and Agents	1
1.1.1 Overview of the concept of immunotherapy.....	2
1.1.2 The Spleen – A Potential Immunotherapeutic Target.....	4
1.1.3 Anatomy and Function.....	5
1.2 Animal Models.....	9
1.2.1 Use of animal models and its importance.....	9
1.2.2 Rat and Pig models in drug discovery and development.....	10
1.2.3 Porcine animal model.....	12
1.2.4 Phylogenetic Analysis.....	13
1.3 Drug disposition	16
1.3.1 Importance of drug discovery.....	16
1.3.2 Overview of the drug disposition process.....	17
1.3.2.1 ADME: Drug Absorption and its influence reaching the target.....	18
1.3.2.2 ADME: Drug Distribution and importance of the role of drug transporters.....	20
1.3.2.2.1 Drug Transporters.....	23
1.3.2.3 ADME: Drug Metabolism and its role in drug discovery.....	26
1.3.2.3.1 CYP450 and UGT enzymes.....	28
1.3.2.3.2 CYP450: Genetic polymorphism.....	31
1.3.2.3.3 CYP450: Possible impact of its regulation.....	33
1.3.2.4 ADME: Drug Excretion and clinical implications.....	35
1.3.2.4.1 Significance of the interplay relationship between drug distribution and metabolism.....	36
1.3.2.5 Predicting hepatic clearance.....	38
1.3.2.5.1 <i>In-Vitro</i> methods.....	38
1.3.2.6 Enzyme kinetics.....	40
1.3.2.7 Generating in vitro clearance, CL_{int} : Substrate depletion approach.....	45
1.4 Aims and Objectives.....	48
2.0 Methods and Materials.....	49
2.1 Assessing the genetic expression of selected phase I and II enzymes including transporter proteins in the spleen that are associated with drug metabolism and distribution.....	50

Table of Contents

2.1.1	Rat and Pig (liver and spleen) tissues.....	50
2.1.2	Isolation of total RNA and cDNA synthesis.....	51
2.1.3	Assessment of purity and quantitation of prepared total RNA and cDNA.....	52
2.1.4	The design of primers for use in PCR analysis.....	53
2.1.4.1	Obtaining FASTA mRNA sequences of selected genes.....	53
2.1.4.2	Assessing the specificity of mRNA sequences.....	54
2.1.5	Single-cell suspension preparation and isolation of primary liver and spleen cells....	56
2.1.5.1	Prepared reagents.....	56
2.1.5.2	Isolation of primary cells from liver and spleen tissue.....	56
2.1.5.3	Cryopreservation of primary cells.....	57
2.1.5.4	Thawing of cryopreserved primary cells.....	58
2.1.5.5	Determination of primary cells yield and viability.....	58
2.1.6	Standard PCR and real-time quantitative PCR analysis (RT-qPCR).....	59
2.1.6.1	Standard PCR.....	59
2.1.6.1.1	Gel electrophoresis of the DNA PCR products.....	59
2.1.6.1.2	Data Analysis of Sanger Sequencing results from the DNA PCR products with the designed primers.....	60
2.1.6.2	RT-qPCR using SYBR Green™ detection method.....	62
2.1.7	Assessment of gene expression using Next Generation Sequencing (NGS).....	63
2.2	Assessing the protein expression of selected phase I and II enzymes including transporter proteins in the spleen that are associated with drug metabolism and distribution.....	64
2.2.1	Preparation of protein lysates.....	64
2.2.2	Preparation of liver and spleen homogenates and microsomes.....	64
2.2.2.1	Homogenisation of liver and spleen tissues from rat and pig.....	64
2.2.2.2	Preparation of microsomes.....	65
2.2.3	Protein concentration determination.....	65
2.2.4	Western Blot and Immunoblotting.....	66
2.2.5	Immunohistochemistry (IHC).....	67
2.2.5.1	Slide preparation (Tissue processing, Fixation, and Sectioning).....	69
2.2.5.1.1	Creating a FFPE block from paraffin wax.....	69
2.2.5.1.2	Preparing tissue sections.....	69
2.2.5.2	Deparaffinisation or Rehydration of tissue sections.....	70
2.2.5.3	Antigen retrieval.....	70
2.2.5.4	Blocking, Washing, Primary Antibody incubation and Immunostaining.....	70
2.2.5.5	Dehydrate, Mounting and Examination.....	71

Table of Contents

2.2.6	Immunofluorescence staining (IF).....	71
2.2.6.1	Prepared reagents.....	71
2.2.6.2	Immunostaining test.....	72
2.2.7	Co-immunoprecipitation (Co-IP).....	73
2.3	Assessing the functional expression of selected phase I and II enzymes in the spleen that are associated with drug metabolism and distribution.....	74
2.3.1	CYP450 content determination.....	74
2.3.1.1	Sample preparation 1.....	74
2.3.1.2	Experimental procedure using autoclave CO chamber.....	75
2.3.1.3	Sample preparation 2.....	76
2.3.1.4	Experimental procedure using cuvettes.....	76
2.3.2	Drug elimination rate determination using substrate depletion method.....	77
2.3.2.1	Sample preparation.....	78
2.3.2.2	Incubation mix plate preparation.....	78
2.3.2.3	Substrate depletion assay.....	79
2.3.2.4	Setting up conditions of Ultra-performance liquid chromatography.....	79
2.3.2.5	Setting up the Mass spectrometry (MS/MS) conditions for analysis.....	80
2.3.2.6	Preparing the MS methods for analysis.....	82
2.3.2.7	Data analysis of MS targetlynx (output) files.....	82
2.3.2.8	Generating curve plot from MS processed data.....	83
3.0	Investigating the gene expression of CYP450, UGT and Transporter proteins associated with drug metabolism and distribution.....	85
3.1	Introduction.....	86
3.2	Results.....	88
3.2.1	Gene expression of CYP450s in rat and pig using standard PCR.....	88
3.2.1.1	Specificity of gene primers using Sanga sequencing.....	91
3.2.2	RNA-seq transcriptome study in rat and pig using NGS.....	94
3.2.3	Gene expression analysis using qPCR.....	106
3.3	Discussion.....	110
4.0	Investigating the protein expression of CYP450, UGT and transporter proteins associated with drug metabolism and distribution.....	114
4.1	Introduction.....	115
4.2	Results.....	119
4.2.1	Protein expression of metabolic enzymes and proteins using Western Blotting.....	119
4.2.2	Immunohistochemistry analysis in rat and pig.....	129
4.2.3	The immunofluorescence staining of pig hepatocytes and splenocytes.....	133

Table of Contents

4.2.4	Co-immunoprecipitation analysis in rat and pig for protein identification.....	139
4.3	Discussion.....	141
5.0	Investigating the functional expression of CYP450 and UGT enzymes associated with drug metabolism in pig spleen.....	144
5.1	Introduction.....	145
5.2	Results.....	151
5.2.1	CYP450 content in pig liver and spleen tissues.....	151
5.2.2	CYP450 and UGT-mediated drug clearance in pig liver and spleen microsomes.....	153
5.2.3	CYP450-mediated metabolite formation in pig liver and spleen microsomes I.....	158
5.2.4	CYP450-mediated metabolite formation in pig liver and spleen microsomes II.....	175
5.2.5	UGT-mediated metabolite formation in pig liver and spleen microsomes I.....	182
5.2.6	UGT-mediated metabolite formation in pig liver and spleen microsomes II.....	190
5.2.7	CYP450-mediated drug clearance in pig hepatocytes and splenocytes.....	193
5.2.8	CYP450-mediated metabolite formation in pig hepatocytes and splenocytes.....	197
5.3	Discussion.....	200
6.0	General Discussion.....	206
	References.....	212
	Appendix.....	231

Table. 1	Examples of the pros and cons of animal models in cardiovascular diseases.....	11
Table. 2	Drug transporters in Tissues: Known ABC and SLC transporters expressed and involved in the distribution of drugs in tissues. Including peptide transporters (PEPT) and Glutathione transporter (GLUT).....	23
Table. 3	Quantified total RNA and cDNA concentration extracted from measured liver and spleen tissues using the Nanodrop spectrophotometer.....	52
Table. 4	Quantified total RNA and cDNA concentration extracted from measured primary liver hepatocytes and splenocytes cells using the Nanodrop spectrophotometer.....	53
Table. 5	The selected primer parameters for optimum primer design.....	54
Table. 6	Designed Rat and Pig primers of selected CYP450 genes including control (housekeeping) genes.....	55
Table. 7	Cycle program for standard PCR.....	59
Table. 8	Cycle program for RT-qPCR.....	62
Table. 9	Prepared standard curve solutions for protein concentration determination.....	66
Table. 10	Reference CYP450 enzymes with their known drug substrates.....	77
Table. 11	Reference UGT enzymes with their known drug substrates.....	78
Table.12	UPLC gradient condition for sample analysis.....	80
Table.13	MS methods of CYP450 and UGT drug substrates and their corresponding marker metabolites.....	81
Table. 14	CYP450 gene expression observations in Rat (Sprague Dawley) by standard PCR and agarose gel electrophoresis.....	91
Table. 15	BLAST searched Sanga sequencing data of rat liver PCR products.....	92
Table. 16	BLAST searched Sanga sequencing data of rat spleen PCR products.....	93
Table. 17	NGS workflow showing quality control and library preparation data.....	95
Table. 18	Identified genes nomenclature.....	96
Table. 19	Top 20 expressed genes identified in rat from the RNA-seq transcriptome data.....	103
Table. 20	Top 20 expressed genes identified in pig from the RNA-seq transcriptome data.....	104
Table. 21	Top 20 expressed genes identified in human spleen obtained from the RNA-seq transcriptome data on ARCHS ⁴ database.....	105
Table. 22	Relative gene expression (C _T values) of selected genes in primary pig hepatocytes.....	108
Table. 23	Relative gene expression (C _T values) of selected genes in primary pig splenocytes.....	108
Table. 24	List of primary antibodies and protein sizes	120
Table. 25	Absorbance readings at 450 nm and 490 nm of pig liver and spleen microsomes including homogenate samples. (autoclave chamber)	151-2
Table. 26	Absorbance readings at 450 nm and 490 nm of pig liver and spleen microsomes including homogenate samples. (cuvette method).....	152

List of Tables

Table. 27	CYP450 content determination in the pig liver and spleen tissues and homogenates.....	152
Table. 28	Literature-based CYP450 enzymes involved in drug metabolism.....	200
Table. 29	Literature-based hepatic microsomal CYP450 content in different species.....	201
Table. 30	Literature-based Michalis Menten parameters for porcine and reference human hepatic CYP450 enzymes.....	202
Table A1	Bioinformatics data from RNA-seq transcriptomic analysis of listed (top 12) phase I and II metabolic enzymes, including transporter proteins identified by NGS in rat and pig.....	239-244
Table A2	The peak area responses of selected drug substrates from UPLC/MS/MS analysis demonstrating CYP450 and UGT-mediated drug disappearance in pig liver and spleen microsomes.....	244-248
Table A3	The peak area responses of selected drug substrates from UPLC/MS/MS analysis demonstrating CYP450 and UGT-mediated metabolite appearance in pig liver and spleen microsomes.....	249-254

Figure. 1	Anatomy of the spleen: The detailed key characterises, components and intended function..4
Figure. 2	Phylogenetic Tree: examples demonstrating the phylogenetic relationship (homology) across different animal models (mouse, rat, pig, dog and monkey) to human using the major contributing cytochrome P450 mRNA sequences known in human liver.....15
Figure. 3	Oral administration of drugs: drug uptake, excretion and first pass metabolism before reaching the systemic circulation.....19
Figure. 4	Mechanism of Action: Drug transporters are either involved in passive or active transport in the distribution of drugs.....25
Figure. 5	Location and topology of CYP450 and UGT on the endoplasmic reticulum membrane in a cell.....27
Figure. 6	Drug metabolism pathway: A simplified illustration of the process of xenobiotic metabolism and detoxification through Phase I and II reactions.....28
Figure. 7	CYP450 Catalytic Cycle: Steps in the CYP450 reaction cycle describing binding of substrate and molecular oxygen to CYP450 and subsequent oxidation of substrate.....30
Figure. 8	Major contributing human CYP450 isoforms: Fraction of clinically marketable drugs metabolised by CYP450 and its factors influencing variability.....32
Figure. 9	Relationship between the interactions of genetic or epigenetic factors and resulting activities of DMEs.....34
Figure. 10	Drug transporters: A. the pie chart of the individual drug transporter proteins in control human livers B. major membrane drug transporters expressed in hepatocytes, enterocytes and proximal tubule kidney cells C. Schematic illustration describing CYP3A4 and P-glycoprotein interplay in the enterocytes.....37
Figure. 11	Preparation of hepatic subcellular fractions.....39
Figure. 12	Relationship between reaction rate and concentration.....42
Figure. 13	A Lineweaver–Burk plot depicting enzyme inhibition.....45
Figure. 14	Scaling human or animal <i>in vitro</i> clearance data to human <i>in vivo</i> hepatic clearance.....47
Figure. 15	Schematic flowchart of total isolation RNA and synthesised cDNA from tissues.....51
Figure. 16	Quality of chromatograms from sanger sequencing data. Data undergo scrutiny of the sequenced chromatograms for potential ‘errors’ 61
Figure. 17	Setup for the samples enclosed in a CO gas chamber for CYP450 content determination.....76
Figure. 18	A simple illustration to define the principle behind the signal to noise ratio.....83
Figure. 19	Agarose Gel Electrophoresis (2% agarose) of PCR amplified products at expected product sizes in rat liver and spleen using designed CYP450 specie-specific PCR primers inclusive of negative controls – (NCT) no cDNA template control and (NRT) no reverse transcriptase control.....90
Figure. 20	The relative abundance of gene expression (%) of Phase I enzymes in rat and pig, liver and spleen tissues. Human liver and spleen genes were used as reference sourced from ARCHS ⁴ database.....98

Figure. 21	The relative abundance of gene expression (%) of Phase II enzymes in rat and pig, liver and spleen tissues. Human liver and spleen genes were used as reference sourced from ARCHS4 database.....	99
Figure. 22	The relative abundance of gene expression (%) of transporter proteins in rat and pig, liver and spleen tissues. Human liver and spleen genes were used as reference sourced from ARCHS4 database.....	100
Figure. 23	Identified promoter genes including related nuclear receptors in untreated rat, pig and human spleen based on their relative abundance bioinformatics RNA-seq transcriptome data.....	101
Figure. 24	Example of gene expression by RT-qPCR: the amplification curve and melt peak of <i>Cyp2d25</i> gene in A. pig hepatocytes B. pig splenocytes.....	107
Figure. 25	Normalised relative gene expression in pig hepatocytes and splenocytes using the Pfaffl method to determine relative quantitation (RQ) of gene expression.....	109
Figure. 26	Protein folding: A. folding driven by local interactions B. folding driven by non-local interactions.....	115
Figure. 27	Total protein identification: A. 10-250kDa pre-stain protein marker or standard, B. rat and pig proteins identified on a nitrocellulose membrane resolved via SDS polyacrylamide gel electrophoresis.....	121
Figure. 28	Western Blot analysis via SDS-PAGE of <i>Cyp2e1</i> probe in rat and pig protein (tissue) lysates with loading control β -actin.....	122
Figure. 29	Western Blot analysis via SDS-PAGE of <i>Cyp20a1</i> probe in rat and pig protein (tissue) lysates with loading control β -actin.....	122
Figure. 30	Western Blot analysis via SDS-PAGE of <i>Cyp27a1</i> probe in rat and pig protein (tissue) lysates with loading control β -actin.....	123
Figure. 31	Western Blot analysis via SDS-PAGE of <i>Ugt8</i> probe in rat and pig protein (tissue) lysates with loading control β -actin.....	123
Figure. 32	Western Blot analysis via SDS-PAGE of CYP450 generic probe in rat and pig protein (tissue) lysates with loading control β -actin.....	124
Figure. 33	Western Blot analysis via SDS-PAGE of <i>Abcb1/P-gp</i> probe in rat and pig protein (tissue) lysates with loading control β -actin.....	125
Figure. 34	Western Blot analysis via SDS-PAGE of <i>Abcc10/Mrp7</i> probe in rat and pig protein (tissue) lysates with loading control β -actin.....	126
Figure. 35	Western Blot analysis via SDS-PAGE of <i>Slc25a6</i> probe in rat and pig protein (tissue) lysates with loading control β -actin.....	126
Figure. 36	Western Blot analysis via SDS-PAGE of Negative (no primary antibody) control in rat and pig protein (tissue) lysates with loading control β -actin.....	127
Figure. 37	Example of CYP450 protein expression in pig: IHC staining probing for target CYP450 generic protein expression in pig.....	130
Figure. 38	Example of drug transporter protein expression in pig: IHC staining probing for target <i>Abcb1/P-gp</i> protein expression in pig.....	131

Figure. 39	Example of CYP450 protein expression in rat: IHC staining probing for target CYP450 generic protein expression in rat.....	132
Figure. 40	Example of drug transporter protein expression in rat: IHC staining probing for target Abcb1/P-gp protein expression in rat.....	133
Figure. 41	Cell count using the trypan blue exclusion test. Live cells are cells not stained with trypan blue and those stained are considered dead cells.....	134
Figure. 42	Immunofluorescence staining of <i>Cyp2e1</i> probe in primary A. pig hepatocytes and B. pig splenocytes.....	135
Figure. 43	Immunofluorescence staining of CYP450 generic probe in primary A. pig hepatocytes and B. pig splenocytes.....	136
Figure. 44	Immunofluorescence staining of <i>Ugt8</i> probe in primary A. pig hepatocytes and B. pig splenocytes.....	137
Figure. 45	Immunofluorescence staining of <i>Slc22a6</i> probe in primary A. pig hepatocytes and B. pig splenocytes.....	138
Figure. 46	Immunofluorescence staining of a (no primary antibody) negative control in primary A. pig hepatocytes and B. pig splenocytes.....	138
Figure. 47	Co-immunoprecipitation analysis via SDS-PAGE of selected CYP450, UGT and transporters target probes.....	140
Figure. 48	Enzyme kinetics: A. Illustration of a single-enzyme single-substrate reaction B. Simplified diagram of an enzyme-catalysed and uncatalyzed reaction C. Michaelis Menten kinetics: rate of reaction order, rate of reaction expression and half-life.....	148
Figure. 49	Mechanisms of enzyme inhibition by drugs.....	149
Figure 50	Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity in pig liver microsomes.....	154
Figure 51	Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity in pig spleen microsomes.....	155
Figure 52	Drug Elimination: Profiles demonstrating UGT-mediated initial rate as a function of enzyme activity in pig liver microsomes.....	156
Figure 53	Drug Elimination: Profiles demonstrating UGT-mediated initial rate as a function of enzyme activity in pig spleen microsomes.....	157
Figure 54	Metabolite Formation: CYP450-mediated metabolite profile of acetaminophen across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	160
Figure 55	Metabolite Formation: CYP450-mediated metabolite profile of hydroxycoumarin across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	161
Figure 56	Metabolite Formation: CYP450-mediated metabolite profile of hydroxybupropion across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	162

Figure 57	Metabolite Formation: CYP450-mediated metabolite profile of desethylamodiaquine across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	163
Figure 58	Metabolite Formation: CYP450-mediated metabolite profile of hydroxytolbutamide across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	164
Figure 59	Metabolite Formation: CYP450-mediated metabolite profile of 5-hydroxyomeprazole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	165
Figure 60	Metabolite Formation: CYP450-mediated metabolite profile of dextrorphan across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	166
Figure 61	Metabolite Formation: CYP450-mediated metabolite profile of 6-hydroxychlorzoxazone across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	167
Figure 62	Metabolite Formation: CYP450-mediated metabolite profile of desmethylastemizole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	168
Figure 63	Metabolite Formation: CYP450-mediated metabolite profile of 1-hydroxymidazolam across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes.....	169
Figure 64	Metabolite Formation: CYP450-mediated metabolite profile of desethylamodiaquine across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes.....	170
Figure 65	Metabolite Formation: CYP450-mediated metabolite profile of 5-hydroxyomeprazole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes.....	171
Figure 66	Metabolite Formation: CYP450-mediated metabolite profile of dextrorphan across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes.....	172
Figure 67	Metabolite Formation: CYP450-mediated metabolite profile of desmethylastemizole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes.....	173
Figure 68	Metabolite Formation: CYP450-mediated metabolite profile of 1-hydroxymidazolam across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes.....	174
Figure 69	CYP450-mediated metabolite profiles of acetaminophen, hydroxycoumarin and hydroxybupropion over time at the selected protein concentration in pig liver microsomes.....	177
Figure 70	CYP450-mediated metabolite profiles of desethylamodiaquine, hydroxytolbutamide and 5-hydroxyomeprazole over time at the selected protein concentration in pig liver microsomes.....	178

List of Figures

Figure 71	CYP450-mediated metabolite profiles of dextrorphan, 6-hydroxychlorzoxazone, desmethylastemizole and 1-hydroxymidazolam over time at the selected protein concentration in pig liver microsomes.....	179
Figure 72	CYP450-mediated metabolite profiles of desethylamodiaquine, 5-ydroxyomeprazole and dextrorphan over time at the selected protein concentration in pig spleen microsomes.....	180
Figure 73	CYP450-mediated metabolite profiles of desmethylastemizole and 1-hydroxymidazolam over time at the selected protein concentration in pig spleen microsomes.....	181
Figure 74	UGT-mediated metabolite profile of serotonin- β -D-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes.....	183
Figure 75	UGT-mediated metabolite profile of 17- β -Estradiol-3-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes.....	184
Figure 76	UGT-mediated metabolite profile of 7-hydroxycoumarin glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes.....	185
Figure 77	UGT-mediated metabolite profile of Naloxone-3-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes.....	186
Figure 78	UGT-mediated metabolite profile of serotonin- β -D-glucuronide across the selected microsomal protein concentration at individual timepoints in pig spleen microsomes.....	187
Figure 79	UGT-mediated metabolite profile of 7-hydroxycoumarin glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes.....	188
Figure 80	UGT-mediated metabolite profile of Naloxone-3-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes.....	189
Figure 81	CYP450-mediated metabolite profiles of Serotonin- β -D-glucuronide, 17- β -Estradiol-3-glucuronide, 7-hydroxycoumarin glucuronide and Naloxone-3-glucuronide over time at the selected protein concentration in pig liver microsomes.....	191
Figure 82	CYP450-mediated metabolite profiles of Serotonin- β -D-glucuronide, 7-hydroxycoumarin glucuronide and Naloxone-3-glucuronide over time at the selected protein concentration in pig spleen microsomes.....	192
Figure 83	Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity in pig hepatocytes.....	195
Figure 84	Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity in pig splenocytes.....	196
Figure 85	CYP450-mediated metabolite profiles of desethylamodiaquine, 5-hydroxyomeprazole, desethylastemizole and dextrorphan over time in pig hepatocytes.....	198
Figure 86	CYP450-mediated metabolite profiles of desethylamodiaquine, 5-hydroxyomeprazole, desethylastemizole and dextrorphan over time in pig splenocytes.....	199
Figure 87	Examples illustrating the metabolites of midazolam and dextromethorphan , 1-hydroxymidazolam and dextrorphan as substrates for UGT and other CYP450 enzymes.....	205
Figure. A1	Primer design procedure: (A-C) Primers designed from rat and pig mRNA sequences using BLAST algorithm to confirm specificity for selected CYP450s.....	232

List of Figures

Figure A2	Test Compound plate layout for the prepared working concentrations of the drug substrates in their corresponding wells (1-11/12).....	233
Figure A3	The amplification curve and melt peak of Abcb1 gene in A. pig hepatocytes B. pig splenocytes.....	233
Figure A4	The amplification curve and melt peak of Cyp2a19 gene in A. pig hepatocytes B. pig splenocytes.....	234
Figure A5	The amplification curve and melt peak of Cyp2e1 gene in A. pig hepatocytes B. pig splenocytes.....	234
Figure A6	The amplification curve and melt peak of Cyp3a29 gene in A. pig hepatocytes B. pig splenocytes.....	235
Figure A7	The amplification curve and melt peak of Ugt8 gene in A. pig hepatocytes B. pig splenocytes.....	235
Figure A8	IHC staining probing for target Cyp2e1 protein expression in A. pig liver tissue section and B. pig spleen tissue section.....	236
Figure A9	IHC staining probing for target Ugt8 protein expression in A. pig liver tissue section and B. pig spleen tissue section.....	236
Figure A10	IHC staining probing for target Ugt1a6 protein expression in A. rat liver tissue section and B. rat spleen tissue section.....	237
Figure A11	IHC staining probing for target Cyp2e1 protein expression in A. rat liver tissue section and B. rat spleen tissue section.....	237
Figure A12	IHC staining of a (no primary antibody) negative control in A. pig liver tissue section and B. pig spleen tissue section.....	238
Figure A13	IHC staining of a (no primary antibody) negative control in A. rat liver tissue section and B. rat spleen tissue section.....	238

List of Equations

Equation 1.....	20
Equation 2.....	21
Equation 3.....	21
Equation 4.....	22
Equation 5.....	22
Equation 6.....	40
Equation 7.....	41
Equation 8.....	41
Equation 9.....	41
Equation 10.....	41
Equation 11.....	42
Equation 12.....	43
Equation 13.....	43
Equation 14.....	44
Equation 15.....	44
Equation 16.....	46
Equation 17.....	46
Equation 18.....	75

List of Abbreviations

Cytochrome P450	CYP450
UDP-glucuronosyltransferase	UGT
New chemical entity	NCE
Drug metabolising enzymes	DME
Less than the limit of detection	< LOD
Not applicable	n/a
Apparent	app
Bovine Serum Albumin	BSA
Fetal Bovine Serum	FBS
Potassium Chloride	KCl
Degree Celsius	°C
Intrinsic Clearance	Cl _{int}
Hepatic intrinsic clearance	Cl _H
Dimethyl Sulfoxide	DMSO
Fraction unbound	f_u
Hepatic blood flow	Q_H or Q
Hank's Balanced Salt Solution	HBSS
Phosphate Buffered Saline	PBS
Gram	g
Milligram	mg
Millilitre	mL
Nanomolar	nM
Micromolar	μM
Nicotinamide adenine dinucleotide phosphate	NADPH
Uridine Diphosphate Glucuronic Acid	UDPGA
No reverse transcriptase	NRT
No cDNA template	NCT
PCR threshold (cycle) value	C _T
Drug-Drug Interactions	DDI
Real time Quantitative Polymerase chain reaction	rt-qPCR
Microlitre	μL

CHAPTER 1:

General Introduction and Aims

1.0 Introduction

1.1 Immunotherapeutic Targets and Agents

An effective immunotherapeutic target enables the activation of T-cells to establish anti-viral, anti-bacterial or anti-tumour adaptive immunity via the use of signalling pathways (D. R. Wang et al., 2022). This signalling process involves specificity of an antigen signal, binding to a surface T-cell receptor and communication with a surface antigen-presenting cell (D. R. Wang et al., 2022). The apparent strong resistance to T-cell activation or auto-immunity by regulatory immune checkpoints accounts for the less favourable clinical outcomes in recent years (Sadeghirad et al., 2023). For example current immunotherapeutic targets observed in cancer research are mainly inhibitory or suppressive immune checkpoint genes, proteins or biomarkers which are blocked with the use of drugs as tumours are shown to also express immune checkpoints in its microenvironment to evade the immune system (Sadeghirad et al., 2023; Sepe et al., 2020). The key aspect here is the use of specific immunotherapeutic agents that aim to enhance the function of the immune system, and these include monoclonal antibodies, drugs designed to be either immune modulators or inhibitors of immune checkpoints (Jain et al., 2022). Specific *in vitro*-produced monoclonal antibodies can direct or indirect the immune response that ultimately leads the immune system to recognise cell abnormalities or infections by various mechanisms (Jain et al., 2022). Immune checkpoints are the primary regulators of the immune system and balances the co-stimulatory and inhibitory signals of the T-cell receptor (Jain et al., 2022). Immunotherapeutic agents like drugs can also play a significant role in manipulating the immune system's ability to recognise and eradicate tumour-cancerous cells for example by inducing or inhibiting specific immune checkpoints; thus, readily amplifying the antigen-specific T-cell responses to cancerous

tumours (Jain et al., 2022). Therefore, having a novel approach that combines the concept of immunotherapy and the specificity of drugs could potentially provide a therapeutic solution for various diseases. The design of precision new chemical entities (NCEs) and the understanding of the drug disposition process of the target are both of critical importance that could aid in accomplishing a successful clinical outcome in patients.

1.1.1 Overview of the concept of immunotherapy

Globally, diseases like cancer often lead to terminal cases in patients and accounts for most deaths as stated by the world health organisation (WHO) (Liu et al., 2020a). Mainstream therapies for example in oncology commonly rely on invasive surgeries, radiotherapy and chemotherapy or a combination of such approaches to increase drug efficacy (Bush & Noe, 2020; Y. Zhang et al., 2020). Clinical outcomes of these approaches have been unsatisfactory due to severe adverse and toxic effects including low selectivity and specificity (Y. Zhang et al., 2020). Hence, recent developments have been spurred to produce new strategies for anti-cancer therapies that are dependent on safety, efficient performance and improved therapeutic efficacy (F. Zhang et al., 2020).

Immunotherapy has revolutionised this treatment paradigm of diseases predominantly in cancer (Conn, 2018). It involves stimulating a patient's immune system to recognise and eliminate tumour cells (Bush & Noe, 2020). Immunotherapy has emerged as a powerful new-generation therapeutic strategy in recent years, showing promising oncology success with improved survival rates for some tumour types, e.g. Cetuximab (in metastatic colorectal cancer) and Pembrolizumab (in advanced lung cancer), indicating a link between immune cells and anti-tumour response (Bush & Noe, 2020; Chen et al., 2019a). Despite the immense

clinical success, immunotherapeutic agents only appear effective on a small portion of cancer patients in many clinical studies (Chen et al., 2019b; Ma et al., 2019). Cancer cells appear to have high mutation rates with circulatory tumours that incites tumour recurrence or metastasis and the ability to produce new antigens which are poorly recognised by e.g. dendritic cells, thereby evading the immune response (Liu et al., 2020; Ma et al., 2019). An improved strategy that not only generates the required immune response but also further develops the ability for antigen presenting cells to recognise e.g. tumour cells, host of viruses and bacterial cells, would be a significant discovery.

Enhancing this key aspect of immunotherapy would increase the activation of the adaptive immune system primarily through effector T cells and cytotoxic T cells (Conn, 2018; J. Li et al., 2020). Moreover, its application to other diseases can be made possible following this concept of immunotherapy in generating the immune response. The challenges the immunotherapeutic approach faces are linked to natural barriers that impedes the adaptive immunity in the body (Liu et al., 2020). Therefore, using a novel strategy like immunotherapy is required that has a pivotal role to address issues or mitigate risks from natural barriers associated with current immunotherapy approaches to improve treatment outcomes in patients (Amin et al., 2020; S. Zhou et al., 2020). In addition, specific drugs that reach the site of action of the intended target is equally important as selecting an appropriate target for the intended indication; with this in mind, the spleen organ is a good example of an immunotherapeutic target where various immune cells reside or a myriad of immune responses are orchestrated and there are no known cell-forming barriers that would impede a drug's access into the spleen (S. Zhou et al., 2020).

1.1.2 The Spleen – A Potential Immunotherapeutic Target

While it is considered a neglected organ as humans can survive without it, the attention on the spleen intensified since the recognition of its role in regulating the immune system (Tarantino et al., 2011). It is the largest of the lymphoid tissues and secondary of the immune organs following primary immune organs e.g. bone marrow (Feleder & Blatteis, 2006). Secondary immune organs are responsible for generating immune responses and tolerance against foreign invaders (Ruddle & Akirav, 2009). In healthy humans, the spleen is approx. 11 cm in length (the size of a cupped hand), weighing 150 g, and has a smooth serosal surface that is attached to fatty ligaments containing a vascular supply (Tarantino et al., 2011). The spleen uniquely hosts a diverse population of stromal, endothelial and immune cells, to perform different immune and homeostasis functions (A-Gonzalez & Castrillo, 2018).

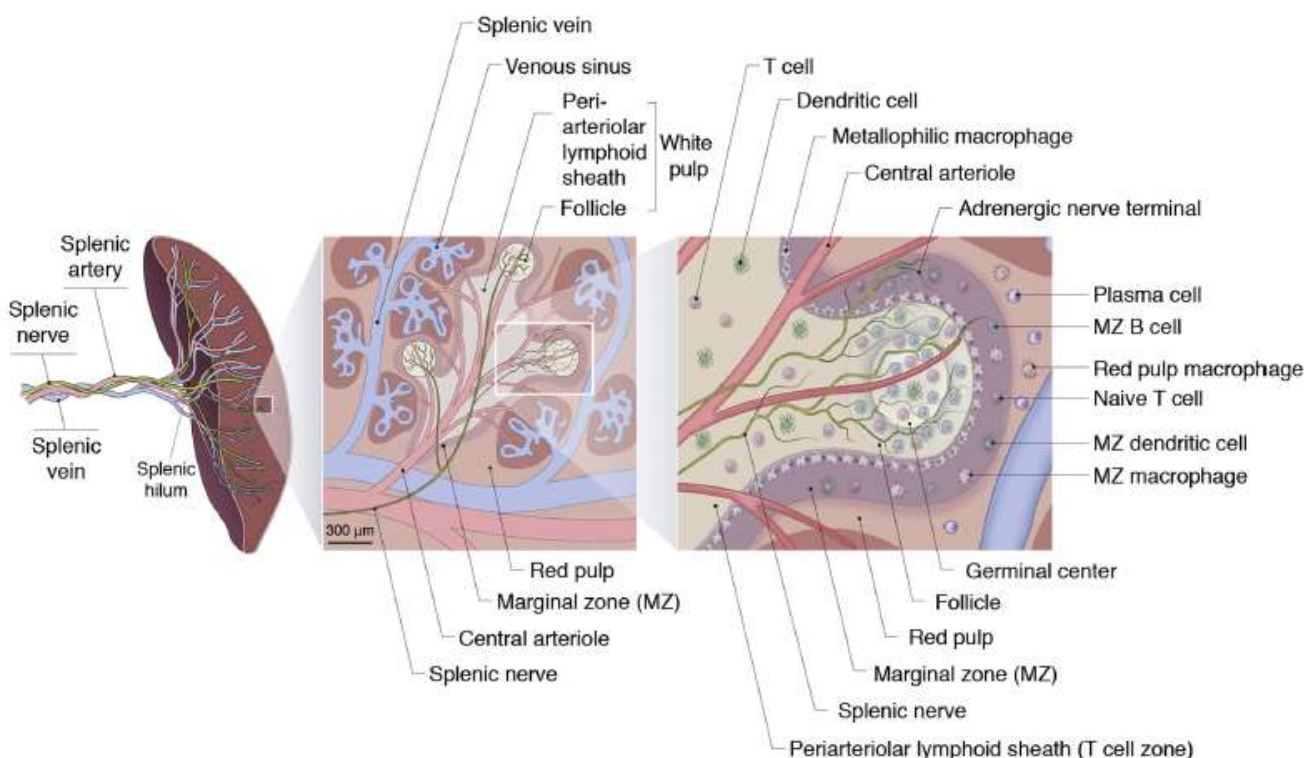


Figure 1 Anatomy of the spleen: The detailed key characterises, components and intended function (Noble et al., 2018).

1.1.3 Anatomy and Function

At the left side quadrant of the abdomen, between the 9 to 11th ribs of the human diaphragm, the spleen is located near the stomach (Burn et al., 2008; Ferrer et al., 2014). The spleen's proximity to the stomach may be significant as it may serve as the first site of attack by different types of bacteria. With a microcirculatory and a highly organised compartmentalised system, the spleen is a complex organ (A-Gonzalez & Castrillo, 2018). Its complex structure (see Figure 1) is formed by the following key macroscopic compartments – the white pulp, which makes up 25 % of splenic volume, including a lymphoid area composed of T and B cell zones where the immune responses are activated (Chadburn, 2000; Ferrer et al., 2014). The red pulp primarily consists of connective tissue filled with sinusoids and blood, making up 75 % of splenic volume, and an important area for blood-filtering of microorganisms, debris and red blood cells (Chadburn, 2000; Ferrer et al., 2014; B. S. Steiniger, 2015). Lastly, the marginal zone (MZ) is a transit area containing a reservoir of distinct cells responsible for antigen surveillance in the splenic blood circulation (Ferrer et al., 2014).

Blood circulation enters the spleen through the splenic artery that originally arises from the branch of the coeliac trunk linked to the hepatic artery (Feleder & Blatteis, 2006; Kraal & den Haan, 2016). There are two types of circulation in the spleen which are known as the 'open circulation' theory and the 'closed circulation' theory (Sheppard et al., 1973). The concept of open circulation illustrates that blood flows from the splenic artery through the arterioles into the splenic cords of the red pulp (B. Steiniger et al., 2011). These splenic cords are the filters of the spleen that consists of macrophages connected by long dendritic processes (Chadburn, 2000). The blood then squeezes through gaps within the cords endothelium to reach the sinusoids (Chadburn, 2000; B. S. Steiniger, 2015). The sinusoids act as a passage for blood flow

to enter the splenic vein and exit the spleen. This process takes a considerable amount of time which is referred to as the 'slow pathway' (Cesta, 2006; Chadburn, 2000). On the other hand, the closed circulation describes a fast process where blood flows from the splenic artery through the arterioles and straight into the splenic vein to exit the spleen via the hilum (Cesta, 2006; Chadburn, 2000). It is not determined whether either of the theories happens independently of each other at a given time, but a recent study portrayed through well-controlled immunohistology-based 3D models, that open microcirculation happens entirely in the human spleens (B. S. Steiniger et al., 2022).

Since the focus is on the spleen's ability to regulate the immune system, a key understanding of its white pulp component is of importance. The white pulp is a lymphocyte-rich area consisting of T cell and B cell zones, where the former is located in the peri-arteriolar lymphoid sheath and the latter in the follicles of the germinal centre as shown in Figure. 1. Moreover, the white pulp mainly supports and generates antigen-specific immune responses against pathogens (Golub et al., 2018; Kraal & den Haan, 2016). The marginal zone that surrounds the white pulp separating it from the red pulp, also contains immune cells e.g. dendritic cells, macrophages and special memory B-cell population as antigen-presenting cells, with the capability to initiate innate and adaptive immunity (Cataldi et al., 2017; Kraal & den Haan, 2016). Such immune cells can be attractive targets to fight against cancer, e.g. targeting dendritic cells in the spleen to activate antigen-specific T-cells which can make a powerful strategy in cancer immunotherapy (Kimura et al., 2019). This approach has demonstrated a promising outcome by Hashemi et al., 2020, in a dendritic cell-based cancer immunotherapy study using nanoparticles to convey an immune response stimulated by a spleen-targeted gene delivery system.

The importance of a spleen's role in fighting bacterial and viral infections has proved significant in cases monitoring malarial infections and therefore should show promise in fighting a host of diseases. The spleen has been regarded as the immune defence against blood-stage malaria and a predominant anti-malaria effector (Wunderlich et al., 2005). Its diminished function in clearance and surveillance of the circulating red blood cell population is more apparent in patients who have undergone splenectomy (Chotivanich et al., 2002). Splenectomised patients showed a high risk of infection, with reduced response to antigens, low response to new antigens and impaired phagocytosis of bacterial cells (Davidson & Wall, 2001). This 'risk' is also exemplified in splenectomised patients with gastric cancer showing no improvement in survival rate compared with a 5-year survival rate of cases with spleen preservation (Son et al., 2017).

Although the complete process of the spleen's development and mechanisms of action is poorly understood, recent studies in animal/murine models have conveyed promising findings in the spleens' dual role of protection and pathology (Burn et al., 2008; Ferrer et al., 2014). The spleen is conveyed as a highly perfused organ with an open arterial blood circulation that introduces lymphocytes into the red pulp and marginal zone where active T-cell migration enters the white pulp compartment and initiates B-cell activation (Cataldi et al., 2017; Chauveau et al., 2020). Activated B-cells secrete antibodies that activate cytokine receptors (of the T-cells) to regulate inflammation and fight infection (A-Gonzalez & Castrillo, 2018; Kimura et al., 2019). This understanding including the splenic blood microcirculation will have profound implications in the drug disposition process or pharmacokinetics in the spleen, where the large blood flux would facilitate the delivery of administered drugs to specifically target receptors of immune cells (Bronte & Pittet, 2013; Cataldi et al., 2017).

Blood-borne molecules (or drugs) can fully permeate through the marginal zone and therefore drugs can have free access to the splenic parenchyma (Cataldi et al., 2017).

In the treatment of autoimmune diseases, activated antigen-specific T-cell processes using dendritic cells in the spleen to target different antigens, or modulating B-cell function (as an antigen-presenting cell) provides a new promising approach as a strategy for immunotherapy (Hashemi et al., 2020; Kimura et al., 2019). In addition, the dynamics of the spleen's distinct cell populations reinforce the spleen as a target for immune activation and mobilised recruitment in response to any diseased state, instigating their production and amplification locally to other tissues (Bronte & Pittet, 2013). Therefore, the spleen has a host of advantages that immunotherapy can rely on for tissue-targeted development of drug therapeutics to produce significant immune response. Thus, the spleen is introduced here as an ideal immunotherapeutic target to potentially establish general immunity in the body by efficiently metabolising drugs that are responsible for producing the immune therapeutic response.

1.2 Animal Models

1.2.1 Use of animal models and its importance

Animal models have been used in research as far back as the 1800s as an essential tool in elucidating pathophysiological mechanisms and developing therapeutic approaches (Huang et al., 2023; Luo et al., 2023). However, this has been met with challenges where either animal models that once had historical significance are no longer in use, and with those conditions that have shown to be difficult to replicate outside the lab (Won et al., 2023). The understanding that there is no omnipotent animal model available as a standard one for all, has led to a common consensus that a criteria is required for animal model selection (Huang et al., 2023). The starting point should be realising the primary purpose of animal models, which is to provide information that translates basic scientific information into human clinical practice (Luo et al., 2023). The next step is to then figure out how to select an appropriate animal model that can be utilised to investigate the research of interest by knowing the characteristics of the animal (Che Has, 2023; Luo et al., 2023). Researchers generally follow a pattern, which stems from the recognition that animal model selection is influenced by multiple factors (Won et al., 2023). Such factors or rather the scenarios that make the model suitable for application includes an animal's preterm viability, ease of creating, manipulating, mimicking and replicating the pathophysiological properties of the research of interest, size, genetic variability, physiological resemblance to humans, and finally associated costs (Che Has, 2023; Huang et al., 2023; Luo et al., 2023; Won et al., 2023). A recent study proposed a systematic flow diagram that can be beneficial to the animal model selection process, which follows (i) Identification (via electronic database searching), (ii) Screening (removal of duplicate records and exclusion from available records), (iii) Eligibility (assessments from full-

text articles), and (iv) Inclusion (of studies in qualitative synthesis) (Palandi et al., 2023). Nevertheless, the use of animal models provides advantages available to researchers (not considering the underlying ethical implications). For example, the models aid in providing clinical relevance; give an insight into manifestations, pathophysiological and neurological understanding of infections, diseases, pain or injuries and the effects of new ones; have the ability to assess the detrimental conditions in question; have the ability to test efficacy of therapeutic approaches, and the ability to detect the effects of interventions made to improve outcomes in patients (Ijuin et al., 2023; Luo et al., 2023; Xiang et al., 2023).

1.2.2 Rat and Pig models in drug discovery and development

Pre-clinical drug development processes aim to determine the potential toxicities and efficacies of novel compounds and to reduce the inherent risk of first-in-man studies with the use of an appropriate animal model (Yaqub et al., 2022). The main critical limitations of animal models are their poor correlation to the human condition even though they are used during studies in drug metabolism and pharmacokinetics (DMPK) and absorption, distribution, metabolism, excretion and toxicology (ADMET) to determine optimal dosages, potential side effects and drug-drug interactions (Yaqub et al., 2022). Rodents like rats have been widely used for disease modelling, drug discovery and disease interventions (Savoji et al., 2019). They fall under the small animals' category and generally provide significant insight into phenotypes and pathways (Savoji et al., 2019). Rodents are commonly used to study liver cancers due to the high success rate, low mortality and the similarity of development progress as in human's liver cancers (L. Wang et al., 2023). The usefulness and validity of rats as rodent models are indicated from possessing physiological similarities to human including factors like

affordability, short lifespan, (large sample numbers in terms of) availability and genetic manipulation (McNally & O'Brien, 2013). The small animal model complies with genome editing technologies such as CRISPR/Cas9 and enables it for far more effective use in drug efficacy studies (Yaqub et al., 2022). Rodent models are great in cardiovascular research for example, whereby mice are extensively used for feasibility and proof of concept studies while rats are the better choice for post-mortem histological and molecular biology analysis (Savoji et al., 2019). There are many positives to using small animal models (see Table 1) but these are often overshadowed by lack of clinical relevance and certain physiological differences to humans (Savoji et al., 2019). The conception of the 3Rs (replacement, reduction, refinement) in the 1950s accelerated the development of models that addresses current scientific questions without the use of animals (Yaqub et al., 2022). With the implementation of the 3Rs, the use of animals has greatly subsided making the scientific community to meticulously consider models based on animal research ethics (Yaqub et al., 2022).

Table 1 Examples of the pros and cons of animal models in cardiovascular diseases (CVD), adapted and sourced from Savoji et al., 2019

	Small Animal Model (Rodents)	Large Animal Model (Swine)
Pros	✓ Easy breeding and handling ✓ Short reproductive cycle ✓ Relatively cheap ✓ Well-defined genome ✓ Ease of genetic manipulation ✓ Large litter number	✓ Closer to human anatomy, better tissue availability and more accurate minimally invasive measurements ✓ Closer lipoprotein profile to human except for human HDL subclasses ✓ Similar vascular lesion structure and lesion distribution to humans ✓ Rare thrombosis due to plaque rupture ✓ Suitable for translational research
Cons	- x Resistance to pathogenesis in wild type - x Different gross anatomy to humans - x Different lipoprotein profile to humans - x Compromised lesion formation - x Absence of plaque rupture and thrombosis	- x Costly and difficult maintenance and handling - x No genetic modifications - x Limited genetic models available - x Rare thrombosis due to plaque rupture - x Ethical concerns

Consequently, large animal models (dogs, pigs, sheep, goats) have gained substantial interest with the added knowledge that the models present a similar spectrum of *in vivo* phenotypes, disease biology, immunology, and pathology to that encountered in humans (N. Kumar et al., 2014). They generally serve as a better model to translate cellular and biological experimental findings from animals to humans in the clinic (Savoji et al., 2019). One that stands out as an alternative and attractive model in the large animal category is the domestic pig since there's a growing ethical concern for the use of canines in research (Savoji et al., 2019). Porcine models have increased in cancer research due to their strong resemblance in anatomy (genetically, metabolically and toxicologically) to humans, and the ability to spontaneously develop cancer like that in humans (Schyman et al., 2021; S. Smith et al., 2022). Typically, piglets or mini pigs are primarily used as models for *in vivo* studies as they are easier to handle and maintain even though it can be significantly more expensive to conduct experiments (Won et al., 2023).

1.2.3 Porcine animal model

Most systems and organs in pigs like the heart, liver, kidney, brain, nasal cavity, reproductive and gastrointestinal system, show analogies to human with significant advantages over other experimental models that may prove pig models superior to classical rodent and primate counterparts (Puccinelli et al., 2011). As large animals, a single pig animal is needed compared to a large quantity of rats or mice required for a certain *in vivo* study, and that same one animal can provide a greater amount of biological resource for research. The use of pigs as an alternative model choice also allows for safe dosage ranges to be defined, for example, in drug development studies and toxicological testing since they have similar responses to

several variety of drugs to that in humans (Lunney et al., 2021). The importance of animal models like pigs can be further illustrated in studies understanding the mechanisms of diseases where observations of drug exposure differences are compared and investigated (Ayuso et al., 2021). Transplanting animal organs or tissues into humans is now possible using genetically modified pig (Groth, 2007; W. Wang et al., 2022). This confirms that overall, though not perfect, the close homology that porcine and rodent models are to humans both anatomically and functionally, gives confidence in using such animal models for experimental *in vitro* research in drug discovery and development.

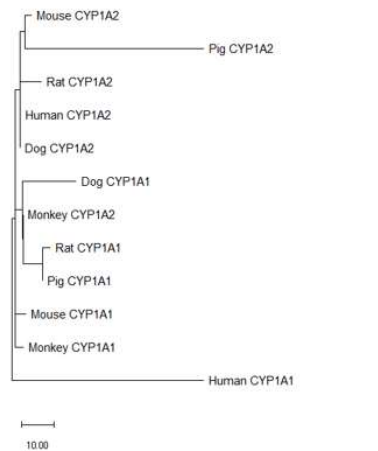
1.2.4 Phylogenetic Analysis

A secondary assessment of the animal model selection involves confirming DNA sequence similarities to humans by conducting a phylogenetic analysis. The basic principle of generating a phylogenetic tree is incorporating the evolutionary process of taxa (biological entities such as genes, proteins, individuals, populations, species or higher taxonomic units) entirely based on their molecular sequence data (Paul & Sahoo, 2014). This allows researchers to prune the resulting phylogenetic tree to include for example the research of interest only from their lists (Jin & Qian, 2023). There are two ways data can be used in the selected method (Neighbour joining, Parsimony, Bayesian or Maximum Likelihood) for constructing a phylogenetic tree, which is either through the distance-matrix method or the character-state method (Paul & Sahoo, 2014). The former uses an algorithm to convert DNA and protein sequences into a distance matrix that represents an estimate of the evolutionary distance between sequences (Paul & Sahoo, 2014). Whereas the latter is based on a stochastic model of the nucleotide

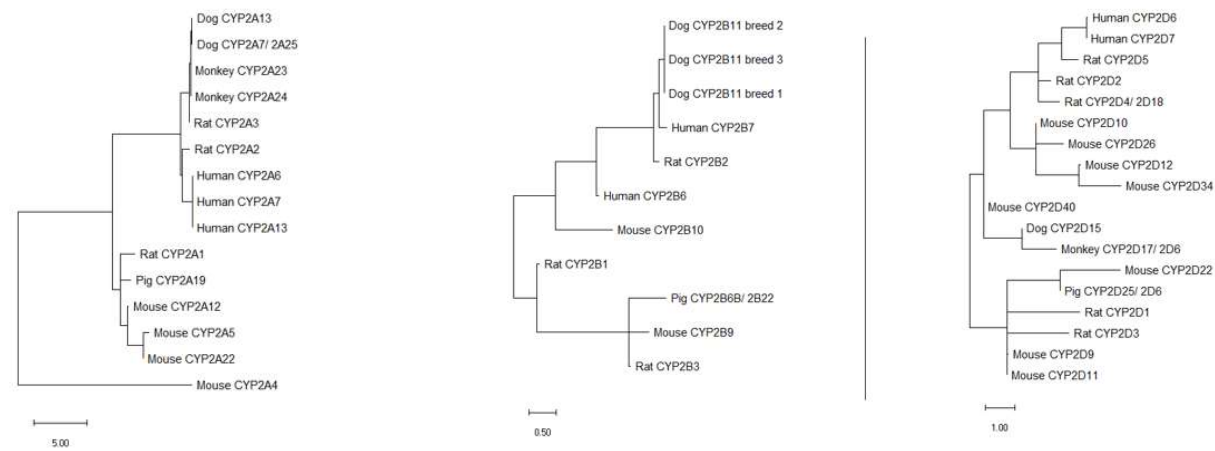
sequences meaning it intends to find the topology that yields the highest probability of the observed data (Paul & Sahoo, 2014).

This study aims to justify the use of rat and pig models in examining enzymes responsible for drug metabolism and distribution as that encountered in humans. The following example is a phylogenetic tree created with a combination of cytochrome P450s (CYP450) mRNA sequences obtained from the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/>), and the MegaX software package using a Maximum Likelihood algorithm (character-state method) to conduct the phylogenetic analysis (Arora et al., 2021; Martin et al., 2020; Z. Wang et al., 2019). The constructed phylogenetic tree (See Figure. 2) was made to determine the close homology or genetic similarity relationship between the selected animal models to human and using the major contributing drug metabolising enzymes observed in human liver as reference.

A.



B.



C.

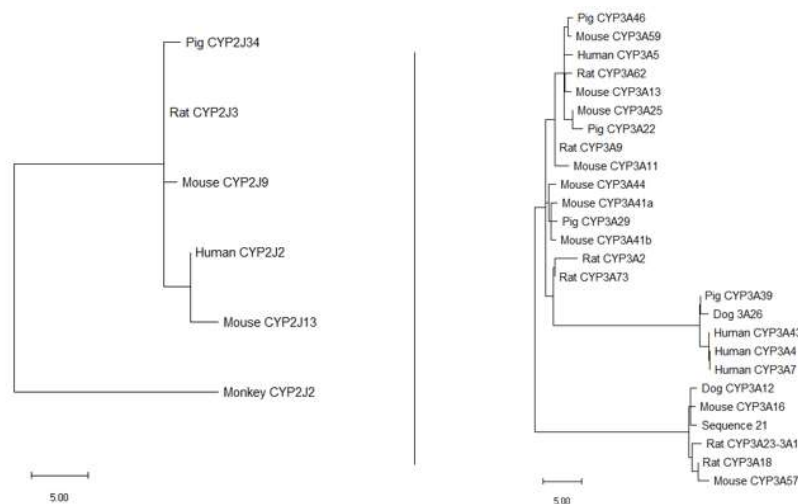


Figure 2 Phylogenetic Tree: examples demonstrating the phylogenetic relationship (homology) across different animal models (mouse, rat, pig, dog and monkey) to human using the major contributing cytochrome P450 mRNA sequences known in human liver. **A.** CYP1A and CYP2A **B.** CYP2B and CYP2D **C.** CYP2J and CYP3A. The phylogenetic analysis was constructed using the Mega X software and the distance scale bar is presented based on the composition distance per site, which examines the composition distance between two nucleotide (or amino acid) sequences divided by the number of positions compared excluding gaps and missing data (Sudhir Kumar et al., 2015).

1.3 Drug disposition

1.3.1 Importance of drug discovery

Drug discovery describes the journey of an NCE towards candidate selection through a series of assessments which includes pharmacokinetics (PK) and pharmacodynamics (PD) studies. The former seeks to establish the administered dose that produces a given exposure by quantifying the drug disposition processes (with plasma and tissue concentrations). The latter aims to determine the range of the observed drug exposure associated with the desired therapeutic response (quantifying the drug effect). In drug discovery, it is important to identify adequate evidence of biological activity at a target, safety and drug-like properties before being introduced into human testing (Mohs & Greig, 2017; Naritomi et al., 2018).

In the 1990s, high attrition rates in drug development were related to unsatisfactory PK or bioavailability profiles which accounted for approx. 40 % of failures – these PK profiles were poorly understood and difficult to predict (Chaturvedi et al., 2001; Mohs & Greig, 2017). High attrition rates also accounted for significant cost for NCEs to become marketable drugs, taking approx. 12 years to develop new medicine from target identification to the market (Gary. W. Caldwell, 2000; Mohs & Greig, 2017). In 2000, Caldwell highlighted a widespread emphasis to addressing and reducing high attrition rates in clinical studies by evaluating PK, efficacy and safety at an early stage which was attempted by pharmaceutical companies. By 2000, this cause of attrition (poor PK properties) had indeed diminished significantly to less than 10 % failures following the approach ‘fail fast, fail early’ aimed to eliminate key risks early before late-stage investments (Mohs & Greig, 2017).

At the discovery stage, there should be clear measures of exposure, target engagement and evidence of biological effects to interpret data correctly and determine early whether a drug

is effective or not (Mohs & Greig, 2017). It is also fundamental to understand that these safety assessments of drugs are largely dependent on the findings in animal models predicting any indicator of human effects (J. Caldwell et al., 1995). The following important considerations should be included in the selection of NCEs or successful drug candidates that would be introduced into the spleen, and then stimulate immune cells: a) the ability to reach the target b) exhibit good distribution into and within the target tissue c) show evidence of its presence at the target and engages with receptors that would induce an immune response.

1.3.2 Overview of the drug disposition process

The study of pharmacokinetics depicts the journey of medication, or xenobiotics as it travels through the body. Pharmacokinetics serves as a mathematical tool that demonstrates what the body does to a drug once administered over time (Stanley, 2017). This concept is understood to be governed by four processes known as absorption, distribution, metabolism and excretion (ADME). For a xenobiotic to enter the systemic circulation of the body, the compound must be introduced (i.e. intravenously, orally, subcutaneously, intramuscularly, inhalation etc) and for example if orally administered, would be absorbed into the body predominantly in the gastrointestinal tract through passive diffusion or facilitated transport mechanisms (J. Caldwell et al., 1995; Ionescu & Caira, 2005). It is subsequently distributed to various organs of the body which is influenced by the blood flow, size and permeability of the organ tissue or its membranes, including binding of the compound to components of blood (i.e. proteins such as albumin) and tissues (Ionescu & Caira, 2005). Metabolism is the third stage of this process and is considered a major elimination pathway of a drug molecule from the body (Khojasteh et al., 2011). The process of excretion of xenobiotics (or as unchanged

compound) is regulated by the kidney primarily through the glomerular filtration as urine, or secretion into the bile (J. Caldwell et al., 1995; Stanley, 2017). Hence, these ADME processes have aided to define the key pharmacokinetic parameters *in vivo* (i.e. clearance, area under the curve (AUC), half-life, volume of distribution, bioavailability etc) that ensures the selection of appropriate and efficacious dosing regimens of suitable drug candidates (Khojasteh et al., 2011).

1.3.2.1 ADME: Drug Absorption and its influence reaching the target

An effective administered drug that is absorbed in the systemic circulation and reaches its target depends on a drug's bioavailability which is primarily influenced by physicochemical and PK properties. Optimising good PK (*in vitro* and *in vivo*) and physicochemical profiles is a prerequisite and crucial to identifying high quality candidate drugs (Chaturvedi et al., 2001; Waring et al., 2015). Early prediction of oral bioavailability of NCEs is also important because it is non-invasive and a preferred choice for patient compliance (Xu et al., 2020).

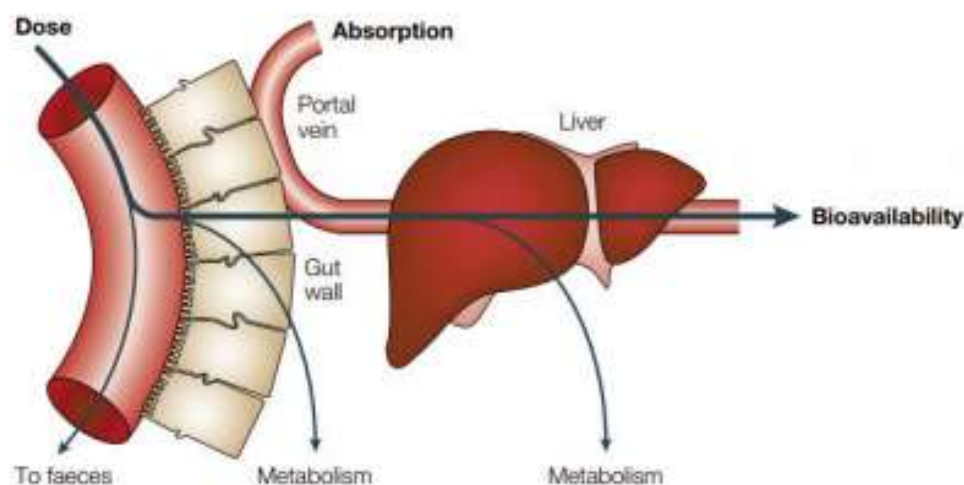


Figure 3 Oral administration of drugs: drug uptake, excretion and first pass metabolism before reaching the systemic circulation (den Braver-Sewradj, 2017; van de Waterbeemd & Gifford, 2003).

Typically, optimised physicochemical properties of drugs are evaluated to assess oral absorption issues such as solubility, lipophilicity, permeability, chemical stability, physical form, formulation abilities and biopharmaceutical characteristics (Lohani et al., 2014). Combination of the 'drug-likeness' approach, *in vitro* human data, and animal ADME (*in vivo*) data is attributed to the prediction of human PK as part of the drug design (Abdelwahab et al., 2020).

Figure 3 demonstrates how oral drugs must be absorbed from the gastrointestinal tract (GI), enter the portal vein through the liver and then reach the systemic circulation (den Braver-Sewradj, 2017; Khojasteh et al., 2011). The drugs may undergo possible first-pass metabolism at the site of administration, for example testosterone and acetaminophen, are known to lower the bioavailability significantly (Neirinckx et al., 2010; Pond & Tozer, 1984). Other

factors can influence the oral bioavailability of drugs, such as pH (drugs susceptible to the acidic conditions in the stomach are destroyed by gastric acid), degree of ionisation (drugs in their ionised form are poorly absorbed via passive diffusion due to their decreased lipophilicity), and drug substrates of p-glycoprotein (P-gp) efflux such as paclitaxel etc (Curry & Whelpton, 2017; Sze et al., 2019).

Oral bioavailability (expressed as F) is a key PK parameter and can be defined as the fraction or percentage of the oral dose reaching the systemic circulation intact (Aungst, 2017). This is dependent on the extent of absorption and metabolism, therefore the collective contribution of both on oral bioavailability can be described as follows in equation [1]:

[1]

$$F = f_{abs} \times f_g \times f_h$$

Where f_{abs} is the fraction of dose absorbed from the GI tract, f_g is the fraction of drug from gut metabolism, f_h is the fraction of drug from hepatic liver metabolism (Aungst, 2017; H. Zhou, 2003).

1.3.2.2 ADME: Drug Distribution and importance of the role of drug transporters

Drugs that are absorbed in the systemic circulation determines how their blood/plasma amount is distributed extensively to organs and tissues in the body (Kok-Yong & Lawrence, 2015). A compound's ability to distribute, interact with transporters, and ultimately engage with the target is dependent on its free and unbound state (Freeman et al., 2019). This concept is generally termed the 'free drug' hypothesis, stating that only the free (unbound) drug concentration at the site of action is responsible for the (*in vivo*) pharmacological activity

at steady state and at equilibrium on both sides of any biological membrane (Freeman et al., 2019). Measurement of the free drug available (i.e. fraction unbound, f_u) or what is bioavailable in blood/plasma can be used as a surrogate to predict drug action in a concentration-effect relationship (Lin, 2006).

This is inter-related with a drug's ability to bind to plasma proteins e.g. albumin, which will affect the free plasma concentration as described by the following equations [2], [3] (Ryu et al., 2020):

[2]

$$\text{Fraction Unbound } f_u = \frac{\text{unbound drug concentration}}{\text{total drug concentration}}$$

Therefore,

[3]

$$C_{up} = C_{tp} \times F_{up}$$

Where C_{up} is the free drug concentration in plasma, C_{tp} is the total plasma concentration *in vivo*, F_{up} is the plasma protein binding (fraction unbound) *in vitro* or *ex-vivo*.

The duration and intensity of drug action is then defined by a key PK parameter known as the Volume of distribution, V_d (the dose or loading dose required to achieve the target plasma concentration in the body), making tissue distribution an important determinant of a pharmacokinetic profile of a drug molecule (Boobis et al., 2002). The extent of drug distribution can be defined by the apparent V_d , and described as in equation [4]:

[4]

$$V_d = \frac{\text{amount of drug in the body}}{\text{plasma drug concentration}}$$

Drug distribution is also related to the elimination half-life ($t_{1/2}$) in plasma, defined as the time it takes for the plasma drug concentration to be reduced by 50% shown in equation [5] (Roberts, 2005):

[5]

$$t_{1/2} = \frac{V_d}{Cl_p} \times 0.693$$

Where Cl_p refers to clearance in plasma. The $t_{1/2}$ represents the duration of drug action and increases when the V_d increases and Cl_p decreases, however a long $t_{1/2}$ and a high V_d is suggestive of drug accumulation in the tissue (Roberts, 2005).

The transfer across cell membranes or movement of drug molecules is vital as they undergo ADME processes to function in the body (Peng et al., 2020). Hydrophilic compounds cannot penetrate cells since cell membranes consists of a lipid bilayer and would only enable compounds with favourable physicochemical properties to permeate freely (Y. Zhang & Hagenbuch, 2019). Drug transporters are required to mediate both the influx and efflux of drugs across cell membranes (S. Chiba et al., 2020a; L. Zhang et al., 2019). This is particularly important to understand the translocation of drugs into target tissues indicating that transporters play a role in drug delivery and drug interaction (Peng et al., 2020).

1.3.2.2.1 Drug Transporters

Drug transporters are membrane transport proteins and are widely expressed in a range of organs, including the kidney, placenta, brain and liver (S. Chiba et al., 2020b). According to their genetic codes, drug transporters are divided into two classes: solute carrier (SLC) transporters and ATP-binding cassette (ABC) transporters (Ren et al., 2020). Table 2 shows ABC transporters as active transporters which are mainly involved in efflux transport such as P-gp, multidrug resistance associated protein (MDR) and breast cancer resistance protein (BCRP) (Khojasteh et al., 2011).

Table 2 Drug transporters in Tissues: Known ABC and SLC transporters expressed and involved in the distribution of drugs in tissues. Including peptide transporters (PEPT) and Glutathione transporter (GLUT). Information adapted and sourced from Peng et al., 2020.

Position	Influx transporter type	Efflux transporter type
Intestine	PEPT; OATPs; OCTNs; OCT1; OCT2	MDR1; MRP1/2/3/4/5; BCRP
Liver	OATP1B1; OATP1B3; OATP2B1; OCT1; OAT2	MDR1/2/3; MRP2/3/4; BCRP; BSEP
Kidney	OCT2; OAT1; OAT3; OAT4; PEPT2; GLUTs	MDR1; MRP2/4; OCTN1/2; MATE1/2; OCT4; OATP1A2
Blood-brain barrier	GLUT1; OAT3; OATP2B1; OCTN2; OCT1/2/3	MDR1; MRP1/2/3/4/5; BCRP

ABC transporters predominantly uses energy to transport endogenous materials into and out of cell membranes (Ren et al., 2020). Most known drug transporters are SLC transporters, they facilitate ion gradient transport produced by active transporters as described in Figure. 4 (Khojasteh et al., 2011). Examples of SLC transporters include organic anion transporters (OAT) and organic cation transporters (OCT) as listed in Table. 2, which function as influx

transporters and are understood to be highly expressed in liver to distribute drugs or xenobiotics from the blood circulation to hepatocytes (Peng et al., 2020; Ren et al., 2020).

Most transporters either establish or maintain the electro-chemical gradients across cell membranes and are further classified as passive or active transporters based on their energy consumption as shown in Figure 4 (Y. Zhang & Hagenbuch, 2019). In different tissues, transporters express differently suggesting that it possible to induce drugs to concentrate at a target site which will subsequently improve efficacy and reduce toxic side effects (Peng et al., 2020). In 2019, Zhang. L et al., demonstrated this in a study that showed altered expression and function of transporters in diet-induced obese patients which may impact the drug disposition process. Monitoring drug uptake by transporters enables the evaluation of its substrate specificity in pharmacokinetic studies (S. Chiba et al., 2020b).

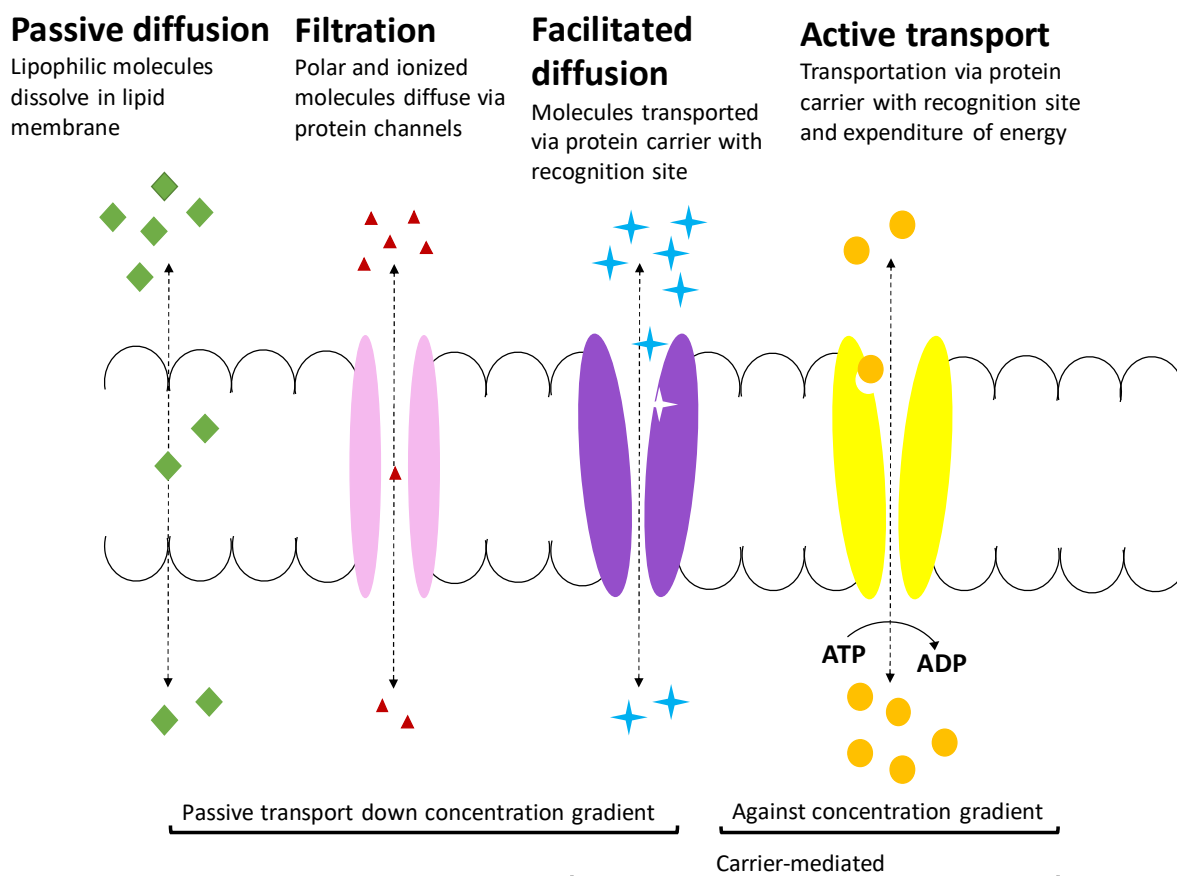


Figure 4 Mechanism of Action: Drug transporters are either involved in passive or active transport in the distribution of drugs. Illustration adapted and sourced from Curry & Whelpton, 2017.

Several factors may influence drug distribution and are central to enhancing PK properties in drug discovery. Diffusion of xenobiotics across cell membranes by transporters will depend on the rate of delivery of perfused blood flow within an organ or active site of the target (Bachmann, 2009; Kok-Yong & Lawrence, 2015). Drug molecules are likely to distribute more rapidly to tissues richly perfused with blood – organs that are well perfused (e.g. liver and heart) will receive a majority of the absorbed drug, while a slower distribution is observed for poorly perfused tissue regions (e.g. fat and skin etc) (J. Caldwell et al., 1995; Gopinath & Wilson, 2020).

1.3.2.3 ADME: Drug Metabolism and its role in drug discovery

Maintenance of chemical homeostasis (or the fate of xenobiotics) in the human body is mediated by the chemical process of metabolism, which is governed by a large family of enzymes referred to as drug metabolising enzymes (DMEs) (Mackenzie et al., 2017). Metabolism represents an elimination mechanism for the majority of drugs and becomes a key determinant of PK, in identifying safe and efficacious therapeutic NCEs (Y. Li et al., 2019; Mackenzie et al., 2017). DMEs are expressed in various tissues (i.e. lung, kidneys, liver, placenta etc), though a large proportion are observed in liver hepatocytes at the smooth endoplasmic reticulum and the main metabolism site as shown in Figure 5 (J. Caldwell et al., 1995; Z. Zhang & Tang, 2018). Most drugs that undergo enzymatic degradation or conjugation will lose their pharmacological activities through metabolic transformation, yielding water-soluble metabolites that are readily excreted (Y. Li et al., 2019). These chemical reaction processes are classified as Phase I (functionalisation) reactions and Phase II (conjugation) reactions as illustrated in Figure 6.

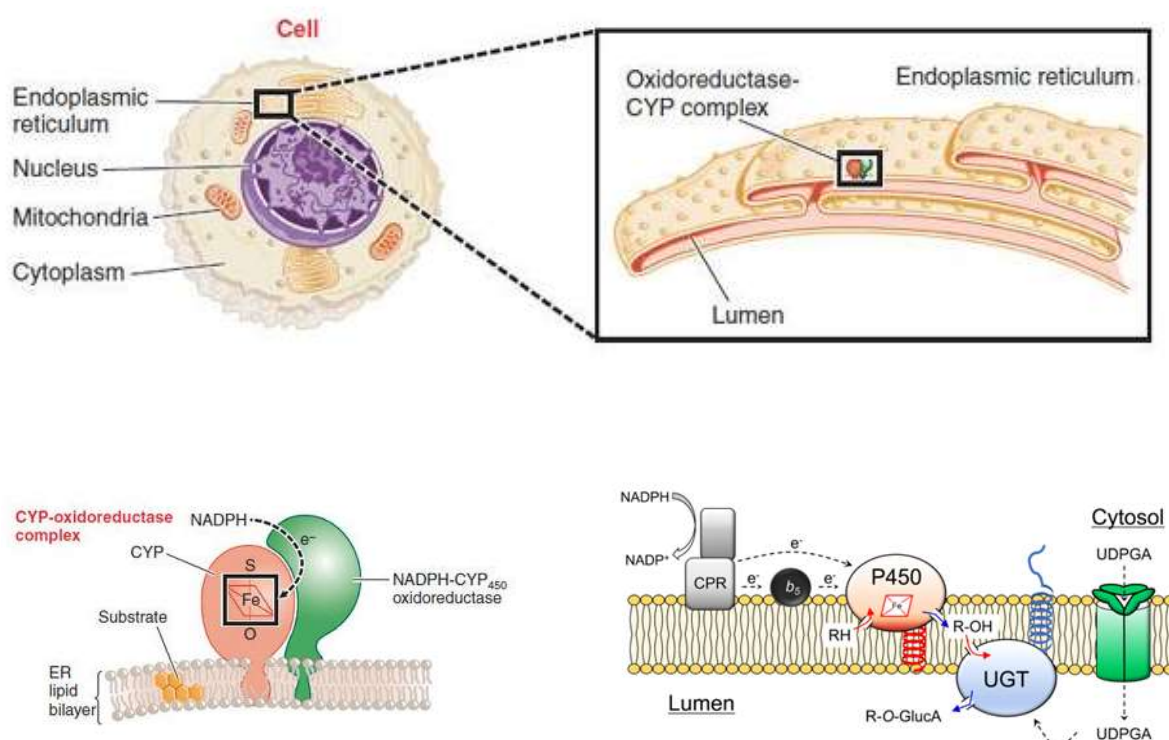


Figure 5 Location and topology of CYP450 and UGT on the endoplasmic reticulum membrane in a cell (Miyachi et al., 2021) and sourced from www.basicmedicalkey.com/drug-metabolism-3/. (NADPH)-cytochrome P450 reductase (CPR), Cytochrome P450 (CYP450), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), Cytochrome b_5 (b_5), Electron (e^-), Substrate (RH), Metabolite formed by CYP450 (R-OH), (UDP)-glucuronosyltransferase (UGT), UDP-glucuronic acid (UDPGA), glucuronide generated by UGT (R-O-GlucA).

However, the biotransformation of drugs may lead to either the generation of active metabolites that could potentially be responsible for the pharmacological response, or production of reactive metabolites with potential toxicological implications (G. N. Kumar & Surapaneni, 2001). Metabolites are products formed from the resulting catalysed reaction, for example between a CYP450 enzyme and a drug substrate. Hence, assessments such as potential drug-drug interactions and interactions due to enzyme inhibition or induction, are

important in drug metabolism studies for observing markers that would be considered as major liabilities in the disposition of a drug candidate (G. N. Kumar & Surapaneni, 2001).

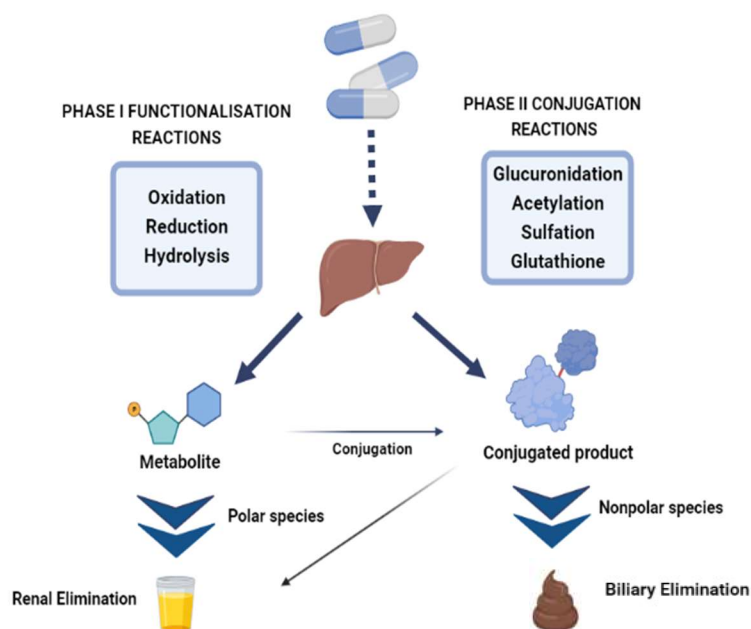


Figure 6 Drug metabolism pathway: A simplified illustration of the process of xenobiotic metabolism and detoxification through Phase I and II reactions. Adapted from Creative Biolabs and created in BioRender.

1.3.2.3.1 CYP450 and UGT enzymes

Cytochrome P450 enzymes (CYP450) are the most widely and extensively studied of the heme-containing DMEs. These resident membrane mixed-function oxidase superfamily of enzymes play a major role in Phase I biotransformation of xenobiotics (foreign chemicals) and endogenous substances, with the highest concentration expressed in the liver (Rodríguez-Antona et al., 2000; Sadler et al., 2016). The name CYP450 emanates from its heme-group – the iron-porphyrin unit (the centre of the oxidising site), and in its reduced state binds with

high affinity to carbon monoxide to form a complex that shows a large UV absorbance at 450 nm (Taxak & Bharatam, 2014). CYP450 enzymes consist of multiple types of structurally distinct isoforms and are responsible for the catalysis of oxidative and reductive hepatic metabolism of approximately 25 % of clinically used drugs (Jiang et al., 2016; Nakasa et al., 1993).

The mechanism of action of CYP450s can be described by the steps in the P450 reaction cycle (Figure 7). Firstly, this involves binding of the substrate to Fe^{3+} of CYP450 isoform with the co-factor NADPH P450 reductase, transferring an electron to CYP450 reducing the heme iron to form Fe^{2+} , ferrous iron (Khojasteh et al., 2011). The resulting O_2 binds to iron and a second electron transfer occurs by another co-factor Cytochrome b_5 , forming reactive iron species FeO^{3+} complex (Khojasteh et al., 2011). The complex rearranges and transfers an oxygen atom to the complex-bound substrate resulting finally in the oxidised substrate being released and the enzyme is being regenerated (Bachmann, 2009).

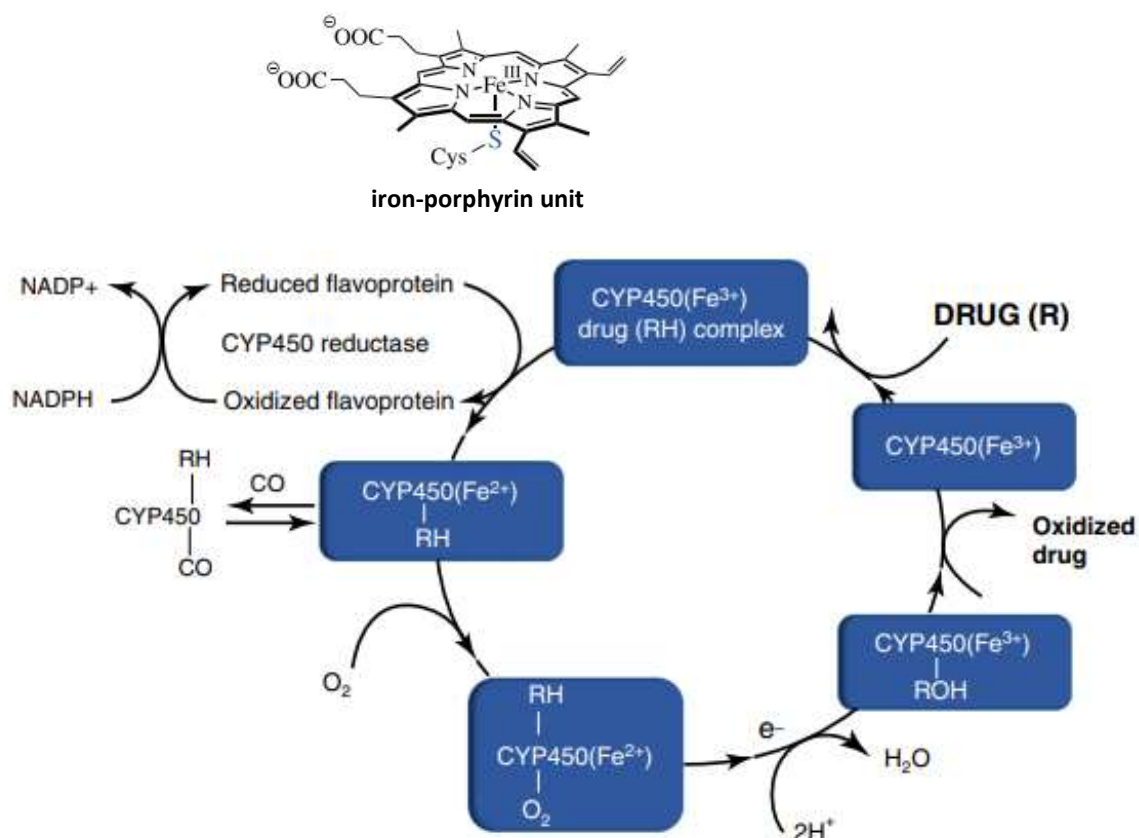


Figure 7 CYP450 Catalytic Cycle: Steps in the CYP450 reaction cycle describing binding of substrate and molecular oxygen to CYP450 and subsequent oxidation of substrate (Bachmann, 2009; Ghosh et al., 2016; Groves, 2015).

UDP-glucuronosyltransferase enzymes (UGTs) also have a significant role in drug metabolism and are responsible for catalysing conjugation reactions of phase II biotransformation of drugs related to glucuronidation (Kaur et al., 2020). Such enzymes are also highly expressed in the smooth endoplasmic reticulum of the human liver, but widespread in other tissues too such as the GI tract, kidney, lung, brains and reproductive organs (Kaur et al., 2020). Together with CYP450s, both types of enzymes metabolise more than 90 % of marketed drugs which are dependent on hepatic clearance for excretion from the body (Rowland et al., 2013). Glucuronidation aims to enhance chemical compounds' solubility in urine, but favours

elimination through biliary excretion as the conjugated compounds tend to be large (Jarrar & Lee, 2021). This chemical reaction mediated by 22 UGT isoforms in human involves the formation of a covalent bond with glucuronic acid from uridine 5'-diphospho-glucuronic acid (UDPGA), and substrate compounds that contain hydroxyl, carboxylic acid, amine and thiol functional groups (Allain et al., 2020; Jarrar & Lee, 2021; Kaur et al., 2020).

These enzymes are known to be concentrated in microsomes as a liver pigment which are harvested (via homogenisation then ultracentrifugation) from the liver tissue, and subsequently used to conduct experiments for example in studying CYP450/UGT enzyme activity to estimate ADME properties of drugs and chemical templates for 'drug-ability' (Bachmann, 2009; Vrbanac & Slauter, 2016).

1.3.2.3.2 CYP450: Genetic polymorphism

CYP450 enzymes exhibit broad overlapping substrate specificities – the ability to interact with most chemical species as a determinant of metabolic clearance (D. Zhang et al., 2012). Despite this, the enzyme activities are influenced by several rate-limiting intrinsic and extrinsic factors such as genetic (variation) polymorphism (Zanger & Schwab, 2013; D. Zhang et al., 2012). Figure 8 shows the major contributing CYP450 enzymes with *CYP3A4/5* being the most involved in the metabolism of drugs at 30 % and the least, *CYP2E1* or *CYP2J2* both at 3 %. There are significant ethnic differences in the metabolism of some drugs due to CYP450 polymorphism particularly *CYP2D6*, and such variations gives rise to alleles conferring no enzyme function, decreased function or increased enzyme function (Rajman et al., 2017). These alleles or variant genes can produce several phenotypes defined as ultra-rapid, normal, intermediate and poor metabolisers (Daniel, 2017). Therefore, CYP450 expression may

significantly contribute to the variation in oral bioavailability, drug response and systemic clearance in individual patients (Thervet et al., 2003; Zhao et al., 2021). Such information is of great interest and highlights that meticulous care is needed when examining the involvement of CYP450 microsomal metabolism in drug interactions, toxicity and bioactivation of carcinogens or relevance in clinical pharmacology (Han et al., 2019; Puccinelli et al., 2011; Zanger & Schwab, 2013).

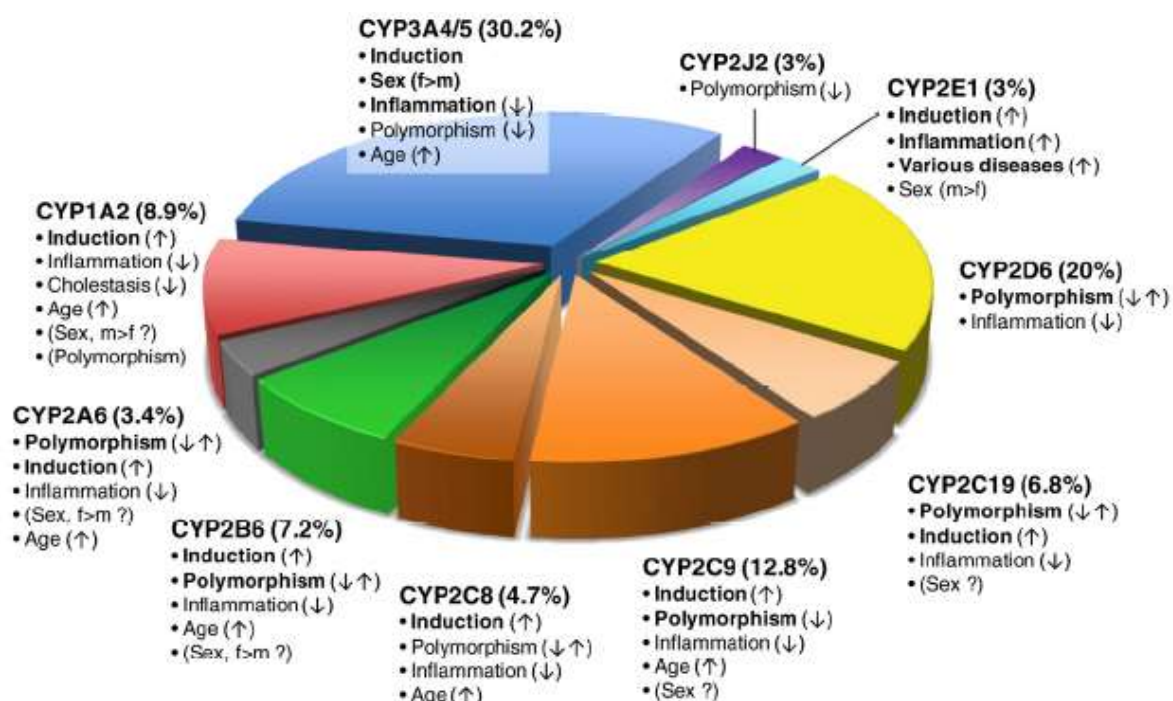


Figure 8 Major contributing human CYP450 isoforms: Fraction of clinically marketable drugs metabolised by CYP450 and its factors influencing variability. % abundance of 248 drug metabolism pathways of CYP450 involvement as indicated (Zanger & Schwab, 2013).

1.3.2.3.3 CYP450: Possible impact of its regulation

The discovery of gene regulatory mechanisms controlled by transcription factors such as microRNA (miRNAs) on CYP450 expression have been extensively studied. These mechanisms can be tissue-specific and influenced themselves by other factors including sex, age and the environment (Zanger & Schwab, 2013). For example, smoking can cause such epigenetic patterns to occur by influencing *CYP1A1* promoter methylation in human lung tissue, with *CYP1A1* expression lowest in tobacco smokers and highest in non-smokers (Zanger & Schwab, 2013). Concerns into the possible influence of epigenetic processes on pharmacologically genes or drug responses is of great interest in this field of research, since interaction of genetic or epigenetic factors determines an individual's capacity to metabolise a given drug (Morgan, 2001). In the past, unpredictable component of drug responses were attributed to genetic polymorphisms, however with the in-depth knowledge of genotypes, it is worth considering that such unpredictability is caused by variation of epigenetic factors between individuals or in the same person (Morgan, 2001). In addition, miRNAs also target nuclear receptors, (together with other factors) these stabilise the corresponding mRNA and post-translational modifications of enzymes (David F.V. Lewis, 2001). It is well documented the association between nuclear factors e.g. Ah receptor nuclear translocator (ARNT), hepatic nuclear factor (HNF-4), constitutive androstane receptor (CAR), pregnane X receptor (PXR), with P450 gene expression and induction (David F.V. Lewis, 2001) and are subject to considerable interindividual variation (see Figure 9). These respective nuclear factors govern the expression of *CYP1A*, *CYP1B*, *CYP2A*, *CYP2B*, *CYP3A* etc. and are controlled by miRNAs, they influence both the CYP450 expression levels including the metabolism of xenobiotic drug substrates of these enzymes (David F.V. Lewis, 2001; Zanger & Schwab, 2013). A study examining P450 down-regulation by Morgan (2001) questions whether variation caused by

epigenetic factors are simply a homeostatic mechanism (e.g. from functional requirements) or a pathophysiological phenomenon (consequence of inflammatory processes), and answers to this might provide the following understanding: which stimuli regulates a particular P450 isoform, how to predict the effects of infection or inflammation on drug metabolism, how to predict the effects of other diseases, drugs or toxins that activate similar pathways on drug clearance or toxicity (Morgan, 2001).

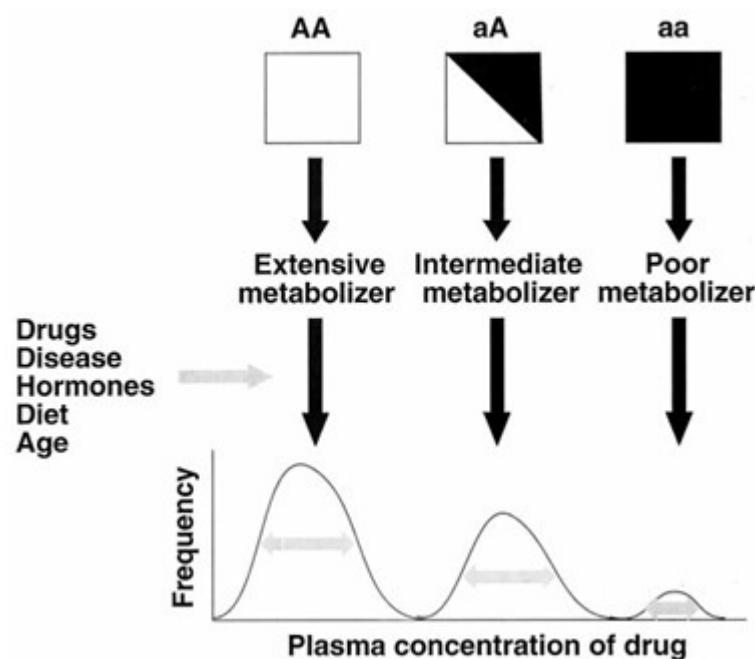


Figure 9 Relationship between the interactions of genetic or epigenetic factors and resulting activities of DMEs in A – wild type allele and a – mutant (deficient) allele. The variance influenced by epigenetic factors are conveyed by the grey arrows (Morgan, 2001).

1.3.2.4 ADME: Drug Excretion and clinical implications

The removal of drugs and its metabolites from the body is referred to as excretion, which includes toxic metabolites, xenobiotic metabolites and metabolite wastes (Greener, 2009; Masereeuw & Russel, 2001). Drug excretion is frequently used interchangeably with the elimination of the active drug but have different meanings; excretion is the irreversible loss of a chemically unchanged compound whereas elimination is the irreversible loss of drug from the site of measurement (namely excretion plus metabolism) (Doogue & Polasek, 2013). The kidney is the main excretory organ expelling drugs in the urine and bile respectively, though other routes of excretion include expired air, sweat, saliva, faeces and milk (Gerald M. Cohen et al., 2020; Greener, 2009). Drug transporters (see Figure 10A) are central to determining the excretion of certain drugs which is carried out predominantly via active transport (carrier-mediated transport of xenobiotics) (Masereeuw & Russel, 2001). This efficient means of transport characterises (see Figure 10B) a high clearance capacity for a diverse number of substances (Ayrton & Morgan, 2001; Masereeuw & Russel, 2001). Thus, indicating the involvement of multiple transporters with a wide substrate specificity (Masereeuw & Russel, 2001).

However, xenobiotics achieve high concentrations within the proximal tubular cell because of active transport (Masereeuw & Russel, 2001). Some drug metabolites (e.g. anionic intermediates – glutathione S conjugates of polyhaloalkanes or hydroquinones, cationic drugs like cisplatin and *para*-aminophenol) may cause the development of nephrotoxicity (Masereeuw & Russel, 2001). Nephrotoxicity is defined as a rapid deterioration in kidney function due to a toxic effect from medications and chemicals, which can have a profound impact on patients already suffering from kidney failure (Al-Naimi et al., 2019). Hence, the

overall mechanisms that regulate the renal excretion of drugs and its clinical implications are closely monitored.

Renal excretion of drugs involves the following processes; filtration by the renal glomerulus, passive absorption in the tubules, and active secretion by the tubules (Greener, 2009). Even though all drugs are filtered by the glomerulus, reabsorption of some drugs (depending on their physiochemical properties) happen in the tubule with the unbound fraction passing into the urine (Greener, 2009). The composition of the urine then becomes a reflection of renal function, and a quantitative method is used to assess the functionality of the kidney by determining the renal clearance of various compounds (Masereeuw & Russel, 2001). Renal clearance is a key PK parameter in the determination of drug elimination and effects, and its pharmacokinetic value reflects the proportion of renal plasma flow that is completely cleared of a compound per minute (e.g. units in mL/min) (Masereeuw & Russel, 2001).

1.3.2.4.1 Significance of the interplay relationship between drug distribution and metabolism

Drug elimination is a complex process of drug influx into an organ and efflux into the systemic circulation involving a combination of cellular metabolism and excretion (Pang et al., 2009). This highlights the 'interplay' between enzymes and transporters as an essential component of drug removal in eliminating organs or tissues (Pang et al., 2009). Historically, a drug exhibiting poor oral bioavailability was attributed to either poor physiochemical properties or extensive first-pass hepatic metabolism (Benet, 2009; Benet et al., 2004). Instead a drug transporter and metabolism alliance, working co-ordinately as a protective mechanism could account for the poor bioavailability observed in certain drugs (Benet et al., 2004).

In 2009, Benet demonstrated the complementary roles and interactions between CYP3A and P-glycoprotein in the intestine, suggesting that a drug can be absorbed by a passive process into the enterocyte and then be metabolised by the enzyme (see Figure 10C). It is understood that basolateral influx and efflux transporters modulate the extent of elimination by regulating drug accumulation in the cell (Pang et al., 2009). The resulting outcome is dependent on the interaction between the drug, and proteins for binding, transport and metabolism (Pang et al., 2009).

With this knowledge there is a paradigm shift in the way drug interactions and absorption problems are considered (Benet et al., 2004). The widely accepted 'well-stirred' hepatic clearance model was developed to mechanistically explained the relationship between the intrinsic capacity of the liver to eliminate drugs (intrinsic clearance) and the actual capacity of the liver to eliminate drugs (hepatic clearance) (Endres et al., 2009). This may need to be modified to take into consideration the role of transporters in the hepatic clearance of drugs (Endres et al., 2009).

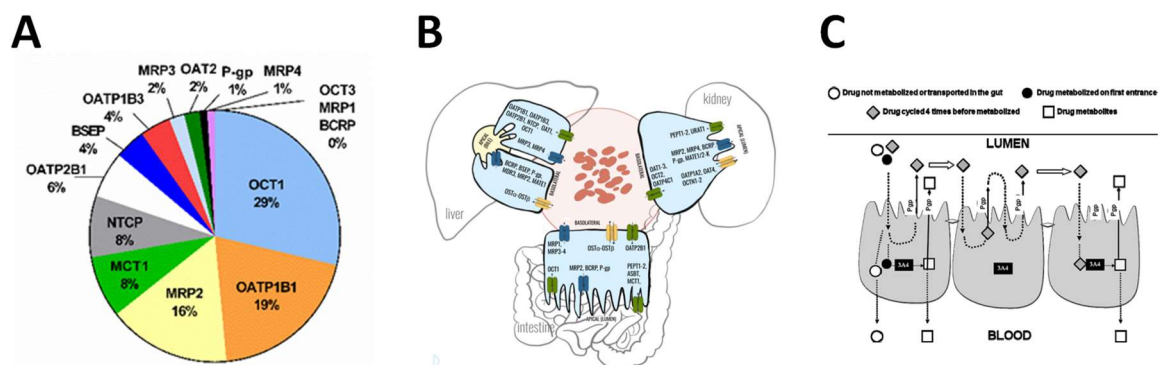


Figure 10 Drug transporters: **A.** the pie chart of the individual drug transporter proteins in control human livers **B.** major membrane drug transporters expressed in hepatocytes, enterocytes and proximal tubule kidney cells **C.** Schematic illustration describing CYP3A4 and P-glycoprotein interplay in the enterocytes. (Benet, 2009; Drożdżik et al., 2020, 2022)

1.3.2.5 Predicting hepatic clearance

One of the most important factors that drives the overall kinetics of chemicals or substances used across processed products today, is the hepatic metabolic clearance (Gouliarmou et al., 2018). Making any information on hepatic metabolic clearance indispensable for the safety assessment of xenobiotics (Gouliarmou et al., 2018). Methods were first described 25 years ago for predicting *in vivo* drug clearance from *in vitro* data (Howgate et al., 2006). The need to understand and predict drug clearance in humans enabled various methods utilizing preclinical animal *in vivo* data and human *in vitro* data to be implemented and refined (Ito & Houston, 2005; Poulin et al., 2012). Clearance (CL) is defined as the volume of drug cleared in plasma per unit of time, and while CL is constant, the rate of drug elimination is concentration-dependent (Smith et al., 2019). This corresponds to the typical exponential (log-linear) decline in drug concentration observed with time (Smith et al., 2019). Hence, CL data can then be incorporated in physiologically based pharmacokinetic models (PBPK) to perform *in vitro* - *in vivo* extrapolation (IVIVE) in support of risk assessments (Gouliarmou et al., 2018). Predicting human PK, therefore has become an invaluable component of assessing a drug candidate's probability of success in the preclinical evaluation stage (Chiba et al., 2009). This has encouraged *in vitro* studies to be widely and routinely used and contributing to the reduction of the attrition rate in human PK during clinical development (Chiba et al., 2009).

1.3.2.5.1 *In Vitro* methods

In vitro methods are biologically based test systems comprised of subcellular fractions (e.g. liver microsomes or S9), or intact cells (e.g. immortalised cell lines or primary hepatocytes) (Gouliarmou et al., 2018). *In vitro* approaches are most convenient as they require a minimal

amount of compound and do not need animal studies (Poulin et al., 2012). Microsomes are the most preferred testing systems over hepatocytes, as they are more readily available (Jia & Liu, 2007). However, microsomes lack representation of the endogenous physiological environment and are artefacts from a subcellular fraction preparation (see Figure 11) (Jia & Liu, 2007; Smith et al., 2019). They require co-factors for CYP-mediated reactions and do not contain phase II enzymes (Jia & Liu, 2007). As a result, hepatocytes have advantages over microsomes, but generally, *in-vivo* results should always take precedence over *in vitro* data (Jia & Liu, 2007). *In vitro* hepatic metabolic methods are specifically designed to estimate the intrinsic clearance (CL_{int}) resulting from the metabolism of the substance under study, or in drug metabolism studies, CL_{int} is obtained as an estimate of metabolic stability of tested NCEs (Gouliarmou et al., 2018; Soars et al., 2002).

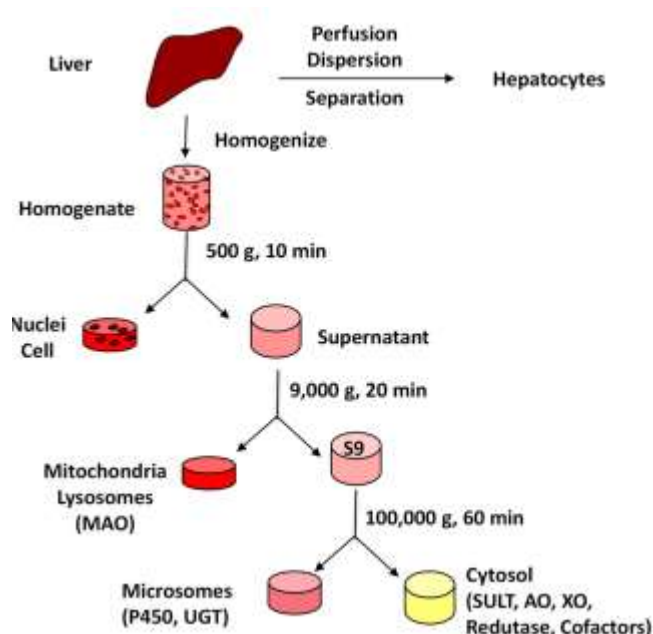


Figure 11 Preparation of hepatic subcellular fractions (Smith et al., 2019).

1.3.2.6 Enzyme kinetics

In order to understand the kinetics of enzyme activity, the basic principles of the nature of enzymes must be realised. Firstly, enzymes are potent catalysts; they have an association with protein molecules; the enzyme activity requires a co-factor or a co-enzyme to function; it engages with a substrate to form a product; they can speed up reactions at low concentrations; and their catalytic activity is expressed by the constant k_{cat} (referred to as the turnover rate) which represents the number of substrate molecules that can be converted by a single enzyme per unit of time (Robinson, 2015). Structurally, enzymes can be arranged with several polypeptide chains that consist of an area known as the active site where the substrate would bind (Robinson, 2015). This led to a theory known as Fischer's 'lock and key hypothesis' meaning that only a key of the correct shape (the substrate) would fit in the keyhole (the active site) of the lock (the enzyme) (Robinson, 2015).

Enzyme-catalysed reactions are described using the following equation [6] and shows the conversion of a S substrate to a P product by the enzyme E using an ES enzyme-substrate complex intermediate, and k_{cat} which is the first order rate constant of the reaction (Nath & Atkins, 2006). Thus, enzyme kinetics is the study of factors that determines the speed or rate of these enzyme-catalysed based reactions (Robinson, 2015).

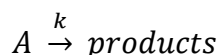
[6]



Moreover, such enzyme-catalysed reactions follow the basic principles of chemical kinetics (non-enzyme reactions). A reaction is classified according to its *order* to describe its kinetics

and it is defined by the number of concentration terms multiplied together to get an expression for the rate of reaction (Athel Cornish-Bowden, 2013). In a *first-order* reaction, the rate of a reaction is proportional to the concentration of the reactant (A) as shown in equations [7] and [8].

[7]

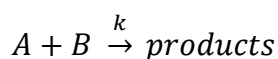


[8]

$$v = -\frac{dA}{dt} = kA$$

The first-order rate constant k , is usually expressed as a function of time (t) and is determined by measuring the initial velocities (v or v_0) of the reaction at different concentration of A and by plotting the velocities versus A to then generate the slope, k (Paul F. Cook & W. W. Cleland, 2007). When two molecules react together, the subsequent rate is proportional to the concentration of both (Paul F. Cook & W. W. Cleland, 2007). This is referred to as the *second-order* reaction [9] [10] [11] and its rate constant k , is expressed as a function of concentration per time.

[9]



[10]

$$v = -\frac{dA}{dt} = -\frac{dB}{dt} = kAB$$

$$v = -\frac{dA}{dt} = kA^2$$

Methods used to observe enzyme activity usually involve measuring the appearance of product over time and typically reflects *first-order* kinetics (see Figure 12A), though the rate of reaction can also be determined by measuring the disappearance of substrate over time (Nath & Atkins, 2006; Robinson, 2015). The relationship between enzyme concentration and the rate of reaction is monitored over a series of studies that specifies the initial rate of reaction (the linear region of a curve plot as shown in Figure 12B). This is indicative of the interval where the enzyme is most active. Therefore, changes in the initial velocity are evaluated when either the substrate or enzyme concentration is altered (Robinson, 2015).

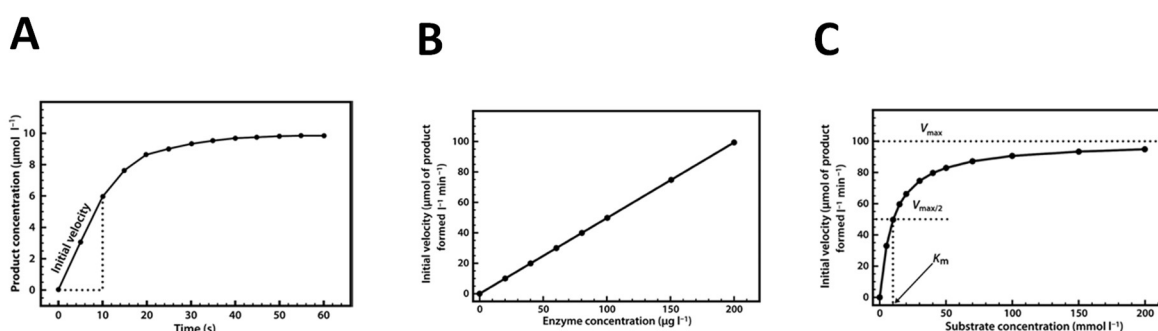


Figure 12 Relationship between reaction rate and concentration: **A.** formation of product in an enzyme-catalysed reaction versus time **B.** relationship between enzyme concentration and the rate of an enzyme-catalysed reaction **C.** Relationship between substrate concentration and the rate of enzyme-catalysed reaction (Robinson, 2015)

It is important to consider that there is a stage where increasing the substrate concentration has little or no further effect on the reaction rate and this is known as substrate saturation (Robinson, 2015). Such a profile demonstrates the enzyme has reached its maximum velocity, V_{max} (see Figure 12C) and the relationship is described by the Michalis Menten equation (a model of enzyme kinetics) [12] (Pant, 2022; Robinson, 2015).

[12]

$$v_0 = \frac{V_{max} \times \text{Substrate concentration}}{\text{Substrate concentration} + k_m}$$

k_m is also known as a Michalis constant, and can be defined as the substrate concentration at which half of the maximum velocity is achieved (Gokulnath & Raveendran, 2023; Pant, 2022). Thus, it becomes a measure of the affinity an enzyme has for its substrate for example, the lower the value of k_m , the more efficient the enzyme is at accomplishing its function at a lower substrate concentration (Pant, 2022; Surti, 2023). Therefore, understanding the mechanisms of enzyme activity can provide insight into the way metabolic enzymes would function in an *in vitro* study. Hence, the Michalis Menten equation can be incorporated to methods predicting *in vivo* metabolic clearance from *in vitro* data. The relationship between the initial metabolic rate (v_0) and the unbound drug concentration (C_u) can be expressed as follows [13] (Ito et al., 1998):

[13]

$$v_0 = \frac{V_{max} \times C_u}{k_m + C_u}$$

From this, the equation thereby assumes that the reaction is in the steady state where the ES concentration remains constant (Surti, 2023). Hence, CL_{int} can be defined as the proportional constant between v_0 and C_u as described by equation [14] (Ito et al., 1998):

[14]

$$CL_{int} = \frac{v_0}{C_u} = \frac{V_{max}}{k_m + C_u}$$

Under linear conditions and when the drug concentration is much smaller than the k_m , the metabolic rate can be expressed by equation [15] indicating that the rate is proportional to the drug concentration and confirming that CL_{int} is expressed as V_{max}/k_m (Ito et al., 1998).

[15]

$$v_0 = \frac{V_{max}}{k_m} \times C_u = CL_{int} \times C_u$$

A Lineweaver–Burk plot is another useful representation of the Michalis Menten kinetics by plotting the inverse of the reaction rate ($1/r$) against the inverse of the substrate concentration ($1/[S]$) (Surti, 2023). This produces a straight line and enables various quantities and values to be easily interpreted (Surti, 2023). The Lineweaver–Burk plot is commonly used when determining the presence of enzyme inhibition (see Figure 13) and its effects on V_{max} and k_m (Surti, 2023).

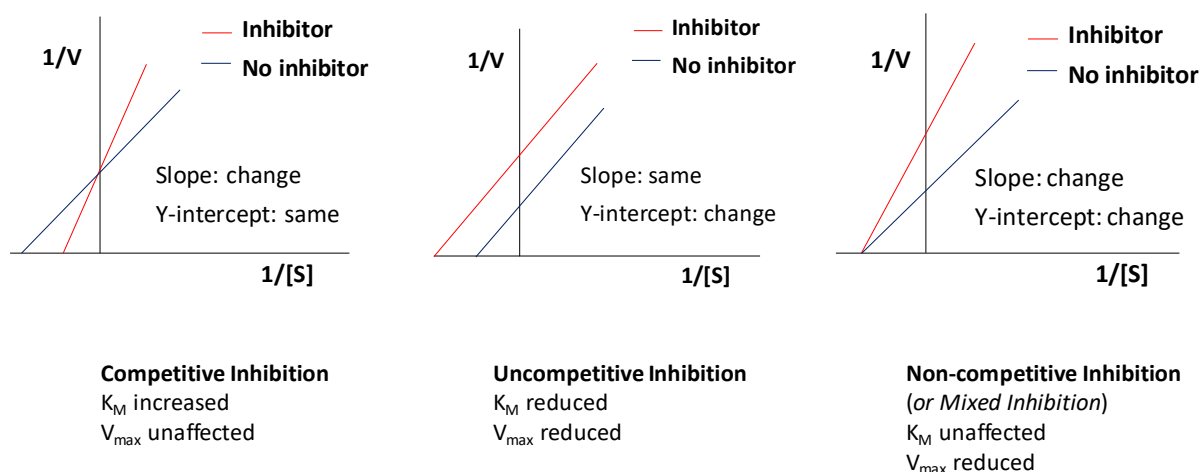


Figure 13 A Lineweaver–Burk plot depicting effects of enzyme inhibition relating to substrate concentration in competitive, uncompetitive and non/ mixed competitive modes of inhibition. Illustration adapted and sourced from Surti, 2023.

1.3.2.7 Generating *in vitro* clearance, CL_{int} : Substrate depletion approach

The determination of CL_{int} is calculated by the activity of enzymes metabolising a substance based on either the disappearance/depletion of the parent compound or the formation of a metabolite over time (Gouliarmou et al., 2018). The substrate depletion approach depicts the disappearance of a compound by metabolism in an *in vitro* incubation completed at a set enzyme-substrate concentration (Smith et al., 2019). The disappearance will follow a first-order process when the substrate concentration is below the k_m (Michalis constant) for the metabolic enzyme (Smith et al., 2019). A plot of the substrate concentration versus time will convey an exponential decline and produce a straight line using a log-linear scale (Smith et al., 2019). The slope of this line relates to the V_{max}/K_m of the molecule, which can be transformed into the apparent intrinsic clearance ($CL_{int\ app}$) of the molecule by considering the protein concentration (microsomes) or number of hepatocyte cells in the incubation as described in equation [16] (Smith et al., 2019).

[16]

$$CL_{int\ app} = \frac{0.693}{t_{1/2}} \times \frac{mL\ incubation}{cell\ number\ or\ [protein]}$$

The calculated $CL_{int\ app}$ is subsequently converted to *in vivo* hepatic metabolic clearance (CL_H) as shown in Figure 14, by taking into account the liver blood flow (Q) and the unbound fraction of the substance in plasma (f_u) (Gouliarmou et al., 2018). This is achieved using the commonly used mathematical model known as the ‘well-stirred’ model of drug clearance and is described by the equation [17] (Smith et al., 2019; Soars et al., 2002).

[17]

$$CL_H = \frac{Q \times f_u \times CL_{int}}{Q + f_u \times CL_{int}}$$

True apparent intrinsic clearance ($CL_{int\ app}$) reflects the outcome of metabolite formation. Since the process of metabolism is a chemical reaction, therefore the resulting product formed is a true indication that the reaction has happened (Z. Zhang & Tang, 2018). Evidence of metabolism can be directly linked to the appearance of metabolites and should provide the level of confidence in using $CL_{int\ app}$ measurements in drug metabolism studies.

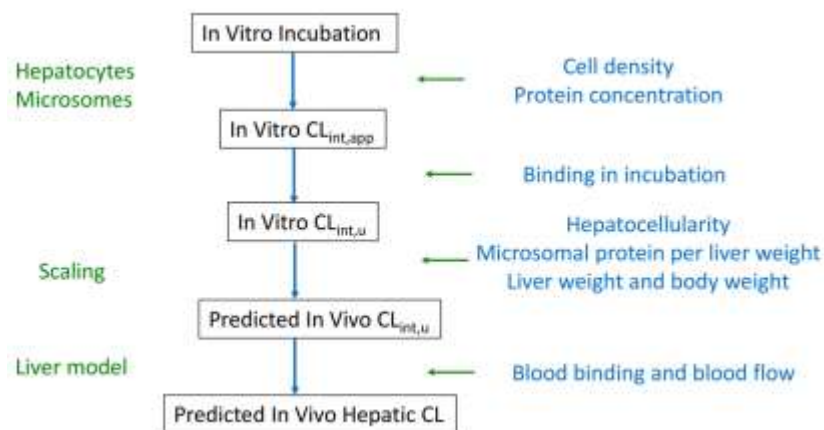


Figure 14 Scaling human or animal *in vitro* clearance data to human *in vivo* hepatic clearance (Smith et al., 2019).

1.4 Aims and Objectives

In vitro experimental assessments in drug metabolism studies serve as key indicators of ADME fate of drugs *in vivo*. The understanding that metabolic enzymes involved in the metabolism of drugs can alter the required drug dose at the site of action key for determining the desired immune therapeutic response. There are pressing questions to be answered in the expectation the goal of this study will bring some clarity which includes (i) whether the spleen would metabolise similar drugs as in the liver, (ii) confirm whether CYP450s or UGTs are located in splenic microsomes, (iii) whether CYP450s and UGT are substrate-specific in the spleen and lastly, (iv) whether a similar metabolite profile would be observed in the spleen compared to the liver tissue. This research aims to investigate the drug disposition process (mainly metabolism and distribution) of therapeutic drugs in the spleen.

The following objectives are set to fulfil this aim:

- To discover the expression of metabolic enzymes and transporters in the spleen.
- To characterise their expression in the spleen compared with the liver tissue (which will act as the control in this study).
- To assess and evaluate their activities when engaged with therapeutic drugs.
- To use rat and pig animal models due to their physiological and genetic similarity to humans.

CHAPTER 2:

General Methods and Materials

2.0 Methods and Materials

This section describes the process of establishing the expression of CYP450 enzymes, UGT enzymes and transporter proteins at genetic, protein and functional level in the spleen compared to the liver. Rat and Pig models were used for methods assessing genetic and protein expression of the metabolic enzymes. The pig model was used as a benchmark to demonstrate the activity of the CYP450 and UGT enzymes in the spleen. Techniques used to obtain such information include Next Generation Sequencing (NGS), Standard PCR and qPCR, Western Blot (WB), Immunohistochemistry and Immunofluorescence staining, and Substrate Depletion Assay (to determine the drug elimination rate).

2.1 Assessing the genetic expression of selected phase I and II enzymes including transporter proteins in the spleen that are associated with drug metabolism and distribution.

2.1.1 Rat and Pig (liver and spleen) tissues

Liver and spleen tissues were harvested from male Sprague Dawley rats and female pig sows (landrace x large White x Duroc) post-mortem, in accordance with the procedures approved in schedule 1 of the Animal Welfare Act 2006. The tissues were obtained from Bio IVT UK and the abattoir at both RB Elliot & Son UK and University of Nottingham (UoN), Sutton Bonington campus respectively and immediately stored in -80° C.

2.1.2 Isolation of total RNA and cDNA synthesis

Total RNA was extracted from rat and pig (liver and spleen) tissues using a Nucleospin RNA Mini kit (Macherey-Nagel, UK) according to manufacturer's instructions. Pipettes and new tip boxes were decontaminated using RNAase Zap solution (Thermo Scientific, UK) and placed under UV light for 20 min in a PCR cabinet prior to performing the isolation process for preventing DNA contamination. The prepared total RNA was measured using a Nanodrop spectrophotometer (Thermo Scientific, UK) and then reverse transcribed into cDNA using the Invitrogen Superscript III Reverse Transcriptase kit (Sigma Aldrich, UK). The remaining total RNA and the prepared cDNA samples were stored at -80°C and -20°C respectively for downstream (mRNA) analysis (see Figure 15).

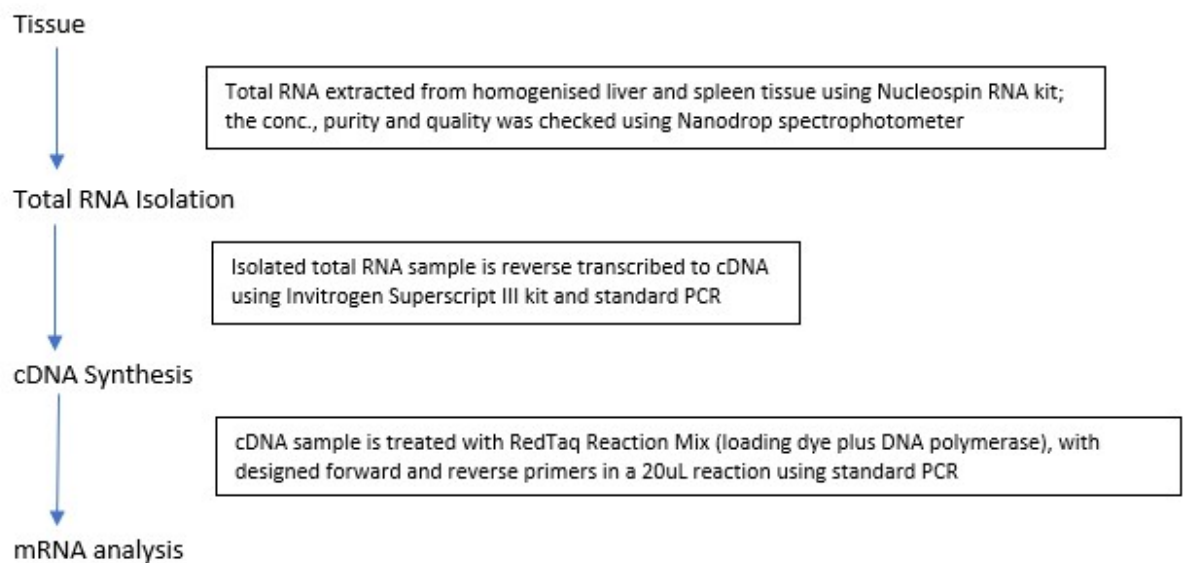


Figure. 15 Schematic flowchart of total isolation RNA and synthesised cDNA from tissues

2.1.3 Assessment of purity and quantitation of prepared total RNA and cDNA

Using the RNA-40 option on the Nanodrop software, 2 μL aliquot of the total RNA or cDNA sample was transferred to the Nanodrop spectrophotometer (Thermo Scientific, UK). The 'measure' button was selected after setting the blank for the instrument with RNase-free water or DPEC water (Thermo Scientific, UK). Using 2 μL of the RNA or cDNA sample in triplicate for quantitation. RNA yield should be above or equal to 100 ng/ μL (see Table 3 and 4). To assess the purity of the total RNA and cDNA samples, the ratio of sample absorbance between 260 and 280 nm wavelength, together with 260 and 230 nm wavelength was monitored. Generally, a ratio of approx. 1.8 is accepted as 'pure' for DNA and a ratio of approx. 2.0 is accepted as 'pure' for RNA (William W. Wilfinger et al., 1997).

Table 3 Quantified total RNA and cDNA concentration extracted from measured liver and spleen tissues using the Nanodrop spectrophotometer.

Tissue	n (n=3)	Weight (mg)	Extracted total RNA conc. (ng/ μL)	Ratio at A260/280 nm	Transcribed cDNA conc. (ng/ μL)	Ratio at A260/280 nm
Rat Liver	1	35	1644	2.07	1563	1.79
	2	34	1876	2.04	1796	1.77
	3	34	2384	1.95	1885	1.79
Rat Spleen	1	36	298	2.08	1422	1.79
	2	44	108	2.05	1430	1.76
	3	56	195	2.12	1433	1.78
Pig Liver	1	47	767	2.04	765	1.80
	2	37	810	2.15	1486	1.76
	3	31	1522	2.06	1395	1.76

Table 4 Quantified total RNA and cDNA concentration extracted from measured primary liver hepatocytes and splenocytes cells using the Nanodrop spectrophotometer.

Cells	n (n=4)	Extracted total RNA conc. (ng/μL)	Ratio at A260/280 nm	Transcribed cDNA conc. (ng/μL)	Ratio at A260/280 nm
Pig Hepatocytes	1	68.1	2.19	2519	1.73
	2	64.6	2.17	2608	1.74
	3	66.6	2.18	2542	1.75
	4	73.0	2.19	1647	1.79
Viability	84 %				
Yield (live)	2.38 x 10 ⁶				
Pig Splenocytes	1	14.2	2.26	1706	1.80
	2	25.7	2.41	1561	1.80
	3	27.8	2.47	1480	1.80
	4	83.9	2.26	1413	1.78
Viability	89.5 %				
Yield (live)	7.63 x 10 ⁶				

2.1.4 The design of primers for use in PCR analysis

2.1.4.1 Obtaining FASTA mRNA sequences of selected genes

Selected CYP450 genes mRNA sequences in rat and pig, were found on the NCBI (national centre for biotechnology information) database to obtain its FASTA (nucleotide) sequences using the URL <https://www.ncbi.nlm.nih.gov/>. The mRNA sequences were submitted to the 'pick primers' option on the software to select appropriate parameters (Table 5) for the design of optimum primers as shown in the appendix (Figure. A1 A and B). These must be on the intended target with a low number of consecutive Gs and Cs at the 3' end of both left and

right primers, and low self-complementarity including the maximum length of a mononucleotide repeat (Borah, 2011). Finally, a no cross-reactivity with other genes was ensured by designing primers aimed to be specific for the desired gene (Borah, 2011).

Table 5 The selected primer parameters for optimum primer design

Primer Design Parameters	
Optimal product size length	200 - 1000 bp (standard PCR) or 75 - 150 bp (real-time PCR)
Primer sequence length	20 bp (base pairs)
Coding or Non-coding region	Exon - Exon (coding)
Species / Organism	Rat or Pig
GC content (%)	40 -60
GC clamp	2
Max Poly X	5
T _m (° C)	55 - 60

2.1.4.2 Assessing the specificity of mRNA sequences

Using a BLAST algorithm, the designed primers (Table 6) went through a ‘primer-blast’ process on the software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the sequences specificity for the gene of interest as indicated in the appendix (Figure. A1 C). The key values are shown with close attention to the percentage identity and the E value. The former refers to the similarity of the target sequences to that of the library sequences on the database; and the latter should give a low number as possible to describe that the likeness of the sequence is not by chance.

Chapter 2: Methods & Materials

Table 6 Designed Rat and Pig primers of selected CYP450 genes including control (housekeeping) genes.

Rat genes	Primer sequence (5' > 3')		Product length (bp)
	Forward	Reverse	
CYP1A2	GACAACGGTGGTCTCATCCC	TGTCCCTCGTTGTGCTGTGG	292
CYP2B1	GCCCTTCTCCACAGGAAAGC	GGATCACCGAGCTGAGAAGC	199
CYP2B2	CATGGATGAGAGAGGAGAAGTCG	GCCTGTGAGAGCCAATCACC	201
CYP2C6	GTCCTGGACACAGTTTTGC	GTTGTGCTTGTGTCTCTGTCC	286
CYP2C7	GTGTGATTGGCAGACATCGC	TTGTTCCCTTCGGGATGAGG	171
CYP2C11	AAAGACAGGAGCCAGATGCC	TCCTCTTCTCTGCTGAGAATGG	283
CYP2C12	CTACTTCATACCCAAGGGAACAGC	CCCTCTCCACACATTTCGG	183
CYP2C13	ACTTCATACCGAAGGGAACAGC	GGCTCTCTCCCAAACACATCC	183
2D1	TCCAAGGTGTGATCCTTGC	GCAGTGAAGGCATCACAGAGG	152
CYP2E1	TTCACCAAGTTGGCAAAGCG	AAACCTCCGCACATCCTTCC	216
CYP3A2	AGCACCGAGTGGATTTTCTCC	TAGGTAGGAGGTGCCTTACTCG	247
GAPDH	TGCTGGTGCTGAGTATGTCG	CTGTGGTCATGAGCCCTTCC	266
B-actin	CAGCCTTCCTTCTGGGTATG	AGGGTGTAACGCAGCTCA	374

Pig genes	Primer sequence (5' > 3')		Product length (bp)
	Forward	Reverse	
CYP1A2	CACAAGCGCCCTATTCAAGC	ACTGTGTCCAGCTCCTTCTGG	201
CYP2B22	AGGGCTTTCTCAAGTCCTTCC	GGAAAAGATCACGCCATATCCC	203
CYP2C33	TACCCAGGGAGGATATGGACTCG	CCACCAGAACTGGGCTTCC	146
CYP2C42	TTCTCCCTCATGACCTTGCG	ATCACAGGGTGAGGCATTGG	120
CYP2C49	CTTGCATGCAGGACAGAAGC	TTGTGCCCTTGGGGATGAGG	147
2D25	CATGATCCTGCACCCAGACG	GTGATAAGTGTGGTCCCCTTGG	231
CYP2E1	TTCACTGTGTACCTGGGTTCG	AATGACCCCTTGTCTTGTGC	144
CYP3A22	GCAAAGAGCAGCACACACG	AAATGCCACATGCCCTTACG	242
CYP3A29	CACAAACCGGAGGTCTTTTGG	ATCACGTCCATGCTGTAGGC	246
GAPDH	TCGGAGTGAACGGATTTGGC	TGACAAGCTTCCCGTTCTCC	189
Villin	TGAGCGCCCAAGTCAAAGG	GCGTCAGACACATGGTACAG	772
HPRT	CCCAGCGTCGTGATTAGTGA	CCACCCAAAGGGAACCGA	861

2.1.5 Single-cell suspension preparation and isolation of primary liver and spleen cells

2.1.5.1 Prepared reagents

1. KCl buffer: 0.5 g KCl was added to 10 mL 1 M HEPES and vortex mix until dissolved
2. Perfusion buffer #1: 2.5 mL 1 M glucose solution, 0.25 mL 0.5 M EDTA and 5 mL KCL buffer were added to 1x phosphate buffered saline (PBS) made up to 500 mL. The solution was mixed, pH adjusted to pH 7.4 with sodium hydroxide (NaOH) and filtered sterilised using a 500 mL filter system. Chemicals purchased from Fisher Scientific and Sigma Aldrich, UK.
3. Perfusion buffer #2: 2.5 mL 1 M glucose solution, 10 mL 1 M HEPES, 1 mL 0.5 M CaCl_2 and 5 mL KCL buffer were added to 1x phosphate buffered saline (PBS) made up to 500 mL. The solution was mixed, pH adjusted to pH 7.4 with sodium hydroxide (NaOH) and filtered sterilised using a 500 mL filter system. Chemicals purchased from Fisher Scientific and Sigma Aldrich, UK.
4. Collagenase solution: weigh 45 mg of Collagenase Type I to 100 mL pre-warmed (37 °C) perfusion buffer #2. Chemical purchased from Sigma Aldrich, UK.

2.1.5.2 Isolation of primary cells from liver and spleen tissue

This methodology was modified as described in the study from Mendoza et al., 2022. Two samples of 5 - 8 g (cut in small pieces) of pig liver and spleen solid fresh tissues were soaked for 30 min in 30 mL pre-warmed (37 °C) perfusion buffer #1 at RT for the initial dissociation and to wash off blood residues from the fresh tissue. The collagenase solution was prepared, and 10 mL added to 30 mL pre-warmed (37 °C) perfusion buffer #2 to make a 25 % v/v strength buffer. The tissue samples were immediately transferred to 30 mL perfusion buffer #2 and

soaked for additional 30 min at RT. The tissue samples were minced and crushed using a piston/ flat end of plunger to release the cells in a petri dish and filtered using a 70 or 100 μm cell strainer (Greiner Bio-One, UK) before incubated on rotation at RT for 30 min. After centrifugation (ThermoFisher Scientific, UK) at 300 $\times g$ for 10 mins, the supernatant was discarded, and the pellet was re-suspended in culture media or PBS ready for use within 1 hr of harvesting or stored by cryopreservation.

2.1.5.3 Cryopreservation of primary cells

Freezing media (10 % v/v DMSO, 50 % v/v FBS, UK, 40 % v/v culture media) was prepared and pre- warmed at 37 °C. All the chemicals were purchased from Sigma Aldrich, UK and Gibco, UK respectively. The centrifuged cell pellet was re-suspended in 20 mL freezing media and 2 mL aliquots were transferred to 2.5 mL cryogenic vials or tubes (Fisher Scientific, UK). The primary cells contained in the cryogenic vials or tubes (Fisher Scientific, UK) were placed overnight in a Mr. Frosty™ freezing container (Thermo Scientific, UK) with a small volume of isopropanol (Fisher Scientific, UK) to control the freezing temperature of the cells and prevent formation of ice crystals. The primary cells were moved to a liquid nitrogen dewar for long term storage. Alternatively, the primary cell pellets can be re-suspended in Bambanker™ Cell Freezing Media (Geneflow, UK) and stored long term in -80 °C freezer without impacting cell viability.

2.1.5.4 Thawing of cryopreserved primary cells

The primary cells in cryogenic vials or tubes (Fisher Scientific, UK) were placed in a 37 °C water bath (Clifton, UK) to thaw for up to 2 min, and immediately transferred into a 50 mL falcon tube (Fisher Scientific, UK) containing pre-warmed (37 °C) culture media or 1x PBS (Thermo Scientific, UK), with the contents gently inverted up and down to mix. The cell solution was centrifuged (Thermo Scientific, UK) at 350 xg for 10 mins and re-suspended in 2 mL PBS (Thermo Scientific, UK) ready for cell counting and dilution for immediate analysis.

2.1.5.5 Determination of primary cells yield and viability

A Luna-II automated cell counter (Logos Biosystems, UK) was used to measure the primary cells yield and to determine its viability in accordance with the Trypan Blue (Sigma Aldrich, UK) exclusion test method. At first instance, the count protocol settings were set as follows: Dilution factor (with Trypan Blue) is 2, Noise reduction is 5, Live cell sensitivity is 8, Roundness is 60 %, Min. and Max. cell size (μm) is 3 and 60 respectively, and declustering level is medium. 100 μL of the primary cell sample was mixed with 0.4 % Trypan Blue stain at 1:1 ratio. 10 μL of the mixture was loaded onto the chamber of the Luna cell counting slide (Logos Biosystems, UK) and inserted into the port of the cell counter. The cells were previewed and magnified to enlarge the images. The Count option was selected to start cell counting. The results displayed the total, live and dead cell count to provide its yield and the calculated cell viability.

2.1.6 Standard PCR and real-time quantitative PCR analysis (RT-qPCR)

2.1.6.1 Standard PCR

The selected CYP450 genes of interest were amplified using standard PCR and the DNA products were resolved using agarose gel electrophoresis for separation based on size and charge. Visualised bands on the agarose gel determines gene expression of the amplified CYP450 gene (Mitchell & Iadarola, 2010). A 20 μ L PCR reaction was carried out that comprised of the following, 1 μ L of both forward and reverse primers, 10 μ L of RedTaq mix (containing a loading dye and DNA *Taq* polymerase purchased from Sigma Aldrich, UK), 2 μ L of cDNA sample (from liver and spleen tissues in rat and pig), and 6 μ L of RNase-free water (Thermo Scientific, UK). The optimised PCR conditions were set as follows in Table. 7:

Table. 7 Cycle program for standard PCR

Step	Temperature	Time	Cycle
Polymerase activation	94 °C	2 min	1
Denaturation	94 °C	1 min	45
Annealing	60 °C	50 sec	
Extension	72 °C	1 min	

2.1.6.1.1 Gel electrophoresis of the DNA PCR products

1 g of agarose powder (Fisher Scientific, UK) was dissolved in 100 mL of 1x TAE buffer (containing a mixture of Tris base, acetic acid and EDTA,) using a microwave to make a 1 % w/v gel. The gel was poured into a prepared gel tray (which acts as a casting block with

inserted combs for 15-well size). 3 μ L of Nancy -520 (Sigma Aldrich, UK), a fluorescent stain for double-stranded DNA (dsDNA) was added to the liquid gel until mixed in and allowed the gel to set. 3 – 5 μ L of pre-stained DNA ladder (NEB, UK) or marker was transferred in the first well of the gel and the DNA PCR products were added to subsequent wells. The set gel was placed in a gel tank filled with fresh 1x TAE buffer to the max. fill line and the gel was run using a power bank (Bio-Rad, UK) at 120 V for 20 -30 minutes initially and then the parameters were optimised to 110 V for 40 minutes. At end time, the gel was place in a Bio-Rad ChemiDoc imaging system to visualise any apparent bands. All the chemicals were purchased from Fisher Scientific, UK.

2.1.6.1.2 Data Analysis of Sanger Sequencing results from the DNA PCR products with the designed primers

The DNA PCR products from Rat (liver and spleen) were purified using a Nucleospin PCR-clean up kit (purchased from Macherey-Nagel, UK following the manufacturer's instructions) and was outsourced to Source Bioscience UK, with including 50 μ L of the designed primers for Sanger sequencing analysis. The Sanger sequencing method is used to determine a nucleotide sequence of DNA and the results generated was a four-coloured sequencing chromatogram for each of the CYP450 gene of interest. The quality of the chromatogram would depend on how clean the sample is (the level of background noise), extent of observed polymorphisms, signs of double peaks or negative samples, or loss of resolution later in the gel (Gomes & Korf, 2018). The data was analysed using FinchTV software obtained from the URL <https://digitalworldbiology.com/FinchTV> to examine potential 'errors' that can impact on the quality of the chromatogram, such errors are base spacing, mis-call/ missing nucleotides ('N')

and interpreting gaps/ background noise as real nucleotides (Figure 16). The sequenced reads were Blast searched (basic local alignment search tool) through the NCBI database to validate the specificity of the designed primers for the intended CYP450 genes. To query the database, the acquired CYP450 sequences were compared to every other sequence in the database until top hits are found and the results were reported with key quality metrics.

These are defined as:

Percent Query Coverage is the percent of the query length that is included in the aligned segments.

E-value is the measure of likeliness that sequence similarity is not by random chance.

Percent Identity describes how similar the query is to the aligned sequences.

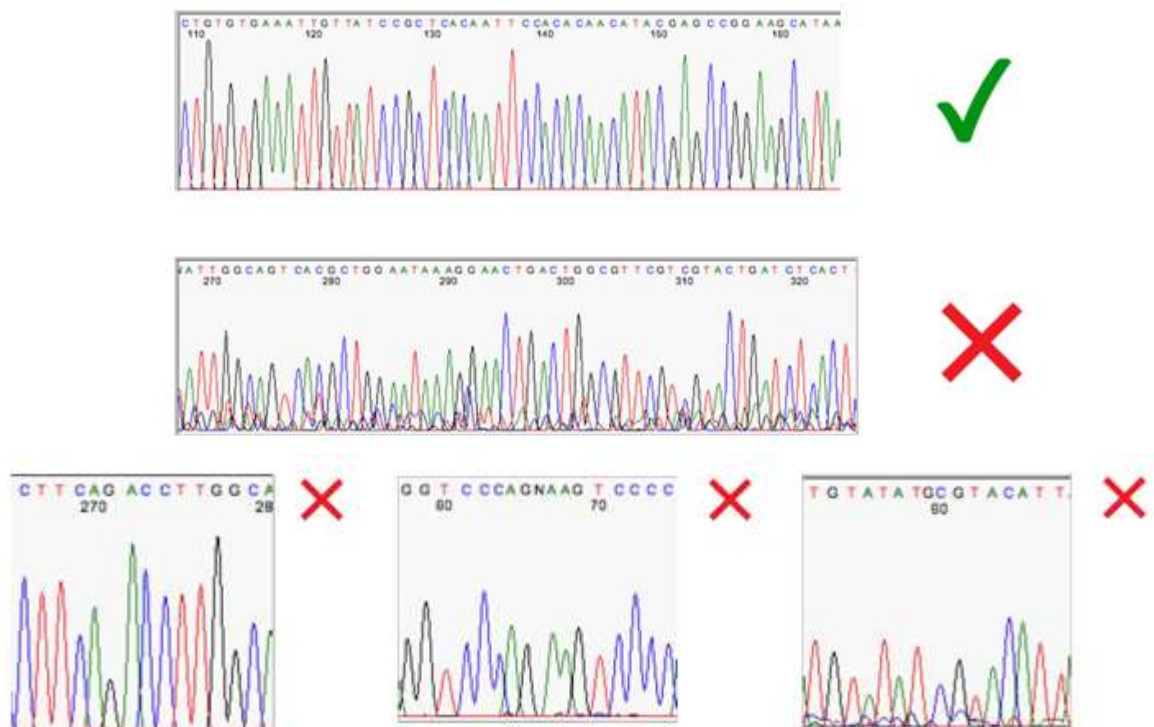


Figure 16 Quality of chromatograms from sanger sequencing data. Data undergo scrutiny of the sequenced chromatograms for potential 'errors' e.g. errors in base spacing, mis-call/ missing nucleotides ('N') and interpreting gaps/ background noise as real nucleotides.

2.1.6.2 RT-qPCR using SYBR Green™ detection method

cDNA template transcribed from RNA of primary pig hepatocytes and splenocytes were used together with the designed primers in a RT-qPCR to confirm the gene expression observed from tissues. The primers of selected genes (*CYP2E1*, *CYP2A19*, *CYP3A29*, *CYP2D25*, *UGT8* and *ABCB1*) including control genes (β -actin and GAPDH) were tested in a RT-qPCR reaction using a 2x QuantiNova Sybr Green PCR Mastermix. In each well of a white light cycler 96-well microplate, the PCR reaction was set up with two technical replicates for each representative gene sample. Briefly, the 20 μ L RT-qPCR mixture contained 5 μ L of cDNA, 1 μ L of 10 μ M forward primer, 1 μ L of 10 μ M reverse primer, 3 μ L of RNase-free water and 10 μ L of SYBR green [™] were mixed and loaded into each well of the 96-well plate. The cycle program for SYBR green [™] RT-qPCR was set as described in Table 8.

Table 8 Cycle program for RT-qPCR

Step		Temperature	Time	Cycle
QuantiNova activation		95 °C	3 min	1
3 step amplification	Denature	95 °C	30 sec	40
	Annealing	55 - 60 °C	30 sec	
	Extension	72 °C	10 sec	
Melting	Denature	95 °C	5 min	1
	Annealing	60 °C	60 sec	
	Extension	97 °C	1 min	
Cooling		40 °C	30 sec	1

2.1.7 Assessment of gene expression using Next Generation Sequencing (NGS)

The NGS analysis used two individual tissue samples from rat and pig (liver and spleen) that was outsourced to Source Bioscience UK, Cambridge. The analysis involved two main steps in the workflow: Sample and Sequencing. The Sample workflow involves quality control and validation of libraries to be sequenced. The Sequencing workflow involves the quantification of gene expression (RNA-seq bioinformatics). The RNA extraction was performed using the Qiagen RNeasy Mini Kit (Source Bioscience UK, Cambridge). The tissue was lysed and then subsequently purified in duplicate. Following extraction, the concentration and integrity of the RNA sample were assessed using Invitrogen Qubit RNA assay (ThermoFisher Scientific, UK) and Agilent BioAnalyzer 2100 (Source Bioscience UK, Cambridge), respectively. For all RNA samples, the RNA integrity number (RIN) was above 8. For the library preparation, 500 ng of total RNA was used. The libraries were prepared using the NEBNext Ultra II Directional RNA Library Preparation Kit (NEB), according to the manufacturer's protocol. During this process, the libraries were indexed using NEBNext Multiplex Oligos for Illumina 96 Unique Dual Index Primer Pairs (NEB). The prepared libraries were quantified via a fluorometric method involving Qubit High Sensitivity (Invitrogen) and qualified using electrophoretic separation on the Bioanalyzer HS Kit (Agilent). The libraries were normalized and pooled before sequencing on the NovaSeq 6000 (Illumina) instrument.

2.2 Assessing the protein expression of selected phase I and II enzymes including transporter proteins in the spleen that are associated with drug metabolism and distribution.

2.2.1 Preparation of protein lysates

Protein lysates from pig liver and spleen tissues was prepared using the TPER Tissue Protein Extraction Reagent (Thermo Scientific, UK) according to the manufacturer's instructions. Briefly, tissues were homogenised with the reagent, 1 g of tissue to 10 mL of T-PER Reagent. After centrifugation (Thermo Scientific, UK) at 10, 000 xg for 5 min, the pellet tissue debris was discarded, and the supernatant was collected. Total protein concentration was determined using the Bradford assay method.

2.2.2 Preparation of liver and spleen homogenates and microsomes

2.2.2.1 Homogenisation of liver and spleen tissues from rat and pig

Approximately, 20 g of liver and 5 g of spleen tissue were thawed on ice and minced in 0.15 M potassium chloride buffer (pH7.4), at a ratio of 10 mL/g of tissue, and homogenised using a Witeg HG-15A set B homogeniser for 2 x 10 s with a 10 s break between. The homogenates can be used immediately or stored in 50 mL falcon tubes (Fisher Scientific, UK) at -80 °C. The chemical was purchased from Sigma Aldrich, UK.

2.2.2.2 Preparation of microsomes

Microsomes were prepared by differential centrifugation following the protocol described by Rasmussen et al., 2011. Liver and spleen homogenates were thawed on ice if frozen and mixed by inverting the 50 mL falcon tube (Fisher Scientific, UK) gently up and down. The homogenate was centrifuged (Beckman Coulter, Avanti J-E series) for 20 min at 10,000 xg, 4 °C. Followed by the ultracentrifugation (Hitachi ultracentrifuge CP80NX) of the supernatant at 105,000 xg for 1 hr at 4 °C. The resultant pellet was suspended in 0.1 M potassium phosphate (Fisher Scientific, UK) buffer, pH 7.4 and ultracentrifuge at 105,000 xg for 1 hr at 4°C. Discarding the supernatant, the microsomal pellet was re-suspended in 0.1 M potassium phosphate (Fisher Scientific, UK) buffer, pH 7.4 (500 µL:1 mL) and stored at -80 °C for later enzymatic assays.

2.2.3 Protein concentration determination

The Bradford assay method is used to determine the protein concentration of the subsequent prepared protein lysates and microsomes extracted from liver and spleen tissues in rat and pig. This procedure involves the binding of Bradford reagent (containing Coomassie Brilliant Blue G-250 dye) to proteins (Kruger, 2009). At first, the dye is in a protonated (cationic) red form, and when bound to protein, it converts to a unprotonated blue form (Kruger, 2009). A standard curve is prepared from a serial dilution of bovine serum albumin (BSA) at 2 mg/mL as described in Table 9. 20 µL of all standards and test or unknown samples were transferred to individual 1.5 mL cuvette in triplicates, and 1 mL of Bradford reagent (Sigma Aldrich, UK) was added to all samples. The protein solutions were measured at 595 nm using a spectrophotometer (Biochrom Libra S22, UK). Either the spectrophotometer is zeroed with a

blank or the blank value is averaged then subtracted from the standards and sample values.

A standard curve plot of the absorbance 595 nm values versus their concentration in mg/mL

is generated to determine the test or unknown sample concentration.

Table 9 Prepared standard curve solutions for protein concentration determination

Tube #	BSA conc. (mg/mL)	Volume of BSA standard solution (μL)	Source of BSA standard	Volume of diluent (μL)
1	10 mg/mL stock	-		
2	2.0	200	Tube 1	800
3	1.5	375	Tube 2	125
4	1.0	250	Tube 2	250
5	0.75	250	Tube 3	250
6	0.50	250	Tube 4	250
7	0.25	250	Tube 6	250
8	0.125	250	Tube 7	250
9	0.025	100	Tube 8	400
10	0	-	Blank	500

2.2.4 Western Blot and Immunoblotting

Aliquots of the protein lysates and microsomes extracted from liver and spleen tissues (10 μL/ well) in rat and pig were resolved on 4-15 % SDS-polyacrylamide electrophoresis (SDS-PAGE) protein gel (Bio-Rad, UK) and transferred onto nitrocellulose (NC) blots (Bio-Rad, UK).

The protein extracts were normalised with tissue-lysis buffer (consisting of the lysates/β-mecaptoethanol (10 % v/v of total volume) /PBS) to the desired protein concentration, and

an equal volume of Laemmli buffer (Bio-Rad UK) to a 1:1 volume ratio. Each reagent here is purposeful for SDS-PAGE and involved in the protein separation process by its (monomeric) molecular weight with the use of a protein ladder or marker (NEB, UK). The prepared samples were heated at 95 °C for 10 mins to denature the proteins to their primary structure. The NC blots were blocked using Everyblot buffer (Bio-Rad, UK) for 1 hr at room temperature (RT), and incubated with primary antibody diluted (1:5000) in Everyblot buffer (Bio-Rad, UK) overnight at 4 °C. Nitrocellulose membranes were washed with 1x Tris-Buffered Saline/ 0.1% Tween® 20 (TBST) (Thermo Scientific, UK) and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Thermo Scientific, UK) diluted 1:5000 in Everyblot buffer (Bio-Rad, UK) for 1 hr at RT. The proteins were detected using Clarity Western ECL Substrate chemiluminescence reagent (Bio-Rad, UK). The chemiluminescence signal was visualised using the Bio-Rad ChemiDoc Imaging System. Negative control (absence of primary antibodies) was also performed to validate the results obtained. All primary (*CYP2E1*, *CYP450* generic, *CYP27A1*, *CYP20A1*, *UGT8*, *SLC22A6*) and secondary antibodies were purchased from Thermo Scientific UK.

2.2.5 Immunohistochemistry (IHC or IHC-P)

With IHC-P, tissues are fixed in formalin, embedded in paraffin, cut into thin sections (about 5-7 µm) which are mounted on microscopic glass slides; following next are multiple steps of tissue processing (including deparaffinisation and antigen retrieval) to which the protein in the tissue section becomes identified using a specific antibody (Polyak K & Meyerson M, 2003). Immunocytochemistry (ICC) involves staining of isolated or cultured intact cells or primary cells, which typically are fixed with paraformaldehyde and permeabilised with e.g.

Saponin or Triton-X-100, to enable specific primary antibody access to antigens or target proteins. An enzyme-coupled secondary antibody (e.g. horseradish peroxidase) is used to recognise the primary antibody to aid visualisation under light microscopy involving the enzyme and a chemical (e.g. substrates of 3,3'-Diaminobenzidine, DAB) to generate a colour stain. This is known as a chromogenic detection, though a fluorescent detection method (IHC-F or IF) can also be used by having the primary antibody (direct detection) or secondary antibody (indirect detection) that is conjugated to a fluorophore/ fluorochrome and would require a fluorescent microscope. These fluorochromes are compounds that contain electrons which when exposed to a light of a certain wavelength achieve an unstable higher energetic state, but on returning spontaneously to the ground state, they emit light of a longer wavelength (Shetty et al., 2017).

In this study, selected polyclonal CYP450 antibodies were used to detect specific CYP450 markers in a tissue section. The antibodies are linked to an enzyme or fluorescent dye and when the antibodies bind to these markers, the enzyme or dye becomes activated. A chromogenic detection system was applied in this experiment using a Leica Novolink polymer peroxidase detection kit (Leica, UK). This kit uses a polymerisation technology that prepares polymeric (horse radish peroxidase) HRP-linker antibody conjugates for DAB (3,3'-Diaminobenzidine) staining. DAB is oxidised by hydrogen peroxide, which is catalysed by HRP, and oxidised DAB forms a brown precipitate at the location of the HRP which can then be visualised using light microscopy.

2.2.5.1 Slide preparation (Tissue processing, Fixation, and Sectioning)

2.2.5.1.1 Creating a FFPE block from paraffin wax

Tissue fixation is carried out to preserve target antigens from degradation and to prevent autolysis with necrosis from the harvested tissues. The process of embedding the tissues provides appropriate support for sectioning making the method more robust. Fresh pig and rat tissues (liver and spleen) were fixed in 10 % neutral buffered formalin (Fisher Scientific, UK) overnight at room temperature and embedded in paraffin wax (making a FFPE block) using an automated tissue processing (Excelsior AS Tissue Processor, UK) and embedded system (Leica EG1160). The FFPE tissue blocks were stored in -80 °C.

2.2.5.1.2 Preparing tissue sections

The Formalin-Fixed Paraffin-Embedded (FFPE) tissue blocks were removed from the freezer and left to equilibrate at room temperature for 15 min. Using a microtome (5 µm), slices of tissue sections (6x from each FFPE block) were carefully transferred to a pre-warm water bath (Clifton, UK) at 37 °C to even out the sections and to remove creases. Pencil labelled poly-adhesive microscopic slides (Fisher Scientific, UK) were slowly placed under the tissue sections at an angle and the water enabled the tissue sections to move sticking to the slides. The slides containing the tissue sections were placed on a plate heating block at 50 °C to dry.

2.2.5.2 Deparaffinisation or Rehydration of tissue sections

The paraffin or wax must be completely removed to rehydrate the tissue for antibody binding and staining. This deparaffinisation/rehydration process involves a series of sequential xylene/ethanol/water washes set as follows:

- Incubate sections in 3 x washes of xylene for 5 min each
- Incubate sections in 3 x washes of 100 % ethanol for 5 min each
- Incubate sections in 3 x washes of 90 % ethanol for 5 min each
- Incubate sections in 3 x washes of 70 % ethanol for 5 min each
- Incubate sections in 2 x washes of distilled water for 5 min each

2.2.5.3 Antigen retrieval

The antigen retrieval step is carried out on fixed tissue sections in order to expose antigens sites and allow antibodies to bind to the target antigens. 1 L of 10 mM sodium citrate buffer pH 6.0 was prepared and the slides containing the tissue sections were boiled in the buffer 3 x 5 min using the microwave, with 1 min in between to maintain the heat while allowing the slides to cool. After the final boil, the slides were left to cool for 30 min. The chemical was purchased from Fisher Scientific, UK.

2.2.5.4 Blocking, Washing, Primary Antibody incubation and Immunostaining

The Leica Novolink polymer peroxidase detection kit was used following the manufacturer's instructions. Blocking solution was prepared (1x Tris Buffered Saline with Tween® 20 (TBST)/ 5 % Normal Goat serum, both from Thermo Scientific, UK) and used to block proteins on the

unmasked tissue sections for 1 hr at room temperature. Blocking enables to prevent non-specific antibody binding and reduce the background signal. Where washes were required, the tissue sections were washed in TBST 2x for 5 min each. The primary antibody was diluted (1:500) in 1x TBST/ 5 % Normal Goat serum and incubated overnight at 4 °C in an IHC chamber. 4 individual primary antibodies (*CYP2E1*, *CYP450* generic, *UGT8* and *ABCB1*) including a negative control (no primary antibody) was used for antibody incubation and staining.

2.2.5.5 Dehydrate, Mounting and Examination

The tissue sections were dehydrated in a series of xylene and ethanol washes. The tissue sections were incubated in 70 % ethanol 2 x for 10 sec each, 90 % ethanol 2 x 10 sec each, 100 % ethanol 2 x 10 sec each and xylene 2 x 10 sec each. The specimens were mounted using a CC Mount (Sigma Aldrich, UK), dropwise to cover the tissue section (optional to use coverslips) and baked in a 50 °C oven for 45 min. After the slides were cooled at room temperature, they were examined using a brightfield Leica upright microscope (Leica, UK).

2.2.6 Immunofluorescence staining (IF)

2.2.6.1 Prepared reagents

1. 2 % BSA solution: 2 g of BSA powder (Sigma Aldrich, UK) was added to 100 mL PBS, using a stirrer to mix until dissolved. This is a component of the blocking solution.
2. 4 % paraformaldehyde (PFA): 4 g of PFA (Fisher Scientific, UK) was added to 80 mL of distilled water and heated gently to dissolve while stirring. Drops of 1 M NaOH was added to

make the solution clear. 10 mL of PBS was added to cool the solution and make a final volume of 100 mL fixing solution for IF test.

3. 0.2 % Saponin solution: 200 mg saponin powder (Sigma Aldrich, UK) was added to 100 mL PBS and mixed with a stirrer until dissolved. This becomes the component for permeabilising the cells for the IF test.

2.2.6.2 Immunostaining test

The immunostaining experiment was carried out in a clean culture hood using aseptic techniques. The cell suspension (containing up to 1×10^6 cells) from pig liver and spleen were washed twice with 1 mL pre-warmed PBS (37 °C) in 1.5 mL microcentrifuge tubes (Fisher Scientific, UK). 1 mL of pre-warmed 4 % PFA (37 °C) was added to re-suspend and fixed the cell pellets and were incubated for 1 hr at RT. Fixing the cells prevent autolysis (immobilising target antigens to allow for the antibodies maximum access to targeted cellular components) and to preserve morphology (Im et al., 2019). 0.05 % saponin in 2 % BSA solution was prepared (12.5 mL of 0.2 % saponin solution in 37.5 mL 2 % BSA solution). The cells were washed twice with pre-warmed PBS (37 °C), and 1 mL of ice-cold 0.05 % saponin/ 2 % BSA solution was added to cell pellets and re-suspended. The cells were permeabilised by incubation for 1 hr at RT or overnight on rotation at 4 °C. Following this step, the cells were blocked with 2 % BSA solution for 30 min at RT. The blocking process prior to immuno-staining aids to prevent non-specific binding of the antibodies to places the target proteins have not attached. After centrifugation (Thermo Scientific, UK) to discard the supernatant, the cells were incubated with primary antibodies (*CYP450 generic*, *CYP2E1*, *UGT8*, *SLC22A6*) for 1 hr at RT. The primary antibodies were diluted in 0.05 % saponin/ 2 % BSA solution at the antibody

concentration recommended by the manufacturers. The cells were washed three times with 2 % BSA solution and incubated with diluted 1:10000 goat anti-rabbit secondary antibody conjugated to a fluorochrome (Alexa Fluor™ 488, Invitrogen) in 0.05 % saponin/ 2 % BSA solution for 1 hr at RT. The cells were washed three times with 2 % BSA solution and incubated with DAPI for 5 – 10 mins at RT. A further two washes with 2 % BSA solution and 2-3 drops of aqueous mounting media was added to the cell pellets and re-suspended. A drop of the immuno-stained cells was placed on microscopic slides covered with coverslips. They were immediately visualised under fluorescent microscope or stored briefly in 4 °C. All the antibodies were purchased from Enzo Life Sciences and Thermo Scientific, UK.

2.2.7 Co-immunoprecipitation (Co-IP)

The Co-IP experiment was used to isolate selected CYP450, UGT and transporter proteins and their co-interacting proteins. The goal of this study was to identify the isolated proteins and confirm the specificity of the antibodies used both for the Co-IP and western blot studies. A Pierce co-immunoprecipitation kit was used according to the manufacturer's instructions. Rat and Pig protein lysates extracted from liver and spleen tissues were used together with approximately 50 µg of primary antibodies (*CYP2E1*, *CYP450* generic, *CYP27A1*, *CYP20A1*, *UGT1A6*, *UGT8*, *MRP7*, *SLC25A6* and *ABCB1*) for the Co-IP experiment. The general principle involved the use of the antibody which was precipitated and contained to a resin in a column, and the purified protein lysates were added to the same column. The column is incubated to enable the antibody to specifically bind to the target protein with its co-interacting protein. The antibody-protein complex is eluted and resolved on 4-15 % SDS-polyacrylamide

electrophoresis (SDS-PAGE) protein gel (Bio-Rad, UK), and subsequently transferred onto nitrocellulose (NC) blots (Bio-Rad, UK).

2.3 Assessing the functional expression of selected phase I and II enzymes in the spleen that are associated with drug metabolism and distribution.

2.3.1 CYP450 content determination

A differential spectroscopy method with dithionite and carbon monoxide (CO) was used on homogenates and microsomes fractions extracted from pig liver and spleen, to determine the CYP450 content. Two different approaches (using an autoclave CO chamber and cuvette method) was carried out. The measured content would enable downstream experiments to demonstrate the functional activities of CYP450 enzymes when exposed to drug substrates. All the chemicals used here were purchased from Sigma Aldrich and Fisher Scientific, UK.

2.3.1.1 Sample preparation 1

2 mL of 1 mg/mL microsome or homogenate solution in 0.1 M potassium phosphate buffer, pH7.4/ 30 % glycerol was prepared to ensure solution is not turbid. 0.5 M sodium hydrosulfite solution was also prepared in 0.1 M potassium phosphate buffer, pH7.4/ 30 % glycerol and should be used on day of preparation.

2.3.1.2 Experimental procedure using autoclave CO chamber

1 mL of the mixture was transferred to two individual 2 mL glass vials containing magnetic stirrers, where one is labelled as reduced P450 solution, **P** and the other as reduced P450-CO complex solution, **PC**. The **P** solutions were left in the fridge and the **PC** vials were incubated in an autoclave CO chamber (Asynt, UK) as shown in Figure 17, at 5 bar gas pressure (or flow rate at ≥ 0.5 L/min) for 30 – 40 min while stirring (IKA, UK). Subsequently, 200 μ L of both **P** and **PC** solutions were transferred to a transparent flat bottom polystyrene microplate in triplicates. 5 μ L of 0.5 M sodium dithionite was added to all samples at a final concentration of 12.5 mM and placed on a plate shaker (IKA, UK) for 5 min. **P** solutions should remain colourless and **PC** solutions should have a slight colour change to yellow. The absorbance was measured in a FLUOstar Omega microplate reader (BMG Labtech, UK) at 450 nm and 490 nm wavelengths respectively. CYP450s show an absorbance peak at 450 nm wavelength, and the difference in optical density between 450 and 490 nm is recorded because at the wavelength region of 490 nm, it should appear flat (i.e. return to baseline). The CYP450 content is calculated following the equation [18]:

[18]

$$nmole\ P450\ per\ mL = \frac{(A450_{PC} - A450_P) - (A490_{PC} - A490_P)}{0.091}$$

Where 0.091 mL/nmole/cm is the CYP450 molar extinction coefficient, A450 is the absorbance at 450 nm, A490 is the absorbance at 490 nm, PC is the reduced P450-CO complex sample, and P is the reduced CYP450 sample.



Figure. 17 Setup for the samples enclosed in a CO gas chamber for CYP450 content determination.

2.3.1.3 Sample preparation 2

1 in 40 dilution of microsome/ homogenate solution in 0.1 M potassium phosphate buffer, pH7.4/ 30 % glycerol was prepared to ensure solution is not turbid. The mixture was prepared in triplicates in a 2 mL glass vials (Fisher Scientific, UK), vortex mixed and infused with CO gas at one bubble per second for 1 min. 0.5 M sodium hydrosulfite solution was prepared in 0.1 M potassium phosphate buffer, pH7.4/ 30 % glycerol and should be used on day of preparation.

2.3.1.4 Experimental procedure using cuvettes

1 mL of the mixture exposed to CO was transferred to two individual cuvettes. One cuvette is placed in the reference cuvette holder of the spectrophotometer (Biochrom Libra S22, UK).

25 μ L of 0.5 M sodium dithionite making a final concentration of 12.5 mM was added to the second cuvette, which was pipette mixed and loaded onto the sample holder of the spectrophotometer (Biochrom Libra S22, UK). The cuvettes were scanned, and the absorbance readings were recorded at 450 nm and 490 nm for each sample respectively. The CYP450 content was calculated as described using equation [17].

2.3.2 Drug elimination rate determination using substrate depletion method

The drug substrates tested in this experiment were substrates of the major contributing CYP450 and UGT enzymes in human liver. These substrates are used as reference to demonstrate the activity of CYP450 and UGT enzymes in pig liver and spleen microsomes and cells (Table 10 and 11). All chemicals were purchased from Sigma Aldrich, UK.

Table 10 Reference CYP450 enzymes with their known drug substrates

#	Reference Human CYP450 gene	Drug Substrate
1	CYP1A2	Phenacetin
2	CYP2A6	Coumarin
3	CYP2B6	Bupropion
4	CYP2C8	Amodiaquine
5	CYP2C9	Tolbutamide
6	CYP2C19	Omeprazole
7	CYP2D6	Dextromethorphan
8	CYP2E1	Chlorzoxazone
9	CYP2J2	Astemizole
10	CYP3A4	Midazolam

Table 11 Reference UGT enzymes with their known drug substrates

#	Reference Human UGT gene	Drug Substrate
1	UGT1A4, UGT2B10	Imipramine Hydrochloride
2	UGT1A1, UGT1A10, UGT2B7	β -Estradiol
3	UGT1A6	Serotonin Hydrochloride
4	UGT1A9	Umbelliferone
5	UGT2B7	Naloxone Hydrochloride Dihydrate
6	UGT2B15	Acetaminophen

2.3.2.1 Sample preparation

10 mM stock solutions of all drug substrates were prepared in Dimethyl Sulfoxide (DMSO). A serial dilution of eight working concentrations for each drug substrate was prepared in a 96-well plate and labelled as the 'test compound plate' (see appendix Figure A2). These working concentrations are 500, 250, 100, 50, 10, 5, 1, and 0.5 μ M and was prepared in 50:50 acetonitrile and water.

2.3.2.2 Incubation mix plate preparation

The microsome incubation mixture was prepared at four different microsome protein concentrations (0.1, 0.25, 0.5 and 1 mg/mL) and consists of the following, 0.1 M potassium phosphate buffer pH7.4, microsomes, the co-factor nicotinamide adenine dinucleotide phosphate (NADPH) or uridine diphosphate glucuronic acid (UDPGA) at the final concentration of 1 mM and 8 mM respectively, and drug substrate at the final concentration of 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5, and 5 μ M, to make a total volume of 500 μ L in each well

of a 96-deep well plate. For the cell incubation mix plate, 250 μL Hank's buffer containing 1x glutamine and 15mM HEPES was pre-warmed in a 1 mL 96-well plate at 37 °C. 250 μL 1×10^6 cell suspension in Hank's buffer (at a final concentration of 5×10^5 cells) was added to make 500 μL total volume of the cell incubation mixture. The prepared incubation mix plate was pre-warmed on a plate shaker (Eppendorf, UK) at 37 °C for 10 mins to equilibrate.

2.3.2.3 Substrate depletion assay

All prepared plates, quenching plates and pipette tips were loaded onto the corresponding platform of the Biomek liquid handling robot (Biomek i7 automated workstation, Beckman Coulter Life Sciences, UK). Addition of the drug substrates to the incubation mixture initiated the reaction. Reactions were quenched at 0, 5, 15, 30, and 45 min by adding 150 μL of ice-cold acetonitrile containing the internal standard (Labetalol, 1 μM). Control incubations were performed using spiked DMSO in microsomes as a blank incubation. After incubation, the samples were centrifuged (3500 rpm, 4 °C) for 15 min, then 100 μL of supernatant was collected and diluted with water (ratio of 1:1). The samples were analysed by UPLC-MS/MS (Waters, Xevo TQS Micro, UK).

2.3.2.4 Setting up conditions of Ultra-performance liquid chromatography (UPLC)

A Waters UPLC system was used to analyse the processed assay samples on an Acquity UPLC HSS T3 C18 column (2.1 x 50 mm, 1.8 μm) which was maintained at 40 °C in a column oven. Mobile phase A consisted of water with 0.1 % formic acid, and mobile phase B consisted of acetonitrile with 0.1 % formic acid. For Coumarin substrate, a buffered mobile phase was

required and a 10 mM ammonium formate in water and in acetonitrile was prepared and loaded onto the second channel mobile phases. The LC gradient is shown on Table 12.

Table 12 UPLC gradient condition for sample analysis

Time	Flow (mL/min)	% A	% B
Initial	1.000	95.0	5.0
0.03	1.000	95.0	5.0
0.80	1.000	5.0	95.0
0.85	1.000	5.0	95.0
1.00	1.000	95.0	5.0
1.10	1.000	95.0	5.0

2.3.2.5 Setting up the Mass spectrometry (MS/MS) conditions for analysis

A triple quadrupole mass spectrometer (Waters Xevo TQS-micro) was used for detection and quantitation in an electrospray (ESI) positive and negative ion mode for all the test compounds (drug substrates), internal standard and the monitored metabolites. The capillary and cone voltage were 0.60 kV and 20 V respectively. The other parameters such as the desolvation and cone gas flow, the collision energy was 900 L/Hr, 20 L/Hr and 20 V respectively. The desolvation temperature applied was 650 °C. The test compounds (drug substrates) were detected using multiple reaction monitoring, with compound specific MS methods (see Table 13).

Table 13 MS methods of CYP450 and UGT drug substrates and their corresponding marker metabolites

Human CYP450 gene probe	Drug Substrate	MS Transitions (m/z)	Ionisation mode	Metabolite Monitored	MS Transitions (m/z)	Ionisation mode
CYP1A2	Phenacetin	180.113 > 109.979	ESI +	Acetaminophen	152.020 > 110.010	ESI +
CYP2A6	Coumarin	146.889 > 105.867	ESI +	Hydroxycoumarin	160.941 > 132.945	ESI -
CYP2B6	Bupropion	240.157 > 184.015	ESI +	Hydroxybupropion	256.200 > 238.000	ESI +
CYP2C8	Amodiaquine	356.098 > 282.984	ESI +	Desethylamodiaquine	328.000 > 283.000	ESI +
CYP2C9	Tolbutamide	268.947 > 169.855	ESI -	Hydroxytolbutamide	285.090 > 185.600	ESI -
CYP2C19	Omeprazole	346.063 > 198.013	ESI +	5-Hydroxyomeprazole	384.042 > 206.020	ESI +
CYP2D6	Dextromethorphan	272.457 > 170.995	ESI +	Dextrorphan	258.000 > 157.000	ESI +
CYP2E1	Chlorzoxazone	167.819 > 131.833	ESI -	6-Hydroxychlorzoxazone	184.000 > 120.000	ESI -
CYP2J2	Astemizole	459.249 > 134.981	ESI +	Desmethylastemizole	445.400 > 121.100	ESI +
CYP3A4/5	Midazolam	326.274 > 291.067	ESI +	1-Hydroxymidazolam	342.100 > 203.100	ESI +

Human UGT gene probe	Drug Substrate	MS Transitions (m/z)	Ionisation mode	Metabolite Monitored	MS Transitions (m/z)	Ionisation mode
UGT1A4, 2B10	Imipramine	281.195 > 85.980	ESI +	2-Hydroxyimipramine glucuronide	472.500 > 457.100	ESI +
UGT1A6	Serotonin	177.055 > 160.059	ESI +	Serotonin-β-D-glucuronide	353.000 > 160.059	ESI +
UGT2B7	Naloxone	328.138 > 212.107	ESI +	Naloxone-3-glucuronide	504.000 > 310.100	ESI +
UGT2B15, 1A9, 1A1, 1A6	Acetaminophen	152.020 > 110.010	ESI +	Acetaminophen glucuronide	326.100 > 150.100	ESI +
UGT1A10, 1A1, 2B7	Beta-Estradiol	273.163 > 145.000	ESI -	17-β-Estradiol-3-glucuronide	447.000 > 271.000	ESI -
UGT1A1 - 6, 2B7, 2B15	Umbelliferone	160.964 > 132.992	ESI -	7-Hydroxycoumarin glucuronide	339.000 > 163.000	ESI +

2.3.2.6 Preparing the MS methods for analysis

Specific MS methods (see Table 13) using multiple reaction monitoring mode (specific parent to daughter ions) were created for each drug substrate to ensure that only the test compounds would be detected and would eliminate detecting other endogenous compounds and artefacts. This is performed by optimising or tuning each compound for fingerprint electron analysis based on the mass-to-charge ratio (m/z) of an ion. Whereby ions are ionised by exposing the molecules in the sample with a high energy electron beam. These are separated by their m/z to present a mass spectrum conveying the relative abundances of the ions and their mass that is subsequently used to calculate the exact masses of atoms or molecules in a sample. 1 mL of 1 μ M solution in 50:50 acetonitrile and water for each drug substrate was prepared in 1 mL glass vials (Agilent, UK). Using the monoisotopic molecular weight of the compound, the mass spec runs a tune analysis to generate the MS method for the compound. These methods were used for the analysis of all samples monitoring the CYP450 and UGT drug substrates and their corresponding metabolites.

2.3.2.7 Data analysis of MS targetlynx (output) files

Each targetlynx file is an analysis output from the Masslynx software (Waters, UK) that corresponds to the initial submitted run-list file producing chromatograms of each sample injection. The MS methods were inclusive of the internal standard and its stable responses at ≤ 20 % correlation variation (CV) would indicate the MS system was working at optimum condition. Each injection was examined that a single chromatogram was detected at the right shape with similar retention time observed for all the sample injections monitoring the same

compound. The signal to noise ratio (S/N) values are provided to act as a measure of the sensitivity of the LC-MS instrument, thereby indicating that a higher S/N value depicts a low background signal that would not impede on the detecting signal for the target compound (see Figure 18).

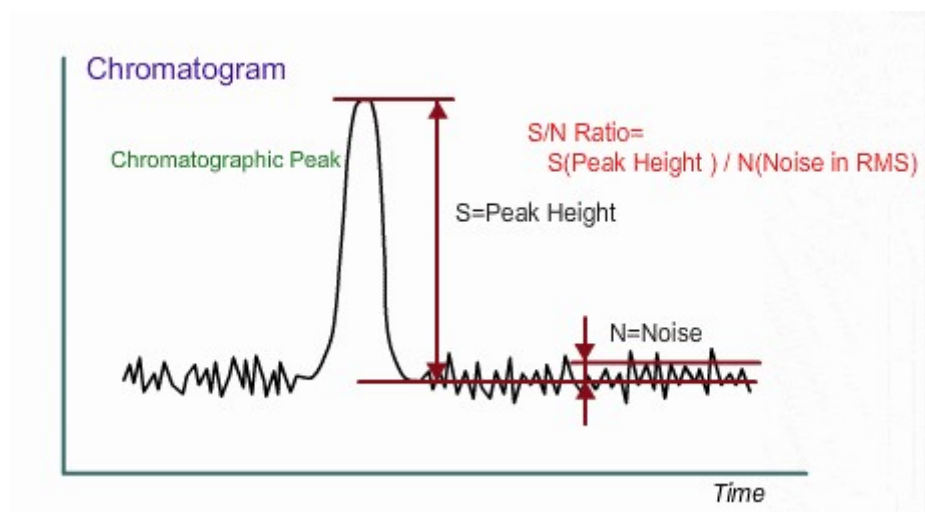


Figure 18 A simple illustration to define the principle behind the signal to noise ratio and its importance in interpreting result data based on the chromatographic peak area response from a single sample injection. The displayed diagram was obtained from the website as shown <https://www.shimadzu.com/an/service-support/technical-support/gas-chromatograph-mass-spectrometry/analysis-results/signal/definition.html>

2.3.2.8 Generating curve plot from MS processed data

The MS peak area responses at each time point for a given concentration were plotted against time to create a regression line. The depletion of substrate will produce a slope and the equation showing the slope with the R-squared was displayed for each chart representing the drug substrate analysed. The given slope or gradient is the elimination rate constant for the

reaction monitored and it is subsequently used to generate the intrinsic clearance (Cl_{int}) for each drug substrate at a given (or tested) concentration. For the monitored metabolites formed, a graph is created using the MS peak area responses (as a function of time) to visualise the shape of the curve over time for each drug substrate.

CHAPTER 3:

**Investigating the gene expression of
CYP450 and UGT enzymes associated with
drug metabolism and distribution in the
spleen tissue**

3.0

3.1 Introduction

Understanding the function of genes and their general expression provides an invaluable insight into human health and disease progression, for example in studies exploring the biological roles of encoded proteins, the causes and consequences of diseases, and possible therapeutic uses of gene products or its targets for therapeutic manipulation (Chengalvala et al., 2007a). Gene expression begins with a deoxyribonucleic acid (DNA) sequence (Alberts et al., 2002a). The process of translating that sequence into function is a fundamental part of gene expression involving ribonucleic acid (RNA) (Alberts et al., 2002b). While transcription involves the production of RNA from a DNA template, the resulting transcribed gene product becomes a key quantitative measurement of gene expression and therefore generating a complete set of RNA transcripts referred to as a transcriptome (Singh et al., 2018). Transcripts of RNA are either classed as protein coding or non-coding, where the latter play a role in transcription and the regulation of post-transcription of RNA (Singh et al., 2018). The former synthesises messenger RNAs (mRNA) which are responsible for making protein products through the process known as translation, that then determines the function of a gene (Singh et al., 2018). A classical approach to genetic studies is followed to discover how a gene functions by observing mutations (either resulting from changes or deletions in their nucleotide sequences) that then disrupts cellular processes (Alberts et al., 2002b). To an extent, cellular phenotype is determined by the set of proteins expressed in a cell, suggesting there is a relationship between a genotype and a phenotype (Henriksen & Kotelevtsev, 2002). Gene expression profiling involves at first a genetic screen (with the use of public databases) to isolate mutants of interest and subsequently the identification of the genes responsible for

the observed phenotype (Henriksen & Kotelevtsev, 2002; Sun et al., 2014). To understand this relationship between genotype and phenotype, it is crucial to understand what controls or regulates gene expression (Mantione et al., 2014). The regulation of gene expression can be cell-specific and involves several complex biochemical processes which are essential both for the development of the organism and its ability to respond to changes in the environment (Singh et al., 2018). Such processes include transcription, epigenetic processes and post-transcriptional modifications, making the need for reliable assessments of RNA transcript abundance in biological samples to drive the development of novel technologies for example DNA microarray, northern blotting, RNA-seq and RT-qPCR etc, that will meet this demand (Mantione et al., 2014).

Two of the techniques have been applied in this study, namely RNA-seq and RT-qPCR (real time quantitative polymerase chain reaction) to determine the expression of genes of interest using extracted purified total RNA from spleen tissues and primary cells. RNA-seq is used more routinely as it quantifies levels of distinct types of RNA by sequencing the RNA directly and counting the number of sequences, while qPCR quantifies RNA through amplification and fluorescent dye intensity (Mantione et al., 2014; Singh et al., 2018). Although RNA-seq has a greater dynamic range to quantify transcripts of thousands of differentially expressed gene isoforms, its high costs and high computational data relative to other technologies is not only a storage burden but also its main limitations (Singh et al., 2018). RT-qPCR allows quantitation of gene expression in real time by measuring its amount in a fluorescently labelled mixture using a spectrophotometer (Singh et al., 2018). mRNA acts as a template for the qPCR reaction that follows a number of steps i.) mRNA is converted to complimentary DNA (cDNA) using reverse transcriptase, ii.) single-stranded cDNA is transformed to a double-stranded DNA using DNA polymerase, iii.) DNA template becomes exponentially amplified with each signal

recorded as the DNA accumulates (Singh et al., 2018). The resulting level of fluorescent signal is directly proportional to the amount of the amplified DNA present (Singh et al., 2018).

In the context of drug development, toxicogenomic or assessment of toxicity is paramount for screening compounds at early stages that are applied in *in-vitro* cultured/ primary cells or tissues, to determine the mechanism of hepatotoxicity in the body (Chengalvala et al., 2007b). For example, expression analysis of CYP450 family of genes are an important focus in toxicogenomics as variations of certain CYP450s and UGTs (*CYP2D6*, *UGT1A1*) have shown to be responsible for toxicity associated with chemotherapeutic drugs like Irinotecan (Chengalvala et al., 2007b). In hindsight, this section aims to identify the genes associated with drug metabolism and distribution in rat and pig spleen. This will also provide insight into the expression levels of such genes and to indicate the spleen's potential ability to metabolise drugs.

3.2 Results

3.2.1 Gene expression of CYP450s in rat and pig using standard PCR

13 CYP450 genes including housekeeping (control) genes (GAPDH, HPRT, Villin and β -actin) were evaluated for positive gene expression in rat and pig liver tissue and followed with the same experiment repeated in the spleen tissue respectively. Bands expressing the target CYP450 genes were all observed in rat liver with exception of one gene *CYP2D1*. In contrast, only six CYP450 genes (*CYP2D25*, *CYP2E1*, *CYP3A22*, *CYP3A29*, *CYP1A2*, HPRT) expressed positive bands in pig liver with very faint bands observed for latter two respectively (see Figure 19). The experiment in pig spleen was not carried out as a result, while the rat spleen

experiment showed positive gene expression in almost all the tested CYP450 genes including the housekeeping genes with exception of three genes, *CYP1A2*, *CYP2B2* and *CYP2D1* (See Table 14). Apparent contamination of the no reverse transcriptase negative control for some samples were observed, but the no cDNA negative control indicated a true control.

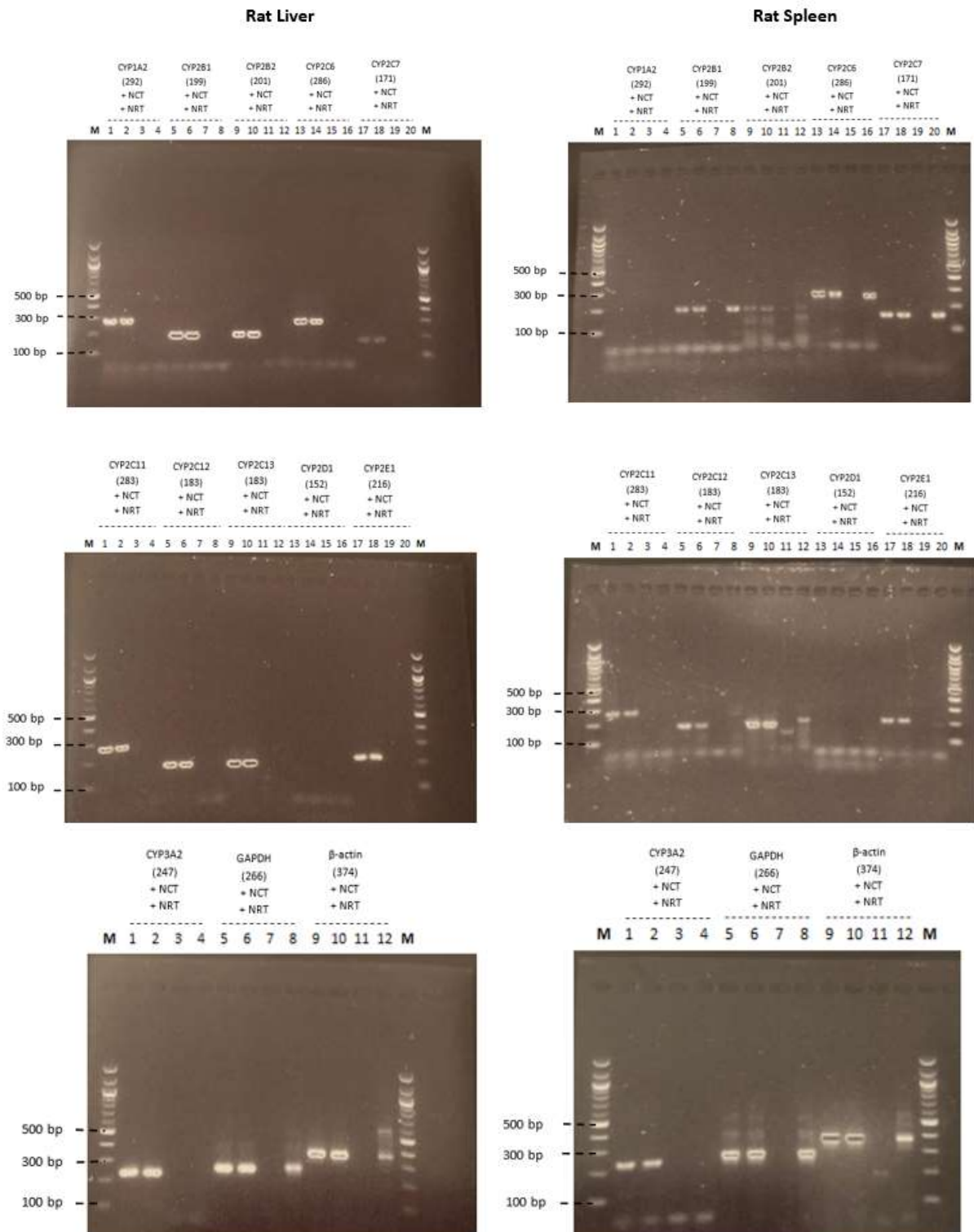


Figure 19 Agarose Gel Electrophoresis (2% agarose) of PCR amplified products at expected product sizes in rat liver and spleen using designed CYP450 specie-specific PCR primers inclusive of negative controls – (NCT) no cDNA template control and (NRT) no reverse transcriptase control. DNA Marker/dye (M)

Table 14 CYP450 gene expression observations in Rat (Sprague Dawley) by standard PCR and agarose gel electrophoresis

Human CYP genes*	Equivalent Rat CYP genes	Liver (Y/N)	Spleen (Y/N)
CYP1A	CYP1A2	Y	N
CYP2B	CYP2B1	Y	Y
	CYP2B2	Y	N
CYP2C	CYP2C6	Y	Y
	CYP2C7	Y	Y
	CYP2C11	Y	Y
	CYP2C12	Y	Y
	CYP2C13	Y	Y
CYP2D	CYP2D1	N	N
CYP2E	CYP2E1	Y	Y
CYP3A	CYP3A2	Y	Y

*major contributing CYP450 gene family in human

3.2.1.1 Specificity of gene primers using Sanger sequencing

All the rat liver and spleen PCR products were used in Sanger sequencing analysis to examine the specificity of the CYP450 gene primers tested and to validate the gene expression observed in rat spleen. Majority of the CYP450 genes from both tissues shown in Table 15 and 16, produced a percent identity above 94 %. However, evidence of broad or double peaks, mis-called or N designated bases at the start of the run, background noise and some loss of resolution towards the end of the run in the sequenced chromatograms were observed. Nonetheless, a very low E value across the CYP450 genes in rat liver and spleen were provided, but there was incidence where either a different gene being identified to the gene of interest or no similarity in sequences found from the sequencing analysis.

Table 15 BLAST searched Sanger sequencing data of rat liver PCR products

Gene	Primer	Query Coverage	E Value	Percent Identity
<i>Cyp1a2</i>	Forward	64 %	2.00E-105	97.00 %
<i>Cyp1a2</i>	Reverse	92 %	9.00E-119	99.19 %
<i>Cyp2b1</i>	Forward	83 %	9.00E-68	100 %
<i>Cyp2b1</i>	Reverse	85 %	8.00E-69	99.32 %
<i>Cyp2b2</i>	Forward	different gene identified		
<i>Cyp2b2</i>	Reverse	94 %	4.00E-72	98.75 %
<i>Cyp2c6</i>	Forward	95 %	3.00E-121	99.60 %
<i>Cyp2c6</i>	Reverse	96 %	4.00E-113	98.33 %
<i>Cyp2c7</i>	Forward	38 %	8.00E-53	99.16 %
<i>Cyp2c7</i>	Reverse	59 %	2.00E-57	99.21 %
<i>Cyp2c11</i>	Forward	95 %	3.00E-114	98.77 %
<i>Cyp2c11</i>	Reverse	93 %	2.00E-115	99.57 %
<i>Cyp2c12</i>	Forward	91 %	8.00E-62	97.26 %
<i>Cyp2c12</i>	Reverse	92 %	6.00E-63	99.28 %
<i>Cyp2c13</i>	Forward	90 %	1.00E-58	97.12 %
<i>Cyp2c13</i>	Reverse	91 %	6.00E-63	99.28 %
<i>Cyp2d1</i>	Forward	74 %	2.00E-36	94.55 %
<i>Cyp2d1</i>	Reverse	80 %	3.00E-40	98.97 %
<i>Cyp2e1</i>	Forward	85 %	7.00E-76	100 %
<i>Cyp2e1</i>	Reverse	91 %	2.00E-76	98.81 %
<i>Cyp3a2</i>	Forward	88 %	1.00E-86	98.93 %
<i>Cyp3a2</i>	Reverse	92 %	2.00E-96	99.01 %
GAPDH	Forward	91 %	3.00E-101	98.60 %
GAPDH	Reverse	88 %	8.00E-102	100 %
β -Actin	Forward	90 %	1.00E-151	98.71 %
β -Actin	Reverse	96 %	3.00E-169	99.70 %

Table 16 BLAST searched Sanger sequencing data of rat spleen PCR products

Gene	Primer	Query Coverage	E Value	Percent Identity
<i>Cyp1a2</i>	Forward	93 %	2.00E-123	100 %
<i>Cyp1a2</i>	Reverse	different gene identified		
<i>Cyp2b1</i>	Forward	87 %	7.00E-69	100 %
<i>Cyp2b1</i>	Reverse	84 %	6.00E-71	98.09 %
<i>Cyp2b2</i>	Forward	29 %	6.00E-70	98.71 %
<i>Cyp2b2</i>	Reverse	no similarity found		
<i>Cyp2c6</i>	Forward	92 %	2.00E-115	99.57 %
<i>Cyp2c6</i>	Reverse	87 %	2.00E-105	99.53 %
<i>Cyp2c7</i>	Forward	88 %	3.00E-54	99.17 %
<i>Cyp2c7</i>	Reverse	90 %	4.00E-58	99.22 %
<i>Cyp2c11</i>	Forward	91 %	4.00E-113	100 %
<i>Cyp2c11</i>	Reverse	86 %	1.00E-100	98.60 %
<i>Cyp2c12</i>	Forward	88 %	2.00E-56	96.38 %
<i>Cyp2c12</i>	Reverse	81 %	6.00E-57	100 %
<i>Cyp2c13</i>	Forward	53 %	2.00E-65	98.65 %
<i>Cyp2c13</i>	Reverse	61 %	1.00E-61	98.56 %
<i>Cyp2d1</i>	Forward	No similarity found		
<i>Cyp2d1</i>	Reverse			
<i>Cyp2e1</i>	Forward	84 %	7.00E-76	100 %
<i>Cyp2e1</i>	Reverse	67 %	7.00E-71	98.73 %
<i>Cyp3a2</i>	Forward	91 %	2.00E-91	98.97 %
<i>Cyp3a2</i>	Reverse	89 %	7.00E-90	97.97 %
GAPDH	Forward	90 %	2.00E-104	100 %
GAPDH	Reverse	87 %	9.00E-102	100 %
β -Actin	Forward	92 %	3.00E-154	98.42 %
β -Actin	Reverse	93 %	2.00E-163	100 %

3.2.2 RNA-seq transcriptome study in rat and pig using NGS (next generation sequencing)

NGS analysis was performed using rat and pig liver and spleen tissue samples at n=2 replicates. A good RNA yield was achieved and confirmed its purity with the 260/230 nm and 260/280 nm absorbance ratios at ≥ 2.0 . The RNA quality was measured using an Agilent bioanalyzer and produced an RNA integrity number (RIN) at ≥ 9.0 . The RIN comes from a ratio of the 28 s/ 18 s ribosomal RNA major bands on fluorescent intercalating-stained agarose gel electrophoresis, in which a Bayesian algorithm utilises various RNA electropherogram trace features in the capillary gel to estimate RIN (Talebi et al., 2021). Hence, the Agilent bioanalyzer simultaneously performs electrophoresis and generates an electropherogram; making this machine as the best-known method to calculate RIN for quality control measurements (Talebi et al., 2021). RIN scores are predefined to a numeric system from 1 through 10 and are classed based on the RNA degradation process, with RIN scores between 8 to 10 indicating highly intact RNA and lower RIN scores (1 to 5) indicating a high degree of degradation (i.e. unsuitable for many experiments) (Schroeder et al., 2006). These steps (Table 17) were part of the quality control and library preparation procedure prior to proceeding with DNA amplification (using adapters for hybridization) and the multiplexed sequencing. The sequenced data was collated and given a mean quality score of ≥ 34.0 for all the tested samples. Subsequently, the relative abundance was determined using a combination of an alignment tool (*Salmon 1.4.0*) and algorithm to map the sequenced files to a reference transcriptome from Ensembl Genome Database and counts (NumRead) that generates RNA transcripts per million (TPM).

Table 17 NGS workflow showing quality control and library preparation data

Animal ID	Total RNA Conc. ng/μL	RIN	260/280 ratio	260/230 ratio	cDNA conc. ng/μL	Adapter 1	Adapter 2
Rat Liver (1)	1528	9.3	2.10	2.23	21.2	CAATGCGA	GACGAACT
Rat Spleen (1)	1343	8.9	2.07	2.22	18.4	GGTTGAAC	TCCACGTT
Rat Liver (2)	1564	9.4	2.11	2.06	24.8	GAATCACC	GAACGAAG
Rat Spleen (2)	806	9.7	2.09	2.21	23.6	GTAAGGTG	CGAATTGC
Pig Liver (1)	592	9.6	2.07	2.04	2.00	CTTCGGTT	AACCAGAG
Pig Spleen (1)	476	9.5	2.08	1.88	1.94	TATGGCAC	TTGCGAGA
Pig Liver (2)	531	9.1	2.08	2.18	1.45	CGAGAGAA	GGAAGAGA
Pig Spleen (2)	354	9.4	2.09	2.01	1.91	GACGTCAT	CCAGTTGA

From both Rat and Pig (liver and spleen) datasets, the identified genes that are associated with drug metabolism and distribution were selected and ranked in order of the relative abundance of gene expression (see Figure 20-22). This was determined by converting the NumRead (estimated relative abundance) as a percentage (% NumRead).

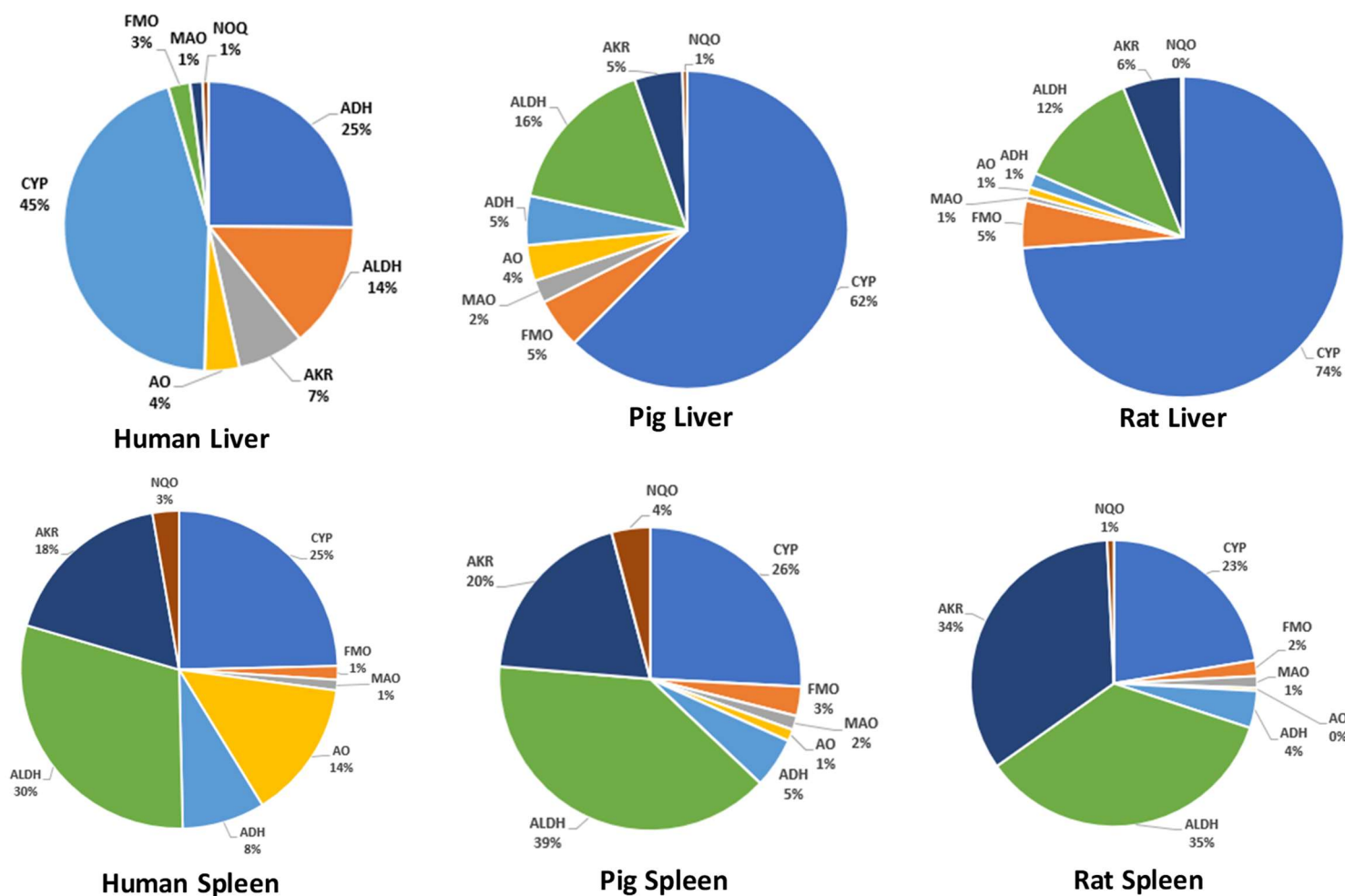
Table 18 Identified genes nomenclature

Gene Symbol	Gene Name
CYP450	Cytochrome P450
FMO	Flavin-Containing Monooxygenases
MAO	Monoamine Oxidases
AO	Aldehyde Oxidases
ADH	Alcohol Dehydrogenases
ALDH	Aldehyde Dehydrogenases
AKR	Aldo-Keto Reductases
NQO	NADPH:Quinone Reductases
UGT	Uridine Diphosphate Glucuronosyltransferases
GST	Glutathione S-Transferases
SULT	Sulfotransferases
NAT	N-Acetyltransferases
ABC	ATP-binding cassette transporters
SLC	Solute carrier transporters
P-gP	P-glycoprotein
ACTB	Actin, β
B2M	β -2 microglobulin
PGK1	Phosphoglycerate kinase 1
RPN1	Ribophorin I
TUBA1	Tubulin
PIIB	Cyclophilin B
HPRT	Hypoxanthine guanine phosphoribosyl transferase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGCX	γ -Glutamyl carboxylase
HMBS	Hydroxymethylbilane synthase
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Gene expression of CYP450 and UGT enzymes are confirmed in rat and pig spleen by the NGS analysis. The metabolic genes identified showed differences in the expression levels between both species compared to human spleen. The CYP450 expression level is over 2-fold greater in liver to the spleen (see Figure 20), but comparable expression levels are observed between the human spleen and that with rat and pig spleen. Where CYP450 enzymes are the most predominant phase I enzymes in pig liver, aldehyde dehydrogenase seems to be the most

expressed in rat, pig and human spleen even though human liver expresses less CYP450 enzymes than observed in pig and rat liver. However, significant differences have been observed with the phase II enzymes particularly for the UGT enzymes. In pig liver, UGT is the second most expressed at 18 % and human liver at 55 % compared to 0.1 % observed in pig spleen, with 9 % in rat spleen and 4 % in human spleen (see Figure 21).

Overall, the gene expression levels of the two drug transporter families, SLC- and ABC transporter proteins are comparable across all species and tissues tested including human spleen (see Figure 22). With most of the expression levels above 80 %, SLC transporter family appears to be the most expressed of the transporter families. However, varying expression levels of individual transporter isoforms from both SLC- and ABC transporters have also been observed.

**Figure 20**

The relative abundance of gene expression (%) of Phase I enzymes in rat and pig, liver and spleen tissues. Human liver and spleen genes were used as reference sourced from ARCHS⁴ database. Identified metabolic enzymes in untreated rat and pig animals obtained from bioinformatics NGS analysis. Rat Liver: 1399738 NumRead (100%) - AO 0.9%, MAO 0.5%; Rat Spleen: 118531.12 NumRead (100%) - NQO 0.8%, AO 0.3%; Pig Liver: 627663.4 NumRead (100%); Pig Spleen: 15550.295 NumRead (100%).

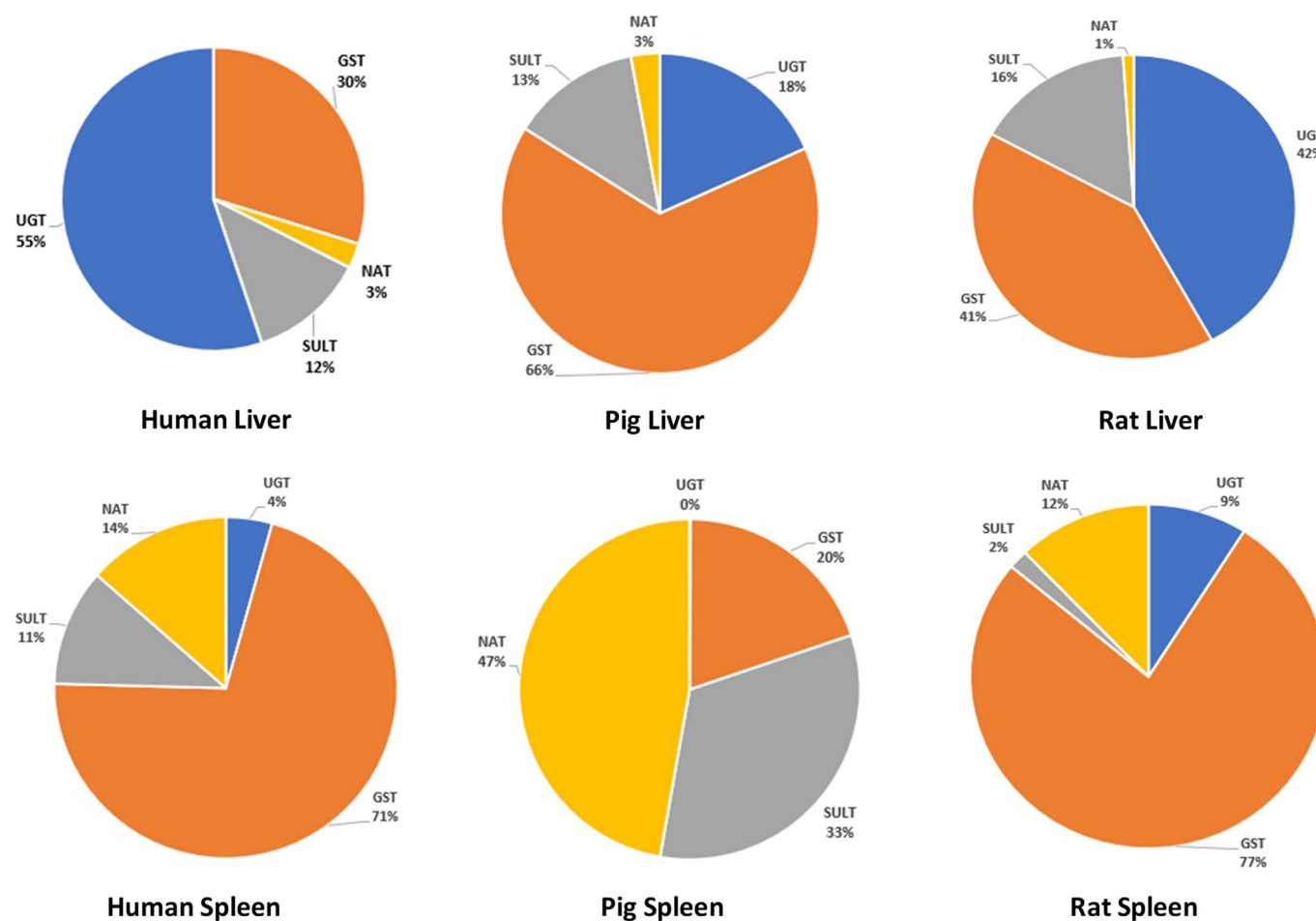


Figure 21 The relative abundance of gene expression (%) of Phase II enzymes in rat and pig, liver and spleen tissues. Human liver and spleen genes were used as reference sourced from ARCHS⁴ database. Identified metabolic enzymes in untreated rat and pig animals obtained from bioinformatics NGS analysis. Rat Liver: 424957.8 NumRead (100%); Rat Spleen: 41798.04 NumRead (100%); Pig Liver: 73673.33 NumRead (100%); Pig Spleen: 6348.077 NumRead (100%) - UGT 0.1%; Human Spleen: 4372297 NumRead (100%).

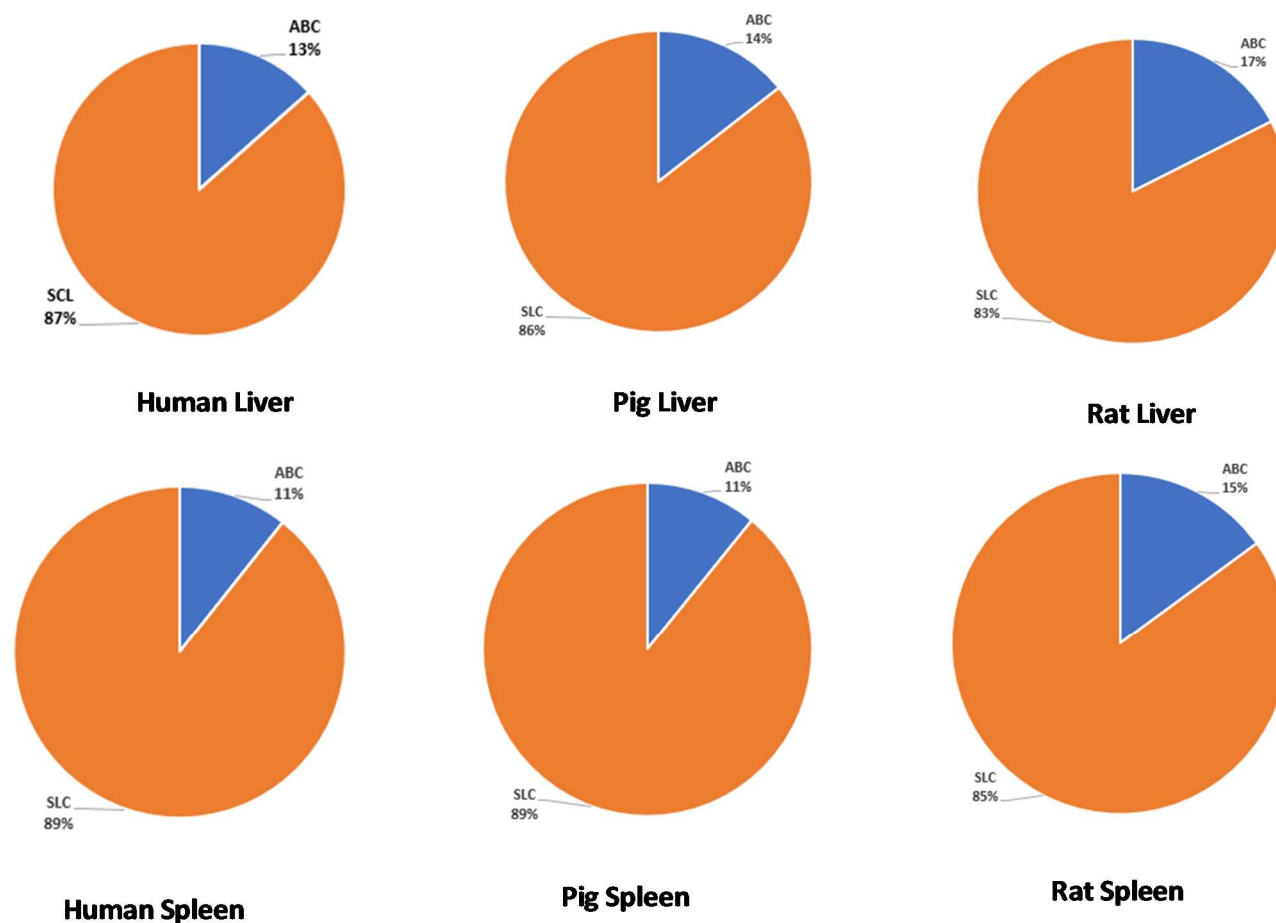


Figure 22 The relative abundance of gene expression (%) of transporter proteins in rat and pig, liver and spleen tissues. Human liver and spleen genes were used as reference sourced from ARCHS⁴ database. Identified metabolic enzymes in untreated rat and pig animals obtained from bioinformatics NGS analysis. Rat Liver: 1023948.6 NumRead (100%); Rat Spleen: 717912.27 NumRead (100%); Pig Liver: 287290.57 NumRead (100%); Pig Spleen: 123148.93 NumRead (100%); Human Spleen: 54518710 NumRead (100%).

The presence of promoter genes (see Figure 23) such as nuclear receptors in the spleen tissue is significant in terms of genes that control gene expression. Varying expression levels of these promoter genes are observed between specie and including human spleen. In rat, pig and human spleen respectively, PAH is expressed at 0.002 %, 0.1 %, 0.1 %; NR1I3 at 0.02 %, 0.5 %, NR1I2 at 0.03 %, 0.2 %; HFN4 α at 0.01 %, 0.03 %, and AHRR in rat spleen at 0.02 %; HFN1 α in human spleen at 0.2 %. Thus, indicating that majority of such genes that are known to activate the transcription of CYP450, UGT and transporters are lowly expressed.

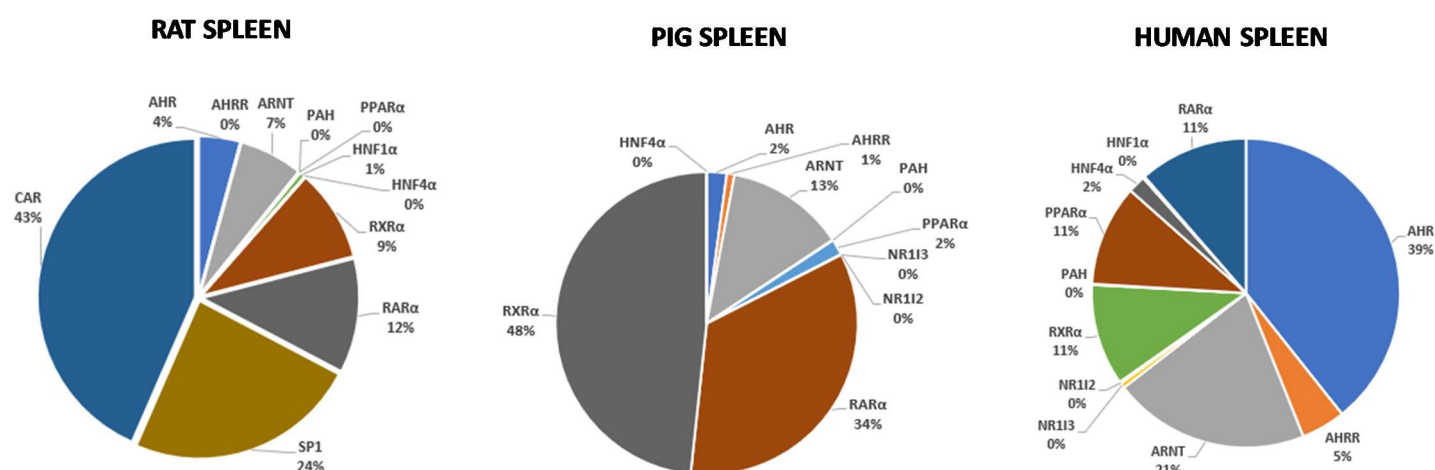


Figure. 23 Identified promoter genes including related nuclear receptors in untreated rat, pig and human spleen based on their relative abundance bioinformatics RNA-seq transcriptome data. AHR (Aryl Hydrocarbon Receptor), AHRR (Aryl Hydrocarbon Receptor Repressor), ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator), CAR (Constitutive Androstane Receptor), PAH (Phenylalanine Hydroxylase), PPAR α (Peroxisome Proliferator Activated Receptor Alpha), RXR α (Retinoid X Receptor Alpha), RAR α (Retinoic Acid Receptor Alpha), HNF1 α / HNF4 α (Hepatocyte Nuclear Factor 1 or 4 Alpha), NR1I2/ NR1I3 (Nuclear Receptor Subfamily 1 Group I Member 2 or 3).

In addition, only few metabolic UGT genes were identified in rat and pig liver and spleen tissues (see Table 19-20). On the other hand, the known drug metabolising UGTs are observed in the top 20 expressed genes identified in rat liver and human spleen. Similarly, some of the major contributing CYP450 enzymes or equivalents to drug metabolism in human liver (Fatunde & Brown, 2020; Zhao et al., 2021) are observed among the top expressed in rat, pig, and human spleen, and these include *CYP3A5/Cyp3a29*, *CYP2E1*, *CYP2D6/ Cyp2d25/ Cyp2d3*, *CYP1A2*, *Cyp2a19/Cyp2ab1* and *Cyp2j34* (see Table 19-20). It may appear that the pig spleen consists of more drug metabolising CYP450s compared to that in the human spleen (see Table 21) but in terms of relative abundance, most of the identified genes are lowly expressed in pig spleen. The variation of expression levels can be observed by the differences of the top expressed genes identified from each specie.

Surprisingly, the two UGT genes identified in pig are not determined for their metabolic function though UGT1A, UGT2A and UGT2B are known to metabolise xenobiotics and endogenous compounds in human (Bhatt et al., 2018). However, there is a significant difference of UGT expression levels between the pig liver (99.9 % *Ugt2b31*, 0.1 % *Ugt8*) and spleen (negligible *Ugt2b31*, almost 100 % *Ugt8*).

Table 19 Top 20 expressed genes identified in rat from the RNA-seq transcriptome data

CYP450		UGT		SLC Transporter proteins		ABC Transporter proteins	
Rat Liver	Rat Spleen	Rat Liver	Rat Spleen	Rat Liver	Rat Spleen	Rat Liver	Rat Spleen
<i>Cyp3a23/3a1</i>	<i>Cyp1b1</i>	<i>Ugt2b15</i>	<i>Ugt1a6</i>	<i>Slc39a14</i>	<i>Slc4a1</i>	<i>Abcb11</i>	<i>Abcg1</i>
<i>Cyp2c11</i>	<i>Cyp51</i>	<i>Ugt2b35</i>	<i>Ugt8</i>	<i>Slc9a3r1</i>	<i>Slc44a2</i>	<i>Abca1</i>	<i>Abcf1</i>
<i>Cyp2c13</i>	<i>Cyp4V3</i>	<i>Ugt1a6</i>		<i>Slc38a3</i>	<i>Slc25a5</i>	<i>Abcc2</i>	<i>Abce1</i>
<i>Cyp2c7</i>	<i>Cyp2s1</i>	<i>Ugt2b10</i>		<i>Slc41a2</i>	<i>Slc25a3</i>	<i>Abcb4</i>	<i>Abca2</i>
<i>Cyp4a2</i>	<i>Cyp20a1</i>	<i>Ugt2a3</i>		<i>Slc27a2</i>	<i>Slc25a39</i>	<i>Abce1</i>	<i>Abca3</i>
<i>Cyp2d2</i>	<i>Cyp2ab1</i>	<i>Ugt2b10</i>		<i>Slco1b2</i>	<i>Slc39a1</i>	<i>Abcc6</i>	<i>Abca1</i>
<i>Cyp2e1</i>	<i>Cyp27a1</i>	<i>Ugt8</i>		<i>Slc25a15</i>	<i>Slc12a6</i>	<i>Abca8a</i>	<i>Abcf3</i>
<i>Cyp2c23</i>	<i>Cyp4f17</i>			<i>Slc38a4</i>	<i>Slc38a10</i>	<i>Abcf1</i>	<i>Abcd1</i>
<i>Cyp2c6v1</i>	<i>Cyp2d3</i>			<i>Slc25a5</i>	<i>Slc38a2</i>	<i>Abca6</i>	<i>Abcf2</i>
<i>Cyp2c22</i>	<i>Cyp4f5</i>			<i>Slc10a1</i>	<i>Slc23a2</i>	<i>Abcd3</i>	<i>Abcc1</i>
<i>Cyp4f1</i>	<i>Cyp2u1</i>			<i>Slc16a1</i>	<i>Slco2a1</i>	<i>Abcf3</i>	<i>Abcb10</i>
<i>Cyp3a18</i>	<i>Cyp4f6</i>			<i>Slc27a5</i>	<i>Slc1a5</i>	<i>Abca3</i>	<i>Abcb4</i>
<i>Cyp2d3</i>	<i>Cyp2u1</i>			<i>Slc38a2</i>	<i>Slc9a3r1</i>	<i>Abcf2</i>	<i>Abcc5</i>
<i>Cyp27a1</i>	<i>Cyp7b1</i>			<i>Slc39a1</i>	<i>Slc25a4</i>	<i>Abcg1</i>	<i>Abca7</i>
<i>Cyp2d5</i>	<i>Cyp2r1</i>			<i>Slc3a2</i>	<i>Slc3a2</i>	<i>Abcb6</i>	<i>Abcd3</i>
<i>Cyp4a3</i>	<i>Cyp2d4</i>			<i>Slc25a47</i>	<i>Slc12a7</i>	<i>Abca2</i>	<i>Abcg3l1</i>
<i>Cyp1a2</i>	<i>Cyp2t1</i>			<i>Slco1a4</i>	<i>Slc20a1</i>	<i>Abcg3l1</i>	<i>Abca8a</i>
<i>Cyp2b1</i>	<i>Cyp4b1</i>			<i>Slc25a10</i>	<i>Slc35b4</i>	<i>Abcb10</i>	<i>Abcb1</i>
<i>Cyp2a1</i>	<i>Cyp4f18</i>			<i>Slc25a3</i>	<i>Slc48a1</i>	<i>Abcg5</i>	<i>Abcb8</i>
<i>Cyp2b3</i>	<i>Cyp4f39</i>			<i>Slc43a3</i>	<i>Slc43a2</i>	<i>Abcb7</i>	<i>Abcc9</i>

Table 20 Top 20 expressed genes identified in pig from the RNA-seq transcriptome data

CYP450		UGT		SLC Transporter proteins		ABC Transporter proteins	
Pig Liver	Pig Spleen	Pig Liver	Pig Spleen	Pig Liver	Pig Spleen	Pig Liver	Pig Spleen
<i>Cyp3a29</i>	<i>Cyp1b1</i>	<i>Ugt2b31</i>	<i>Ugt8</i>	<i>Slc27a5</i>	<i>Slc25a6</i>	<i>Abcb11</i>	<i>Abcc10</i>
<i>Cyp2e1</i>	<i>Cyp27a1</i>	<i>Ugt8</i>		<i>Slc38a3</i>	<i>Slc40a1</i>	<i>Abca6</i>	<i>Abcf1</i>
<i>Cyp2a19</i>	<i>Cyp2s1</i>			<i>Slc7a2</i>	<i>Slc25a3</i>	<i>Abca8</i>	<i>Abcf3</i>
<i>Cyp2d25</i>	<i>Cyp20a1</i>			<i>Slc10a1</i>	<i>Slc11a1</i>	<i>Abcc2</i>	<i>Abca1</i>
<i>Cyp27a1</i>	<i>Cyp51a1</i>			<i>Slc37a4</i>	<i>Slc25a39</i>	<i>Abcf1</i>	<i>Abcc5</i>
<i>Cyp1a2</i>	<i>Cyp2r1</i>			<i>Slc38a4</i>	<i>Slc9a3r2</i>	<i>Abcd4</i>	<i>Abcd1</i>
<i>Cyp2c42</i>	<i>Cyp2e1</i>			<i>Slc5a6</i>	<i>Slc38a10</i>	<i>Abcf3</i>	<i>Abcg1</i>
<i>Cyp4a24</i>	<i>Cyp4v2</i>			<i>Slc26a1</i>	<i>Slc5a10</i>	<i>Abcd3</i>	<i>Abcg4</i>
<i>Cyp51a1</i>	<i>Cyp7b1</i>			<i>Slc6a12</i>	<i>Slc43a2</i>	<i>Abcb8</i>	<i>Abcc1</i>
<i>Cyp4v2</i>	<i>Cyp2U1</i>			<i>Slc17a2</i>	<i>Slc44a2</i>	<i>Abca1</i>	<i>Abcb7</i>
<i>Cyp7a1</i>	<i>Cyp2a19</i>			<i>Slc22a1</i>	<i>Slc9a3r1</i>	<i>Abcg8</i>	<i>Abcb8</i>
<i>Cyp7b1</i>	<i>Cyp3a29</i>			<i>Slc25a39</i>	<i>Slc48a1</i>	<i>Abcb7</i>	<i>Abcd4</i>
<i>Cyp1a1</i>	<i>Cyp2j34</i>			<i>Slc22a7</i>	<i>Slc35C2</i>	<i>Abca3</i>	<i>Abce1</i>
<i>Cyp2b22</i>	<i>Cyp4a24</i>			<i>Slc39a14</i>	<i>Slc12a4</i>	<i>Abcc10</i>	<i>Abca3</i>
<i>Cyp4f8</i>	<i>Cyp39a1</i>			<i>Slc6a20</i>	<i>Slc49a3</i>	<i>Abcb1</i>	<i>Abcg2</i>
<i>Cyp2u1</i>	<i>Cyp2d25</i>			<i>Slc2a2</i>	<i>Slc25a29</i>	<i>Abcg5</i>	<i>Abcb1</i>
<i>Cyp39a1</i>	<i>Cyp11a1</i>			<i>Slc25a6</i>	<i>Slc38a2</i>	<i>Abcd1</i>	<i>Abca5</i>
<i>Cyp2j34</i>	<i>Cyp26b1</i>			<i>Slc46a3</i>	<i>Slc12a7</i>	<i>Abce1</i>	<i>Abcc3</i>
<i>Cyp8b1</i>	<i>Cyp2b22</i>			<i>Slc25a4</i>	<i>Slc44a1</i>	<i>Abcg4</i>	<i>Abca7</i>
<i>Cyp26a1</i>	<i>Cyp46a1</i>			<i>Slco1a2</i>	<i>Slc25a28</i>	<i>Abcc8</i>	<i>Abcb9</i>

Drug transporters (efflux transporters) that mediate the export of drugs or drug metabolites out of cells (e.g. *ABCB1*, *ABCG2*, *ABCC2*, *SLC47A1*, *ABCB11*), and transporters (uptake transporters) that mediate the uptake of drugs into cells (e.g. *SLCO1B1*, *SLCO1B3*, *SLC22A1*, *SLCO1A2*) have a specific pattern of tissue expression in human and are important for drug disposition and drug-drug interactions (König et al., 2013). The gene expression of the efflux pump *ABCB1/Abcb1* also known as P-glycoprotein is observed as one of the top expressed gene across rat, pig, and human spleen. Pig spleen additionally, also expresses the bile salt efflux pump *Abcg2* gene (breast cancer resistance protein, BCRP) in its top 20 expressed

genes. In contrast, pig and rat liver expressed *Slc22a* (Oct1), *Slco1a* (Oat1a), *Slco1b* (Oatp1b) in its top 20 genes respectively, but none of the known major contributing uptake transporters were observed in the top 20 expressed genes in human spleen.

Table 21 Top 20 expressed genes identified in human spleen obtained from the RNA-seq transcriptome data on ARCHS⁴ database

CYP450	UGT	SLC Transporter proteins	ABC Transporter proteins
Human Spleen			
<i>CYP20A1</i>	<i>UGT3A2</i>	<i>SLC2A3</i>	<i>ABCC1</i>
<i>CYP51A1</i>	<i>UGT8</i>	<i>SLC38A1</i>	<i>ABCE1</i>
<i>CYP1B1</i>	<i>UGT1A1</i>	<i>SLC25A6</i>	<i>ABCF1</i>
<i>CYP4V2</i>	<i>UGT2B17</i>	<i>SLC25A3</i>	<i>ABCA7</i>
<i>CYP2R1</i>	<i>UGT2B4</i>	<i>SLC38A2</i>	<i>ABCA2</i>
<i>CYP3A5</i>	<i>UGT1A6</i>	<i>SLC25A5</i>	<i>ABCD4</i>
<i>CYP2U1</i>	<i>UGT1A7</i>	<i>SLC20A1</i>	<i>ABCB1</i>
<i>CYP2S1</i>	<i>UGT2B11</i>	<i>SLC44A2</i>	<i>ABCC5</i>
<i>CYP7B1</i>	<i>UGT3A1</i>	<i>SLC3A2</i>	<i>ABCF3</i>
<i>CYP46A1</i>	<i>UGT1A3</i>	<i>SLC9A3R1</i>	<i>ABCA1</i>
<i>CYP27A1</i>	<i>UGT2A1</i>	<i>SLC35E2B</i>	<i>ABCB7</i>
<i>CYP1B1-AS1</i>	<i>UGT2B10</i>	<i>SLC40A1</i>	<i>ABCF2</i>
<i>CYP2D7</i>	<i>UGT2B15</i>	<i>SLC7A5</i>	<i>ABCC10</i>
<i>CYP4A22-AS1</i>	<i>UGT1A10</i>	<i>SLC5A3</i>	<i>ABCG1</i>
<i>CYP4F12</i>	<i>UGT2B7</i>	<i>SLC25A39</i>	<i>ABCA5</i>
<i>CYP4F3</i>	<i>UGT2B28</i>	<i>SLC1A5</i>	<i>ABCD3</i>
<i>CYP2E1</i>	<i>UGT2B27P</i>	<i>SLC25A36</i>	<i>ABCB10</i>
<i>CYP2D6</i>	<i>UGT2B25P</i>	<i>SLC38A10</i>	<i>ABCB8</i>
<i>CYP1A2</i>	<i>UGT1A5</i>	<i>SLC7A6</i>	<i>ABCF2-H2BK1</i>
<i>CYP4F8</i>	<i>UGT2B24P</i>	<i>SLC35A4</i>	<i>ABCC4</i>

3.2.3 Gene expression analysis using qPCR

Six selected genes including two control genes respectively (*Cyp2a19*, *Cyp2d25*, *Cyp2e1*, *Cyp3a29*, *Ugt8*, *Abcb1*, β -actin and GAPDH) were analysed for gene expression in primary pig splenocytes using RT-qPCR. These genes have shown to be in the top 20 expressed genes in pig, and this experiment would validate the expression observed from the pig spleen tissue NGS analysis. Relative gene expression of the tested CYP450, UGT and transporter protein genes was confirmed in viable splenocytes (see Table 4) from the RT-qPCR analysis, but the assay required further optimisation. Apart from GAPDH in pig hepatocytes, *Abcb1* and β -actin in pig splenocytes, the amplification efficiency for most of the tested samples was < 90 % (see Table 22-23). *Ugt8* and *Abcb1* in pig hepatocytes, *Cyp2a19* and *Cyp2d25* in pig splenocytes showed > 105 % amplification efficiency indicating a possible co-amplification of non-specific products. Overall, the $R^2 > 0.980$ was observed and is within the linear range across all samples except for *Ugt8*, which might indicate poor primer design or suboptimal reaction conditions.

The melt curve analysis as a function of temperature was implemented at the end of the RT-qPCR reaction to validate the specificity of the primers used by identifying any different reaction products including non-specific products present. For each gene sample, a prominent single peak curve and aligned at the same position should indicate specificity (see Figure 24 and appendix Figure A3-A7). The spacing between each amplification standard is important for ensuring that no pipetting errors had occurred with the preparation of the cDNA template serial dilutions. In pig hepatocytes, only *Cyp2d25* and *Cyp3a29* showed a robust and reproducible assay; while some showed a good amplification curves in pig splenocytes (e.g. *Abcb1*, *Cyp2d25*, *Cyp3a29*), only *Cyp2e1* showed good primer specificity.

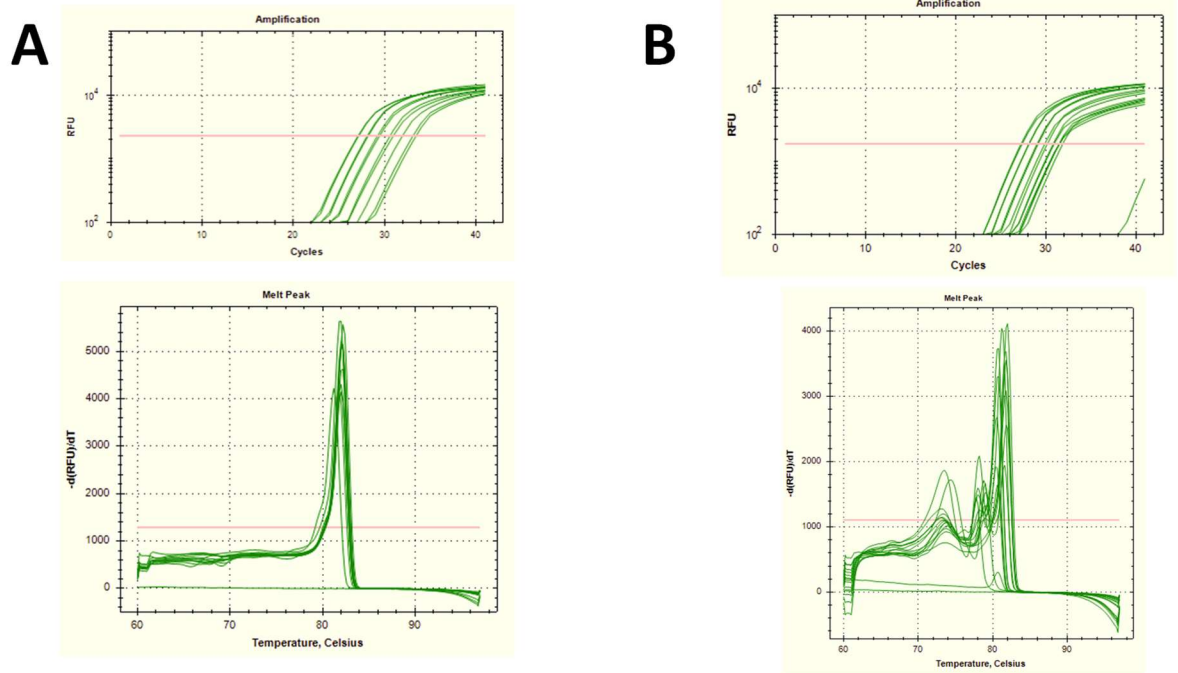


Figure 24 Example of gene expression by RT-qPCR: the amplification curve and melt peak of *Cyp2d25* gene in **A.** pig hepatocytes **B.** pig splenocytes. The accumulated target DNA (expressed as fluorescence) is detected and measured as the reaction progresses. C_T values define the intersection between the amplification curve and the threshold line, which becomes a relative measure of the concentration of the target in the PCR reaction. The red line of the amplification curve indicates the threshold point where a significant increase of fluorescence above the baseline is detected; therefore, the amplification curve represents the accumulation of the DNA template across the whole qPCR experiment. Information sourced from www.goldbio.com.

A Pfaffl method (the amplification efficiency of the target gene, E_{target}) was applied using the C_T values to normalise the gene expression data using a combined mean C_T values of the two reference genes (GAPDH and β -actin) by generating a gene expression ratio ($2^{-\Delta C_T(\text{target}-\text{reference})}$) as shown in Figure 25. C_T values corresponds to the detectable fluorescence signal above background for the expressed gene.

Table 22 Relative gene expression (C_T values) of selected genes in primary pig hepatocytes

cDNA Dilution	Sample #	Target genes, Mean C_T values						Reference genes, Mean C_T values	
		Cyp2a19	Cyp2d25	Cyp2e1	Cyp3a29	Ugt8	Abcb1	β -actin	GAPDH
1/2	Sample 1	29.5	27.1	28.1	27.6	33.3	33.1	27.7	26.8
1/4	Sample 2	33.4	28.1	29.4	29.1	35.2	35.5	29.0	27.8
1/8	Sample 3	35.4	29.3	30.7	31.5	40.2	34.8	30.4	29.1
1/16	Sample 4	35.5	30.8	32.4	33.8	36.8	34.4	31.6	30.4
1/32	Sample 5	39.7	32.0	34.0	37.4	38.2	36.8	34.6	31.5
1/64	Sample 6	38.4	33.3	35.4	38.0	36.3	36.0	37.8	31.8
% Efficiency		50.4	75.7	63.8	32.6	190	157	43.6	97.7
R^2		0.829	0.997	0.993	0.990	0.244	0.838	0.974	0.978

Table 23 Relative gene expression (C_T values) of selected genes in primary pig splenocytes

cDNA Dilution	Sample #	Target genes, Mean C_T values						Reference genes, Mean C_T values	
		Cyp2a19	Cyp2d25	Cyp2e1	Cyp3a29	Ugt8	Abcb1	β -actin	GAPDH
1/2	Sample 1	29.5	27.3	25.7	26.5	36.9	26.5	23.5	20.3
1/4	Sample 2	31.4	28.1	27.0	27.8	37.1	27.6	24.8	21.3
1/8	Sample 3	31.5	29.1	28.4	29.0	36.2	28.9	25.7	22.8
1/16	Sample 4	31.6	30.0	29.9	30.2	35.5	29.8	26.7	23.6
1/32	Sample 5	31.6	30.8	31.4	31.6	37.2	31.3	27.9	25.5
1/64	Sample 6	32.1	31.6	33.3	33.4	34.7	31.7	29.6	26.5
% Efficiency		372	115	61.0	70.1	-66.2	92.8	90.6	75.3
R^2		0.709	0.997	0.996	0.996	0.611	0.995	0.990	0.975

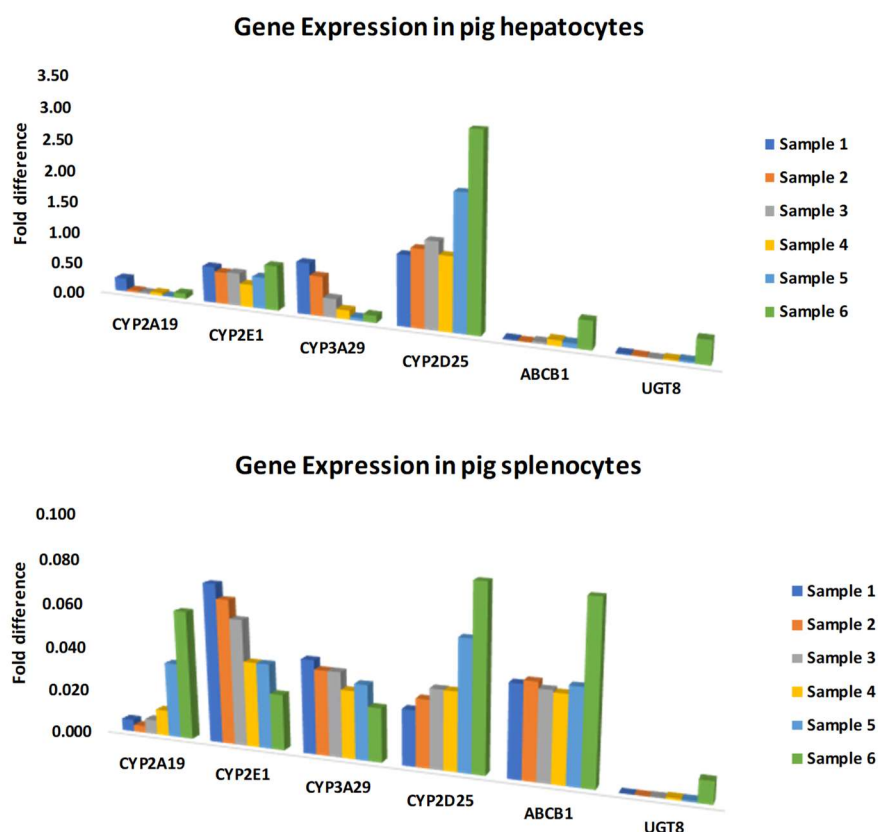


Figure 25 Normalised relative gene expression in pig hepatocytes and splenocytes using the Pfaffl method ($2^{-\Delta C_T}$) to determine relative quantitation (RQ) of gene expression for the tested genes (*Cyp2a19*, *Cyp2e1*, *Cyp3a29*, *Cyp2d25*, *Abcb1*, *Ugt8*). The fold change or difference of RQ is compared to the calibrator (i.e. reference genes, β -actin and GAPDH). The RQ value for the calibrator is 1, and the RQ is deemed significant where there is minimum of two-fold change. Information sourced from Genomic Platform (www.genomique.irc.ca).

3.3 Discussion

The liver was used in all experiments alongside the spleen to provide an expected profile of a gene expression from a positive control perspective. The initial mRNA gene analysis using standard PCR was carried out at first on both rat and pig liver to confirm positive CYP450 gene expression and assess the working conditions of the assay. Confirmation of the CYP450 gene expression for all the selected genes but one was observed in rat liver, whereas in pig liver only a few of the selected genes showed gene expression. As the selected CYP450 genes were chosen based on the literature search of CYP450 genes expressed in rat and pig, there was little consideration that the tissue used may not express the gene tested. Therefore, this halted the same experiment in pig spleen but continued with the experiment in rat spleen. Only three genes showed no expression in rat spleen which included the same gene (*Cyp2d1*) not expressed in rat liver. This provided a strong indication that the gene expression observed in rat spleen was valid. However, to further validate these observations, the designed primers used were tested for their specificity for the CYP450 genes of interest by Sanger sequencing and mapping to the reference sequenced genome on NCBI database. With a percent identity $\geq 94\%$ and a low E value for the majority of the selected CYP450 genes tested in rat liver and spleen tissues, there is reasonable confidence in the CYP450 gene expression observed in rat spleen.

Although, NGS is considered second to Sanger sequencing as a 'gold standard' for clinical research sequencing in DNA or RNA genetic studies, it has a higher throughput capability and a fast turnaround time (ThermoFisher Scientific, UK) (McCourt et al., 2013). NGS analysis of rat and pig (liver and spleen tissues) was used to produce an RNA-seq transcriptome in both species. This would enable the identification of all genes expressed in the tested tissue using

the Ensembl genome database as reference. The bioinformatic data analysis of the untreated tissues identified the genes associated with drug metabolism and distribution and their counts (or expression levels) based on the relative abundance of the mRNA. This revealed that CYP450 and UGT genes were highly expressed in liver compared to the spleen in both specie, which could largely be due to the size of the organs and the proportion of the enzymes contained within them. Comparable gene expression levels of transporter proteins were observed across the tissues in both rat and pig including human spleen. Genome-wide association studies (GWASs) have showed that genes coding for a variety of transporters are susceptible genes for many diseases; therefore, understanding the physiological roles of transporters in normal and patho-physiological conditions is important for the development of new drugs and therapies (Liang et al., 2015). It is interesting that the identified top 20 expressed genes of CYP450, UGT and transporters were different (plus ranking position based on expression levels) across specie including human spleen. However, the known major metabolic contributors observed in human liver, were also identified in rat, pig and human spleen. Also, *Cyp2d1* was not identified by NGS as a gene expressed in both the rat liver and spleen tissue tested which confirmed (no expression present for the gene in) the initial gene expression observations by standard PCR. In addition, most of the selected genes analysed in pig liver and spleen via standard PCR were not identified in the tested pig tissues by NGS and confirms the reasons behind no expression was observed with the standard PCR analysis. Not only are genes of interests including isoforms identified in the NGS analysis, the RNA-seq transcriptome also reveals the identity of promoter genes that govern or regulate the expression of CYP450, UGT and transporter genes. For example, nuclear receptors like Aryl Hydrocarbon Receptor (AhR), Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) which are known to promote the expression of genes involved in xenobiotic metabolism in human

and are identified in both rat, pig and human spleen. Moreover, the promoter genes Nuclear Receptor Subfamily 1, Group I, Member 3 (NR1I3) and (NR1I2), as part of the CAR (Constitutive Androstane Receptor) protein are known to be key regulators of transcriptional target genes (like CYP450) involved in drug metabolism. These genes were identified in pig and human spleen with low expression levels but was not identified in rat spleen, though the CAR protein is moderately expressed in rat spleen. Other identified promoter genes include Hepatic Nuclear Factor 4 Alpha (HNF4 α) in pig spleen, Hepatic Nuclear Factor 1 Alpha (HNF1 α) in rat spleen, and both expressed in human spleen in which are known to activate transcription of *Cyp2c38* and *Cyp1a2*, *Cyp2e1*, *Cyp3a11*, respectively.

Gene expression study was tested in a different matrix to further validate the expression observed in tissues. Pig hepatocytes and splenocytes were analysed for the relative gene expression and hence confirmed the gene expression that was previously observed in pig tissues by NGS. The CYP450, UGT and transporter genes were selected based on the NGS analysis. The prepared primary cells were checked for viability $\geq 75\%$ prior to isolating total RNA. The quality of extracted RNA is important for sensitivity and specificity of an experiment; therefore, it is crucial that a robust, reliable, reproducible protocol is developed for extracting high quantities of RNA with acceptable quality (Gandhi et al., 2020). It appears that there is a significant correlation between RNA quality and yield that may cause variations in gene expression levels, thereby suggesting that RNA yield is also a dominant factor to be considered for gene profiling studies (Gandhi et al., 2020; Walker et al., 2016). 'Pure' total RNA (given that both A260/280 and A260/A230 ratio is 2) and the synthesised cDNA (of acceptable yield e.g. pig spleen 424 and 1876 ng/ μ L respectively) were achieved, however due to resources constraints, the RNA RIN could not be determined and verified. The experiment with the selected genes were tested at n=2 replicates in a RT-qPCR reaction using

the same designed primers in the initial standard PCR analysis. It was noted that optimal RT-qPCR primers should be in the range between 75 – 200 bp so that a higher amplification efficiency can be achieved and for it to be distinguishable from primer-dimers, but the used primers were above 200 bp. Overall, time limitations also impacted in producing optimal reaction conditions for the tested genes of interest (*Cyp2a19*, *Cyp2d25*, *Cyp2e1*, *Cyp3a29*, *Ugt8*, *Abcb1*). It was somewhat clear that all the selected genes indicated gene expression to an extent, but the assay required further investigation.

In summary, this gene expression study has highlighted the genetic make-up of the rat and pig spleen and what constitutes as stored genetic information of an organism. It does not necessarily mean that the expressed genes would translate to functional genes, and there is a high probability that some expressed genes would remain benign or dormant. However, it signifies the importance of understanding what regulates gene expression since that appears to govern the activation of specific gene expression and perhaps in addition the same dormant genes (for example by nuclear receptors that regulate CYP450 or UGT expression). What is known is that some RNA (e.g. microRNAs) are key for regulating gene expression to uphold normal homeostasis by aiding the body to maintain a stable internal environment. Therefore, gene expression studies are indirectly good markers for functional drug metabolism; genes that are embedded in the genetic code of an organism becomes a strong indicator that those genes would highly likely be translated to functional gene products.

CHAPTER 4:

**Investigating the protein expression of
CYP450 and UGT enzymes associated with
drug metabolism and distribution in the
spleen tissue**

4.0

4.1 Introduction

With the completion of the Human genome project, defining the biological significance and the approx. 30, 000 human gene products (e.g. proteins) have been critical to clinical human health research (Ferguson et al., 2005). Genetic information stored in DNA is translated into proteins (involving mRNA molecules) to become a functional gene (Guo et al., 2008). Proteins are made from a chain of amino acids that are covalently linked by peptide bonds, and these precise sequence of amino acids determines both its 3D structure and resulting function in a cell (Alberts et al., 2002; Laura Vargas-Parada, 2014). Proteins are also known as polypeptides and called ‘the building blocks of life’ since they are involved in almost all processes of life by carrying out various biological functions (Alberts et al., 2002; Guo et al., 2008; Marth, 2008).

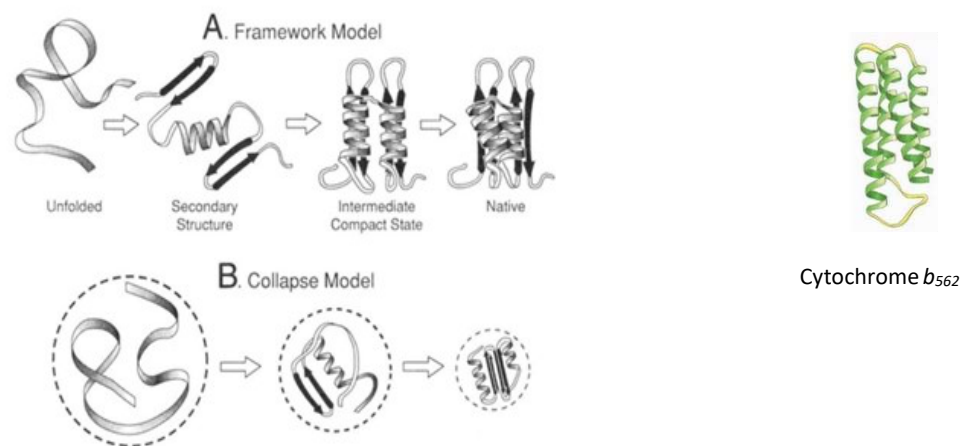


Figure 26 Protein folding: **A.** folding driven by local interactions **B.** folding driven by non-local interactions. Example of a protein structure (cytochrome *b*₅₆₂) composed entirely of α -helices (Alberts et al., 2002; Dill, et al., 1995).

The protein ‘folding’ is an essential element of its structure (see Figure 26). Local and non-local interactions among neighbouring amino acids play a key role in protein folding, where the former is dominated by hydrogen bonding and the latter by hydrophobic forces (Dill, et al., 1995). The protein structure dictates where the protein will act and what it will do, making the study of gene expression also a focus on where the expression is modulated in the cell, and the impact of proteins and RNA molecules in those regions (Alberts et al., 2002; Laura Vargas-Parada, 2014). Human diseases (including Alzheimer’s disease, Huntington’s disease, Parkinson’s disease etc.) show a consequential link with defects or mutations in the protein’s 3D structure, therefore highlighting that changes at the molecular level has a reverberating impact (Laura Vargas-Parada, 2014). Any alteration of the amino acid sequence in a protein create changes in the folding and stability of the protein (Prabantu et al., 2021). However, it may not necessarily alter the protein’s function, and instead it may develop its susceptibility to a disease (Prabantu et al., 2021).

Protein molecules and non-coding RNA molecules are known to be involved in a very large and complex regulation system including controlling the activity of complexes involved in transcription, post-transcription and ‘feedback’ regulation (Gokhale et al., 2011). In addition, proteins mediate the regulation of its own expression levels by controlling both the elongation phase of translation and the process of protein degradation (Gokhale et al., 2011). The intricate details of the complex regulatory processes involving proteins are not fully understood, but there is an assumption that the mRNA expression (or abundance of mRNA) is a prediction of protein expression (Guo et al., 2008). Apparently both mRNA and protein expression provide useful readouts in connecting genes and phenotype (Buccitelli & Selbach, 2020). Positive correlation between differentially expressed mRNA and protein expression levels has been demonstrated in a few studies to an extent, indicating that measurements of

the abundance of mRNA is directly linked to the expression of proteins (Guo et al., 2008; Koussounadis et al., 2015).

In protein profiling studies, detection techniques like western blotting (or immunoblotting) and immunohistochemical staining are most commonly used for the determination of protein expression in isolated cells or tissues. In western blotting, cell or tissue protein lysates are denatured by boiling, fractioned using SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel), and the proteins are transferred to a membrane to be identified using a specific antibody (Liu et al., 2016). Proteins binds to SDS in a uniform way as they migrate in the presence of an electric field; thus, making the proteins become polyanion and the negative charges supplied by the sulphate group in SDS to be directly proportional to size or molecular weight of the protein (Polyak K & Meyerson M, 2003). SDS-PAGE is an important integral component of western blotting technique as it also tests the purity of the protein preparation with use of the reducing agent β -mecaptoethanol that binds separate proteins together (Polyak K & Meyerson M, 2003). Proteins generally remain bound to a membrane support and when exposed to a solution containing a specific antibody, the antibody binds to the protein of interest. The bound protein can be detected and visualised if the antibody is labelled with a catalysing colour or light-emitting reaction (Polyak K & Meyerson M, 2003). A protein marker (or standard) is used in SDS-PAGE alongside the protein lysate samples as a molecular weight calibrator for the protein in kilo-Dalton (kD).

Immunohistochemistry (IHC or IHC-P) or immunocytochemistry (ICC) on the other hand involves the labelling of cellular proteins in tissue samples or cells with a specific primary antibody (either monoclonal or polyclonal antibody) and visualisation of the target protein of interest with detection reagents (Cell Signaling Technology, 2024). Immunohistochemistry

and immunofluorescence (IF) are powerful imaging methods and invaluable in clinical histopathological diagnosis. They enable the detection of localised, distributed and abundance of proteins at cellular and intracellular level (Shetty et al., 2017).

The IHC-P method suffers from high background staining resulting from non-specific antibody binding either from endogenous biotin or peroxidase activity (Magaki et al., 2019). This can be decreased by blocking the tissue sections with normal serum from the same species and using an inhibitor containing hydrogen peroxide to curb endogenous enzyme activity (Magaki et al., 2019). Although IHC/ ICC is known for its ability to visualise subcellular localisation/co-localisation of protein of interests (to access protein expression and post-translational modifications), the technique cannot distinguish non-specific protein(s) from the target protein based on the theoretical molecular mass of the protein of interest (Liu et al., 2016). For this reason, both western blotting and IHC studies are used together and seen as a 'golden pair' in biomedical research from the useful information that is obtained determining protein products to further understand gene function and its underlying mechanisms (Liu et al., 2016).

Other techniques used to detect and analyse protein expression includes ELISA (enzyme-linked immunosorbent assay), Co-IP or IP (co-immunoprecipitation/ immunoprecipitation), sequencing, mass spectrometry, and the use of engineered protein expression systems. One of the methods used in this study includes the Co-IP method which uses a specific antibody as a probe to detect a certain protein in a complex mixture of proteins (Polyak K & Meyerson M, 2003). An antibody directed against a protein of interest is added to a mixture of proteins that can bind to the protein of interest through pre-mediated conditions; subsequently all the immunoglobulins are collected in that mixture using an antibody that binds to the immunoglobulins such as Staphylococcal protein A (Polyak K & Meyerson M, 2003). The

proteins with their immunoglobulins are bound to a solid support such as polystyrene beads or a resin and are removed by gentle centrifugation to be eluted in solution (Polyak K & Meyerson M, 2003). Immunoblotting method is incorporated here, as boiling the protein solution containing β -mecaptoethanol, not only allows the protein complexes to dissociate but to be electrophoretically separated and identified through SDS-PAGE and staining reactions respectively (Polyak K & Meyerson M, 2003). Co-IP can be paired with mass spectrometry to further assess the specificity of the antibody through a peptide digestion and quantitation process to identify the polypeptide sequences of the protein(s) in solution. This is mapped to a known or reference peptide sequences on a database to provide the identity of the protein or target protein of interest based on the acquired peptide sequence.

This study section aims to examine the protein expression of CYP450, UGT and transporter proteins identified from the RNA-seq transcriptome data that was obtained by the NGS analysis in the spleen tissue. In addition, to ascertain whether the determined protein levels if any, in the spleen tissue are comparable to the liver (control) tissue.

4.2 Results

4.2.1 Protein expression of metabolic enzymes and proteins using Western Blotting

Selected CYP450, UGT enzymes and transporter proteins were examined in a western blot analysis to determine the protein expression in the spleen tissue. Polyclonal antibodies (see Table 24) were used as a probe to bind to the target proteins from rat in three individual animals and pig in four individual animals (liver and spleen tissues) respectively. Figure 27A demonstrates the protein standard used for the (relative) quantification of the target protein

of interest based on size and, Figure 27B visually represents the total protein contained in the tested samples after gel electrophoresis.

Table 24 List of primary antibodies and protein sizes obtained from the Human protein atlas database

Polyclonal antibody	ThermoFisher Catalogue #	Protein size (kDa)
CYP450 generic	AD1-MFO-100-F	between 50 and 55*
CYP20A1	PA5-109611	52.4
CYP27A1	PA5-75271	60.2
CYP2E1	PA5-35351	56.8
UGT8	PA5-37282	61.4
MRP7/ABCC10	PA5-75402	158.8
SLC25A6	PA5-120868	32.9
P-gp/ABCB1	PA5-119958	141.5
b-actin	PA1-46296	41.7
UGT1A6	PA5-116726	60.7
SLC22A6	PA5-115910	60.3

*manufacturer validated given protein size

Across the tested antibodies in rat and pig, non-specific binding was observed and particularly seen with ABCB1/P-gp probe antibody. The proteins sizes obtained from the human protein atlas database provided guidance to the expected protein sizes observed from rat and pig protein lysates. For most of the antibodies, the manufacturer did not specify or give an actual protein size for the supplied antibodies. *Cyp2e1* showed to be expressed in all the pig liver samples including liver microsomes sample at the theoretical protein size of 57 kDa, but bands

expressing the enzyme were observed at ≥ 72 kDa for all the pig spleen tissue lysate samples. In rat, all the liver and spleen tissue lysate samples showed varying intensity of bands expressing *Cyp2e1* apart from the spleen microsomes sample. Similarly, in rat, this is observed for *Cyp27a1* whereas, *Cyp20a1* and *Ugt8* seems not to be expressed in both the liver and spleen microsomes sample (see Figure 28-31). Expression of *Cyp20a1*, *Cyp27a1* and *Ugt8* almost appear identical in the pig tissue lysate samples, except that pig liver and spleen microsomes were not expressing *Cyp20a1*.

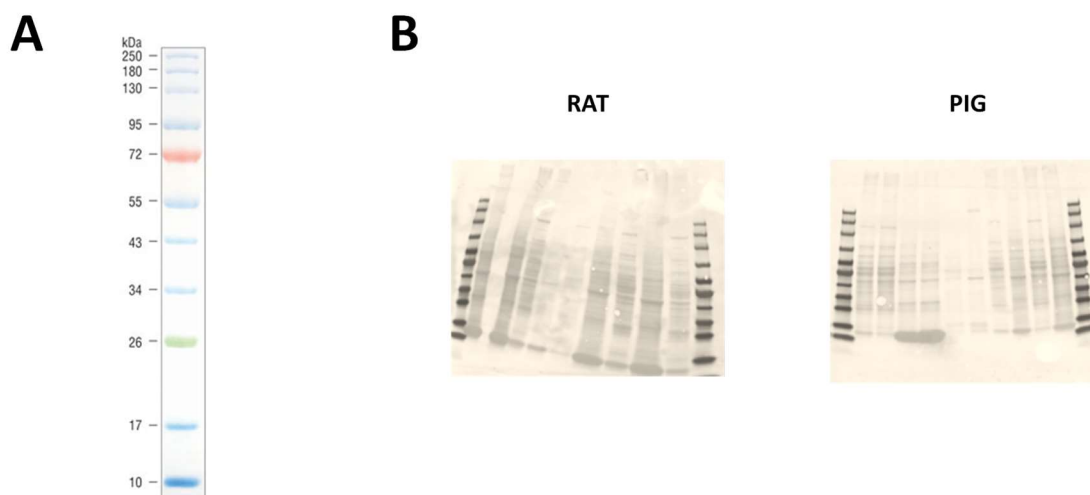


Figure 27 Total protein identification: **A.** 10-250kDa pre-stain protein marker or standard, **B.** rat and pig proteins identified on a nitrocellulose membrane resolved via SDS polyacrylamide gel electrophoresis.

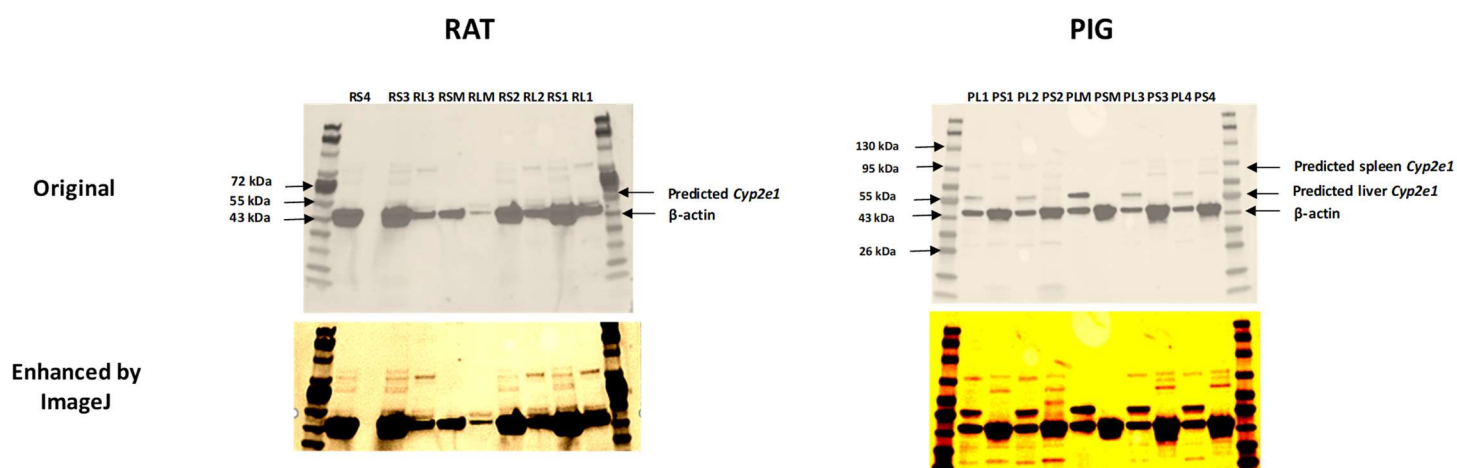


Figure 28 Western Blot analysis via SDS-PAGE of *Cyp2e1* probe in rat and pig protein (tissue) lysates with loading control β-actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

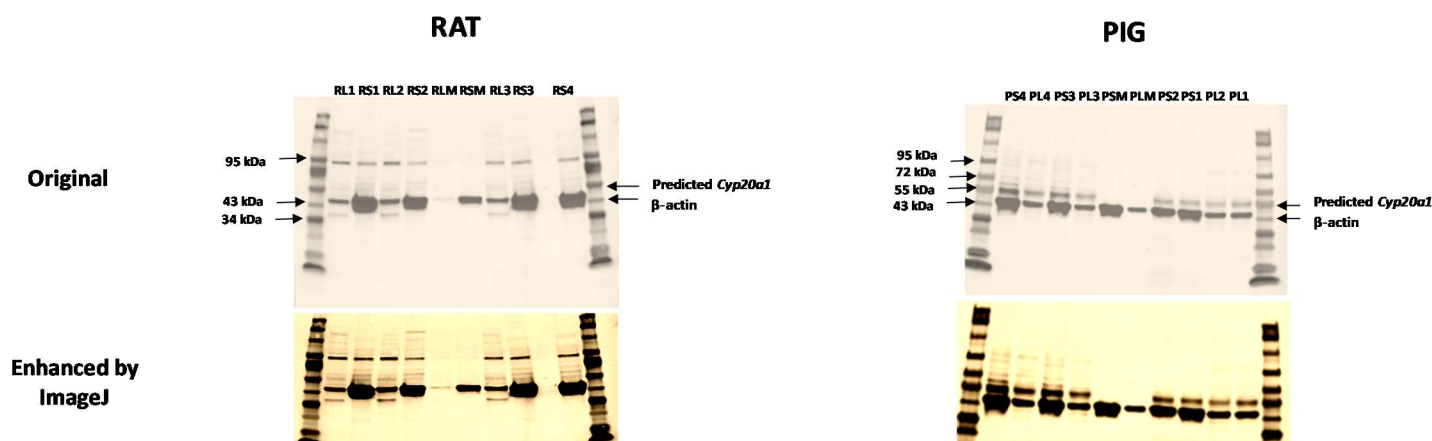


Figure 29 Western Blot analysis via SDS-PAGE of *Cyp20a1* probe in rat and pig protein (tissue) lysates with loading control β-actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

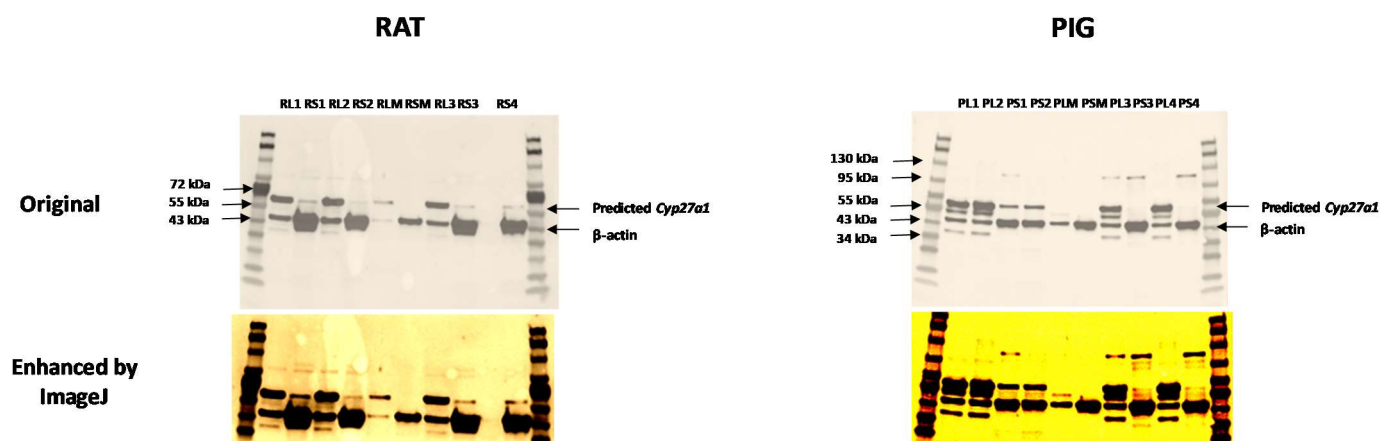


Figure 30 Western Blot analysis via SDS-PAGE of *Cyp27a1* probe in rat and pig protein (tissue) lysates with loading control β -actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

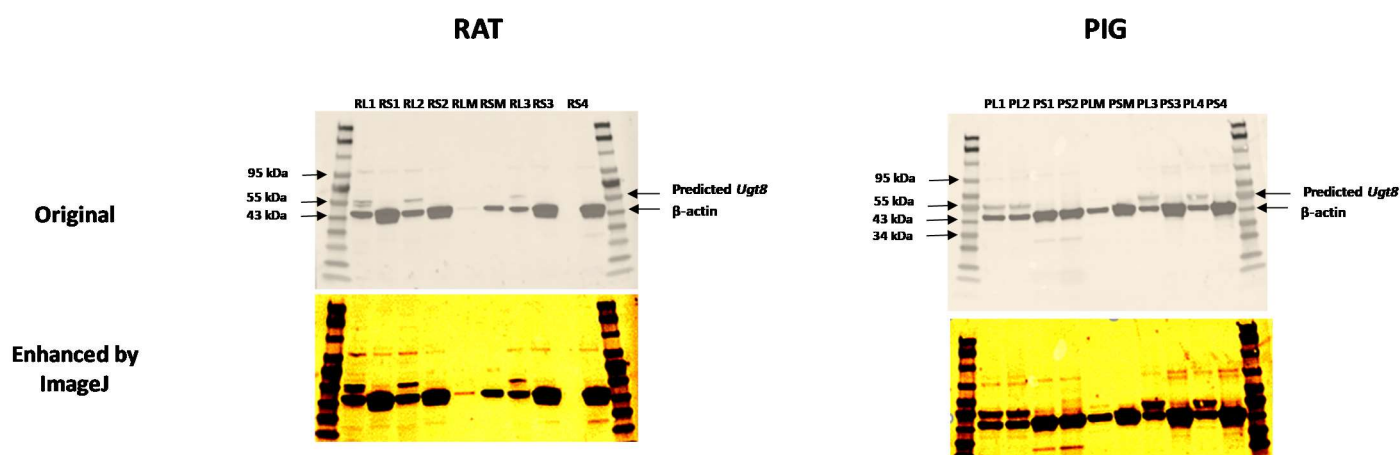


Figure 31 Western Blot analysis via SDS-PAGE of *Ugt8* probe in rat and pig protein (tissue) lysates with loading control β -actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

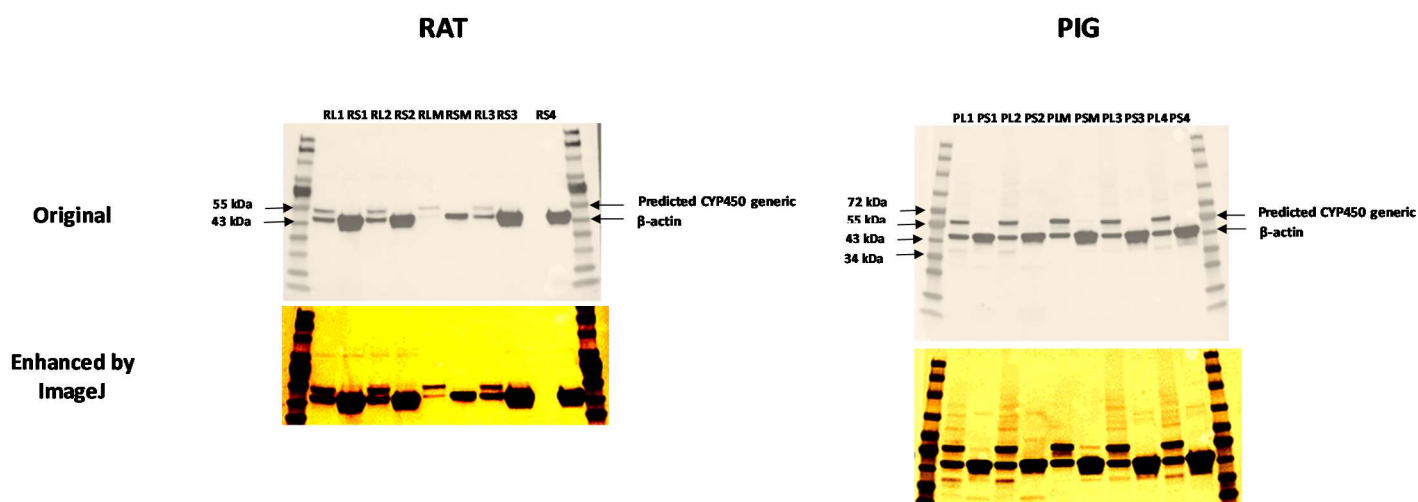


Figure 32 Western Blot analysis via SDS-PAGE of CYP450 generic probe in rat and pig protein (tissue) lysates with loading control β -actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

Although, several bands were observed to express the CYP450 generic probe antibody, very intense bands were observed in all the rat and pig liver tissue lysate samples include the liver microsomes sample around 55 kDa (see Figure 32). Faint bands expressing CYP450 generic were also observed in three pig spleen tissue lysate samples including the pig spleen microsomes sample, but in the rat, expression was only observed in three rat spleen tissue lysate samples. The expression study targeting the selected transporter proteins (*Abcb1*, *Abcc10* and *Slc25a6*) was somewhat ambiguous as strong non-specific binding was observed from the blots. The selected transporter proteins were identified in the NGS analysis, with *Abcc10* and *Slc25a6* at the top 20 of expressed transporter genes in pig spleen. For two pig liver tissue lysates targeting *Abcb1*, protein expression was unclear due to smear signal

observed in the corresponding lanes indicating very high protein concentration detected, but two strong bands were observed in the other pig liver tissue lysates including a third faint band for the liver microsomes sample (see Figure 33).

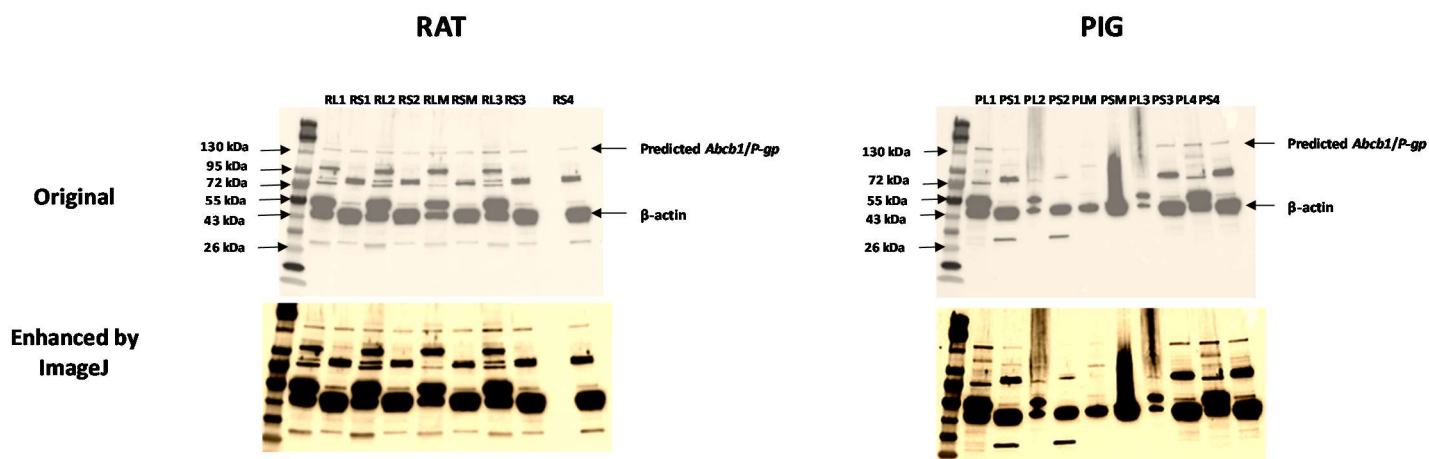


Figure 33 Western Blot analysis via SDS-PAGE of *Abcb1/P-gp* probe in rat and pig protein (tissue) lysates with loading control β -actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

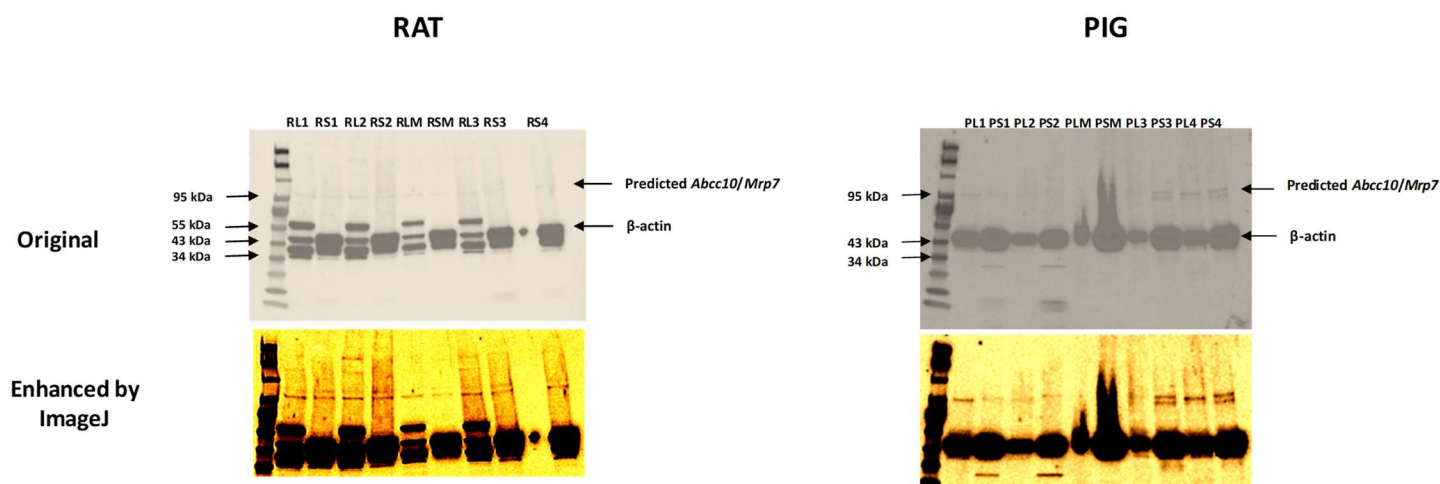


Figure 34 Western Blot analysis via SDS-PAGE of *Abcc10/Mrp7* probe in rat and pig protein (tissue) lysates with loading control β -actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

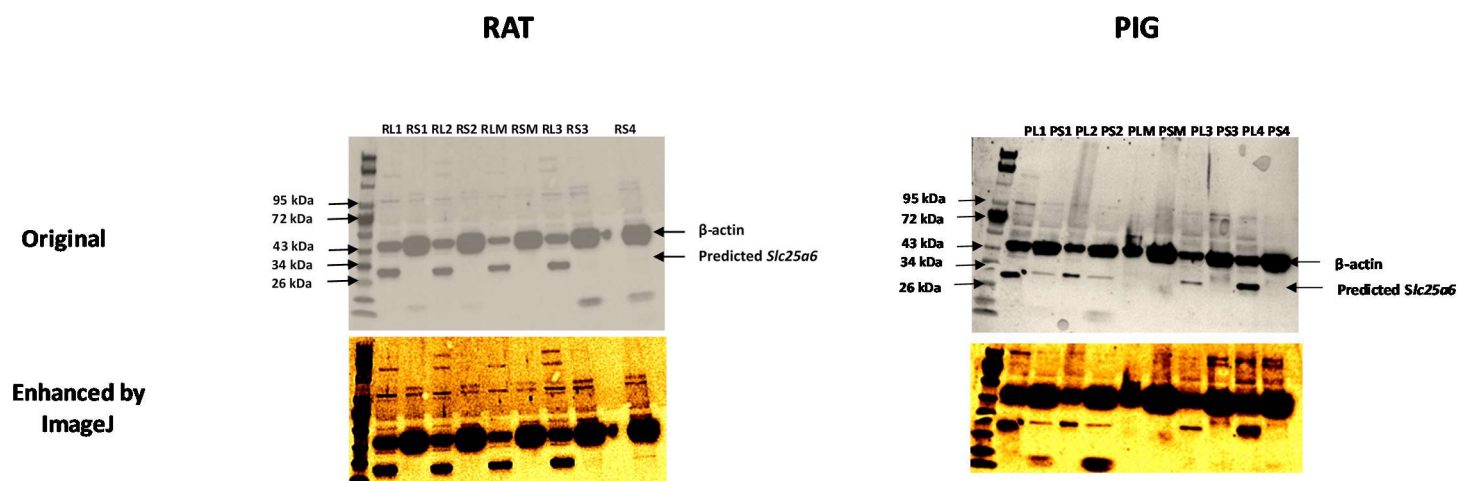


Figure 35 Western Blot analysis via SDS-PAGE of *Slc25a6* probe in rat and pig protein (tissue) lysates with loading control β -actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

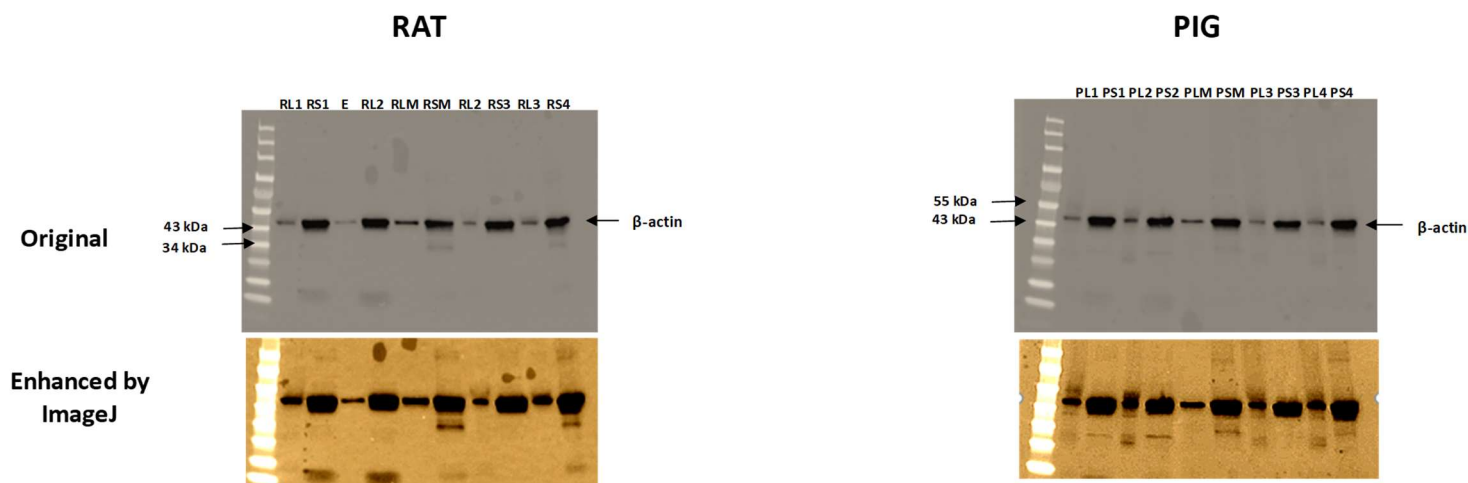


Figure 36 Western Blot analysis via SDS-PAGE of Negative (no primary antibody) control in rat and pig protein (tissue) lysates with loading control β -actin. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>). Pig liver animal 1 (PL1), Rat liver animal 1 (RL1), Pig spleen animal 1 (PS1), Rat spleen animal 1 (RS1), Pig liver animal 2 (PL2), Rat liver animal 2 (RL2), Pig spleen animal 2 (PS2), Rat spleen animal 2 (RS2), Pig liver animal 3 (PL3), Rat liver animal 3 (RL3), Pig spleen animal 3 (PS3), Rat spleen animal 3 (RS3), Pig liver animal 4 (PL4), Pig spleen animal 4 (PS4) and Rat spleen animal 4 (RS4).

Three moderately intense bands for *Abcb1* was observed in the pig spleen tissue lysates but was difficult to determine expression for the spleen microsomes sample due to concentrated protein signal detected. *Abcb1/P-gp* is low to moderately expressed in untreated rat, pig and human spleen as noted from the RNA-seq transcriptome analysis. Bands expressing *Slc25a6* were observed in all pig liver and spleen tissue lysates, though more intense for the liver tissue lysate samples (see Figure 35). There appears to be a discrepancy with the predicted protein size for *Abcc10/Mrp7*, the human atlas database records a protein mass at 158.8 kDa, whereas the manufacturer validated study in mouse spleen using the antibody targeting *Abcc10/Mrp7* showed a size > 180 kDa. The blot results conveyed bands at varying intensity

with 95 kDa and two bands at 34 kDa potentially expressing the protein in rat and pig (see Figure 34). The former mass was chosen as the predicted *Abcc10/Mrp7* since bands were observed in all pig spleen tissue lysates, in three pig liver tissue lysates and unclear for one pig liver lysate including both microsomes samples. However, the discrepancy of the observation of the lower protein size compared to the typical protein size (158 kDa) for *Abcc10/Mrp7* could be explained that the identified protein may not be the target protein of interest and possibly needed more time to be fully resolved in the gel. Smaller proteins appear to migrate faster down the gel than larger proteins as apparent with the tested CYP450 genes, where a better resolution was established and a close homology to the calculated protein size as observed in the human atlas database.

Based on the NGS analysis, *Abcc10* was just outside the top 20 expressed genes in rat spleen and *Slc25a6* was very lowly expressed. The blots identified strong expression of *Slc25a6* in all the rat liver tissue lysates, a faint expression for just one rat spleen tissue lysate and no expression detected for the remaining rat spleen tissue lysates including the microsomes samples. Positive expression of *Abcb1/P-gp* at the expected protein size of approx. 141 kDa was observed for all rat liver and spleen tissue lysates including both (liver and spleen) microsomes samples. Due to the non-specific binding observe on the blot showing expression of *Abcc10*, it was difficult to predict the protein size in rat. Likewise (compared to pig), strong bands expressing *Abcc10* were observed at 34 and 55 kDa, present in all the rat liver tissue lysates including the liver microsome sample, and in all rat and spleen tissue lysates including both microsomes samples respectively. Faint bands potentially expressing *Abcc10* at 95 kDa was also observed in all the tested tissue lysate samples. However, it is important to note that some post translation modification (e.g. ubiquitination, glycosylation) might change the migration of a protein on a gel due to conformational changes to its structure or ‘folding’

(Proteintech®, 2024; Zhong et al., 2023). This could result in detecting the modified protein on the Western blot appearing as a new band or a different molecular weight band(s) (Proteintech®, 2024; Zhong et al., 2023). In this instance, *Abcc10/MRP7* is a higher molecular weight protein but appeared at a lower molecular weight on the western blot (Figure 34), which strongly suggest that the protein has been cleaved off by caspase-3 enzyme (endoproteases) or the antibody has detected an isoform of the protein with a shorter sequence length (Proteintech®, 2024; Zhong et al., 2023).

Figure 36 demonstrated a clean blot as a negative control, though there appears to be non-specific binding at the lower protein sizes but could have resulted from the β -actin loading control incorporated in the experiment to ensure the assay conditions were working as expected.

4.2.2 Immunohistochemistry analysis in rat and pig

Immunohistochemistry staining was performed in rat and pig (liver and spleen tissue sections), to probe for *Cyp2e1*, CYP450 generic, *Ugt8*, *Ugt1a6* and *Abcb1/P-gp* protein expression including to observe where the expression is localised. The selected protein targets were chosen based on the identified genes associated with drug metabolism and distribution obtained from the NGS analysis. In pig liver, strong protein expression was confirmed for all the tested probe enzymes and transporter compared to the spleen tissue. On the other hand, a varying level of protein expression was observed in pig spleen with *P-gp* showing the highest level of protein expression and the least for CYP450 generic (see Figure 37 and 38, in appendix Figure A8-A10).

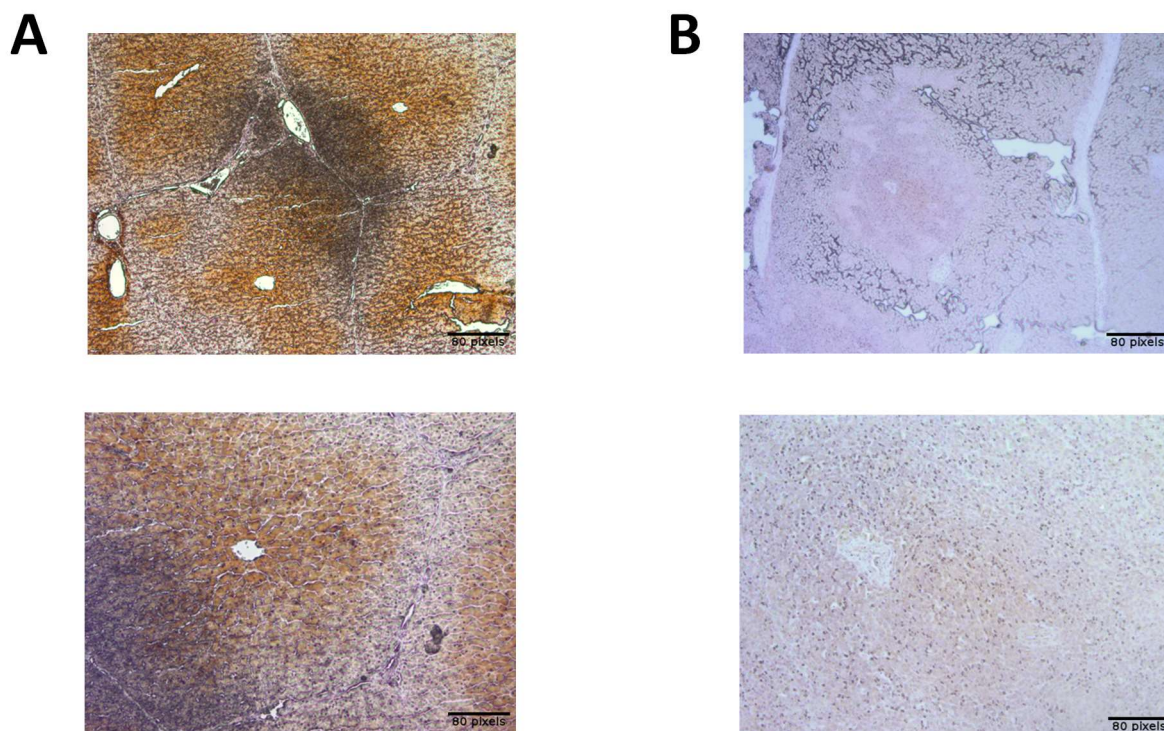


Figure 37 Example of CYP450 protein expression in pig: IHC staining probing for target CYP450 generic protein expression in **A.** pig liver tissue section and **B.** pig spleen tissue section. Two images of the selected region (with one a close-up image) were captured for both species. The brown stain represents the localised expression of the target protein of interest at the selected region captured. Lower CYP450 expression observed in pig spleen compared to pig liver. Scale bar is at 80 pixels.

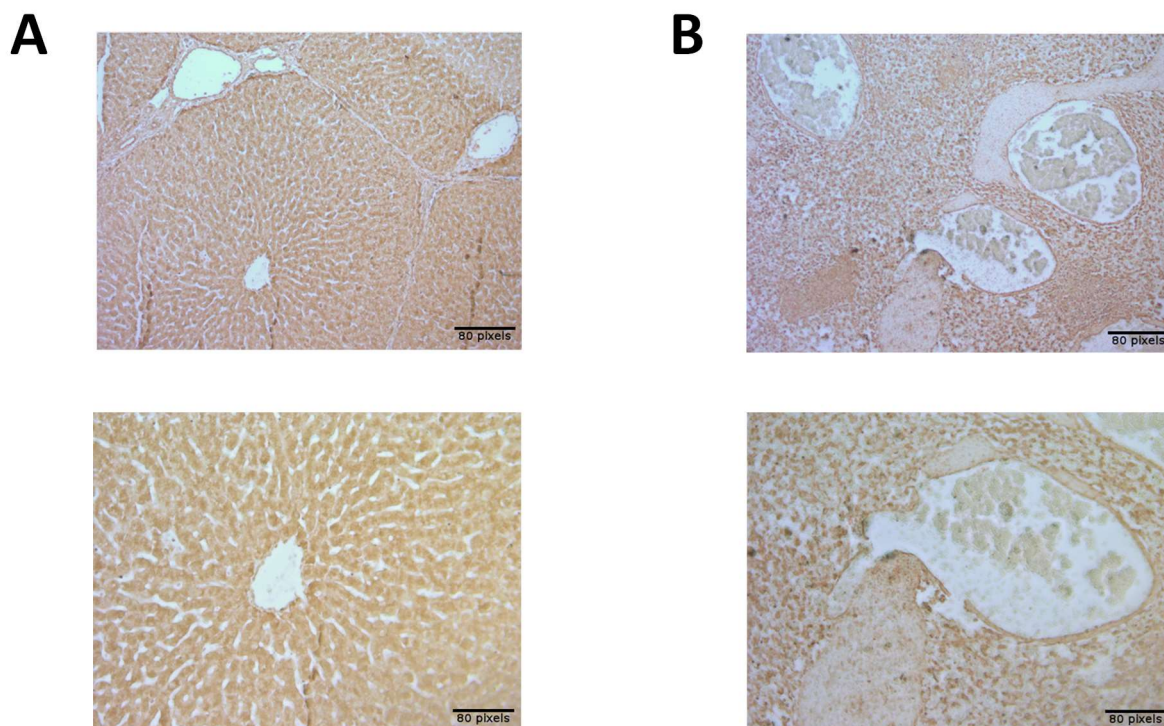


Figure 38 Example of drug transporter protein expression in pig: IHC staining probing for target *Abcb1/P-gp* protein expression in **A.** pig liver tissue section and **B.** pig spleen tissue section. Two images of the selected region (with one a close-up image) were captured for both species. The brown stain represents the localised expression of the target protein of interest at the selected region captured. Similar *Abcb1/P-gp* expression observed in both liver and spleen tissues.

In rat liver, *Cyp2e1* and *Abcb1/P-gp* appears to be highly expressed, with a moderate CYP450 generic expression and a low to moderate expression of *Ugt1a6*. Protein expression in rat spleen was low to moderately expressed for CYP450 generic and *Ugt1a6*, whereas *Cyp2e1* and *Abcb1/P-gp* appears to be highly expressed in some regions of the tissue section (see Figure 39 and 40, in appendix Figure A11-A13). In general, the CYP450 protein expression observed in both rat and pig spleen appear similar, and likewise for the strong expression observed for P-gp in the spleen tissue section for both species.

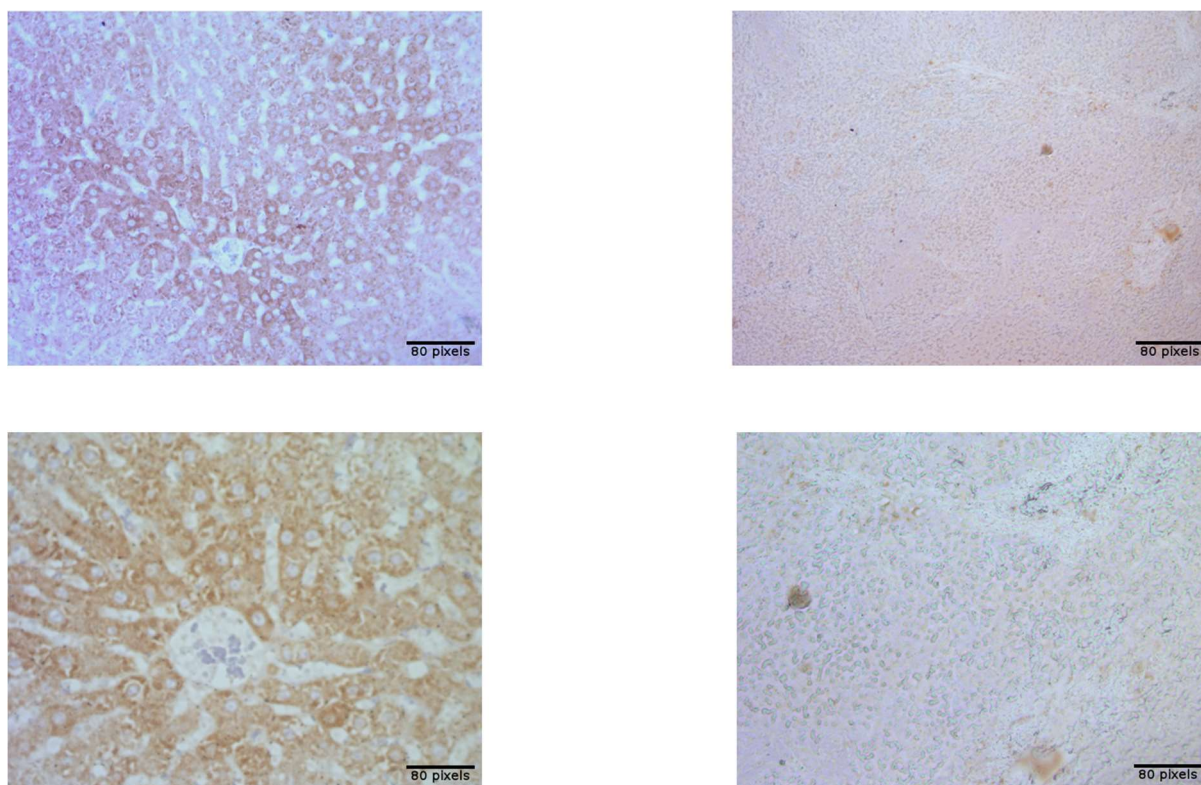


Figure 39 Example of CYP450 protein expression in rat: IHC staining probing for target CYP450 generic protein expression in **A.** rat liver tissue section and **B.** rat spleen tissue section. Two images of the selected region (with one a close-up image) were captured for both species. The brown stain represents the localised expression of the target protein of interest at the selected region captured. Low CYP450 expression observed in rat spleen compared to rat liver.

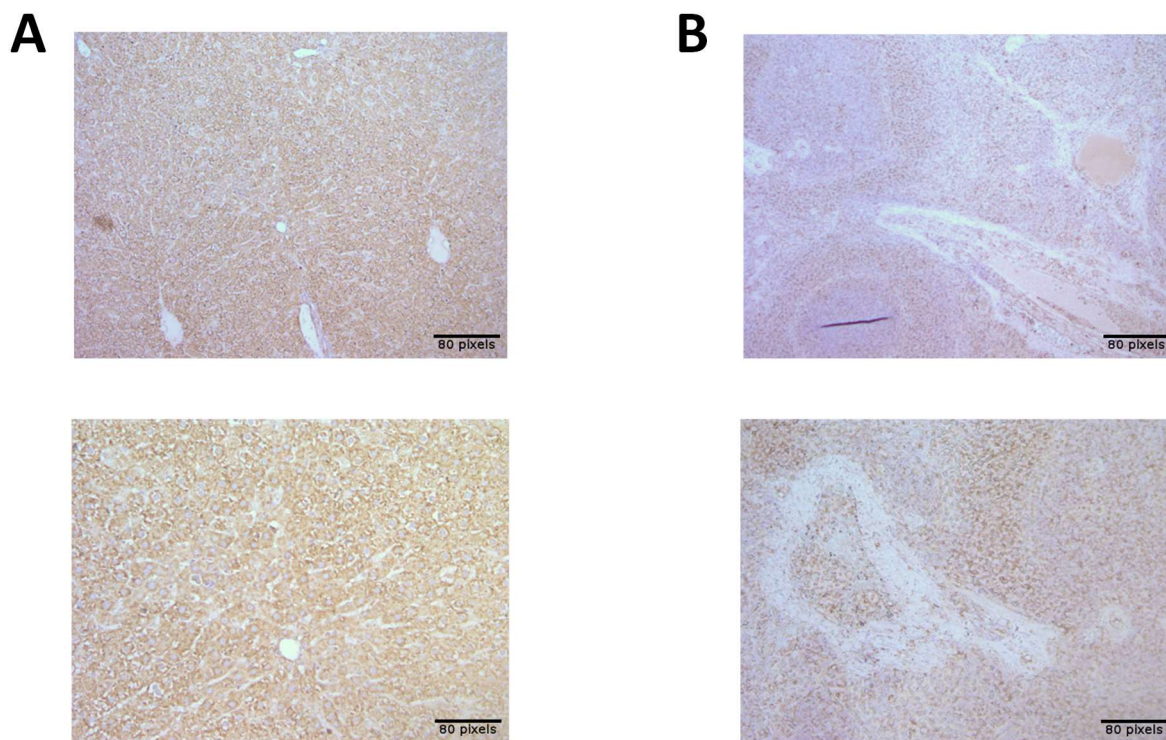


Figure 40 Example of drug transporter protein expression in rat: IHC staining probing for target *Abcb1/P-gp* protein expression in **A.** rat liver tissue section and **B.** rat spleen tissue section. Two images of the selected region (with one a close-up image) were captured for both species. The brown stain represents the localised expression of the target protein of interest at the selected region captured. Low to moderate *Abcb1/P-g* expression observed in rat spleen compared to rat liver.

4.2.3 The immunofluorescence staining of pig hepatocytes and splenocytes

Cryopreserved pig hepatocytes and splenocytes at 3.77×10^6 cells/mL, 76.5 % viable and 1.70×10^7 cells/mL, 95.2 % viable (see Figure 41), was stained with a goat anti-rabbit secondary antibody tagged to Alexa Fluor 488 dye to probe for the selected targets *Cyp2e1*, CYP450 generic, *Ugt8* and *Slc22a6* including a (no primary antibody) negative control. The genes associated with drug metabolism and distribution were identified in the pig liver and spleen tissues through the NGS analysis. Moreover, these genes were a representation of phase I, II

enzymes and transporter protein. The aim for this experiment was to determine the protein expression of these genes in primary cells compared to the expression observed in liver and spleen tissue sections. The fluorescent DAPI signal observed for all the tested targets indicated a presence of a double-stranded DNA of a cell. In addition, the green channel showing an Alexa 488 fluorescent signal was confirmation of protein expression observed for all the tested targets in both the pig hepatocyte and splenocyte cells (see Figure 42-46). However, background fluorescent signal has been noted for the negative control tests in both pig hepatocytes and splenocytes.

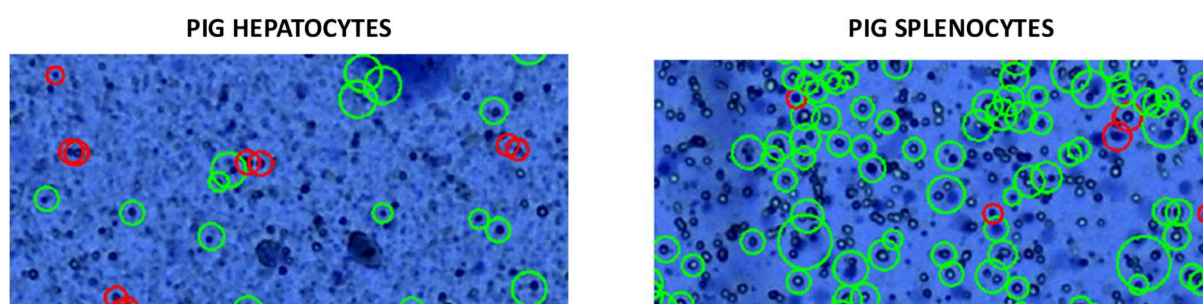


Figure 41 Cell count using the trypan blue exclusion test. Live cells are cells not stained with trypan blue and those stained are considered dead cells. The green circles on both the images represent cells that are valid or referred to as live cells. The red circles indicate cells stained with trypan blue and are considered dead cells. Cell viability is determined by having a large proportion of live cells counted or present in the cell mixture, typically in excess of $\geq 75\%$ is considered suitable for downstream analysis.

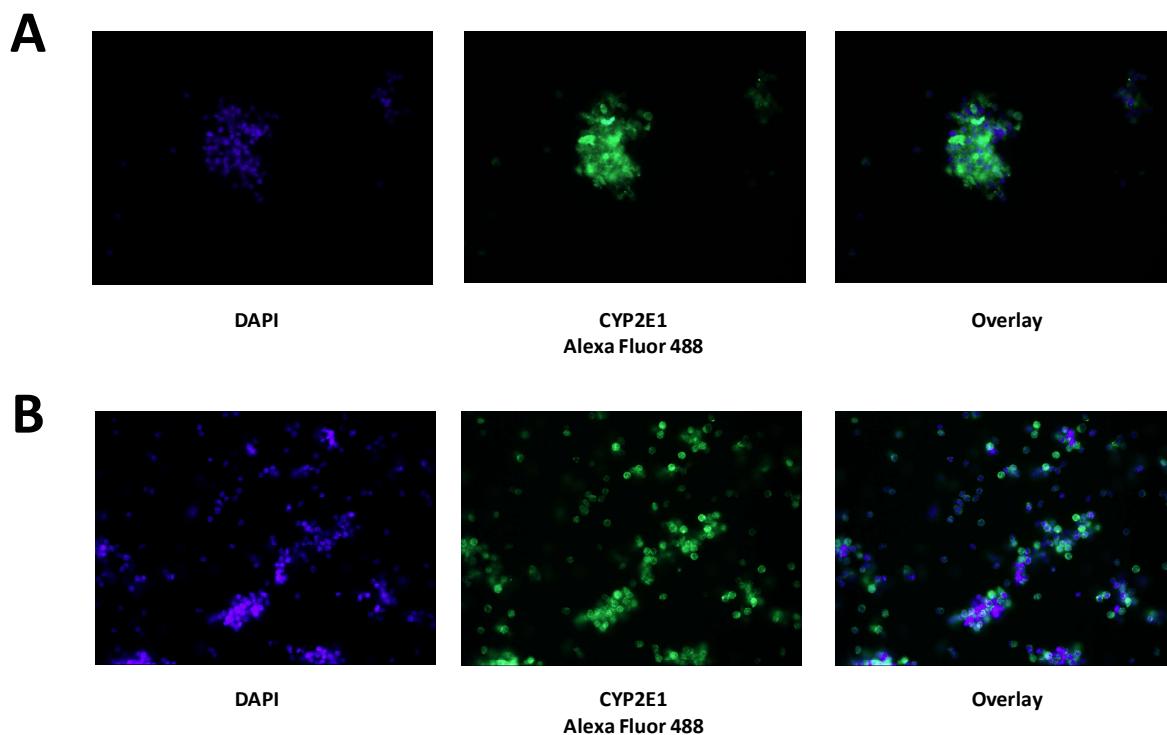


Figure 42 Immunofluorescence staining of *Cyp2e1* probe in primary **A.** pig hepatocytes and **B.** pig splenocytes. Immunofluorescence staining using goat anti-rabbit secondary antibody tagged to Alexa Fluor 488 (green), DAPI (blue label acting as a DNA-binding fluorescent probe used to localize the nuclei of a cell) and an overlay image to show a merge of both stains. 2592x1944 pixels; Wavelength 470 nm (blue stain), 527 nm (green stain). The leica upright microscope 5000 and x45 objective was used to capture the images.

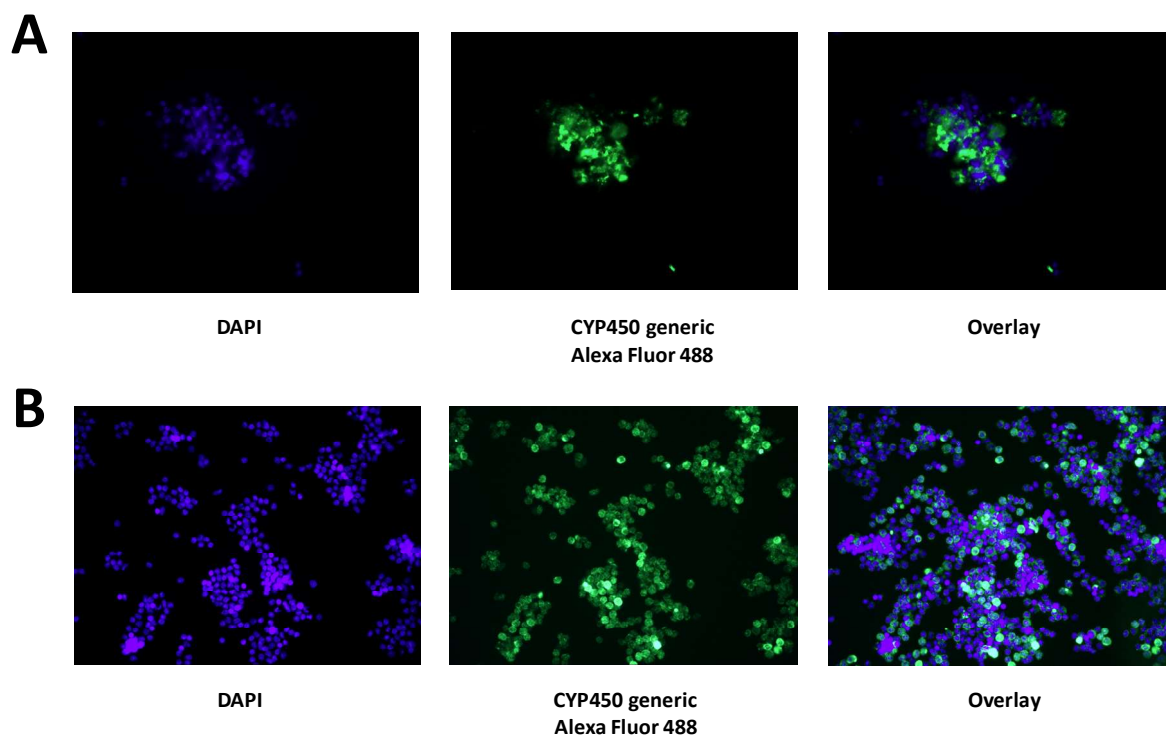


Figure 43 Immunofluorescence staining of CYP450 generic probe in primary **A.** pig hepatocytes and **B.** pig splenocytes. Immunofluorescence staining using goat anti-rabbit secondary antibody tagged to Alexa Fluor 488 (green), DAPI (blue label acting as a DNA-binding fluorescent probe used to localize the nuclei of a cell) and an overlay image to show a merge of both stains. 2592x1944 pixels; Wavelength 470 nm (blue stain), 527 nm (green stain). The leica upright microscope 5000 and x45 objective was used to capture the images.

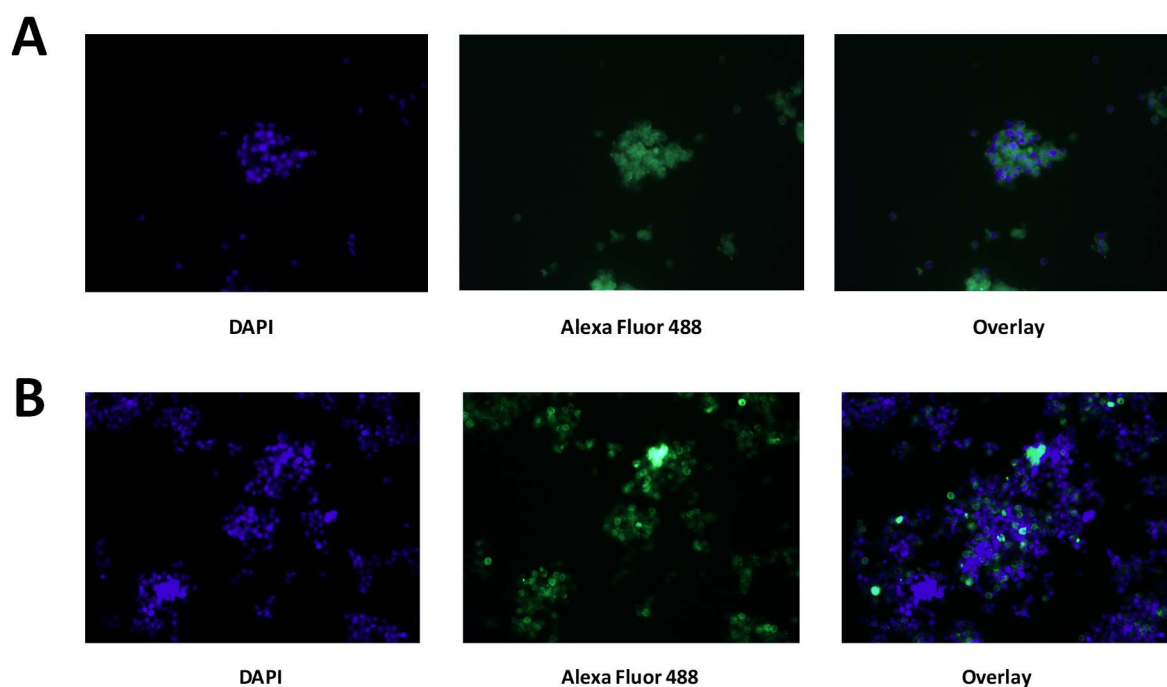


Figure 44 Immunofluorescence staining of *Ugt8* probe in primary **A.** pig hepatocytes and **B.** pig splenocytes. Immunofluorescence staining using goat anti-rabbit secondary antibody tagged to Alexa Fluor 488 (green), DAPI (blue label acting as a DNA-binding fluorescent probe used to localize the nuclei of a cell) and overlay image to show a merge of both stains. 2592x1944 pixels; Wavelength 470 nm (blue stain), 527 nm (green stain). The leica upright microscope 5000 and x45 objective was used to capture the images.

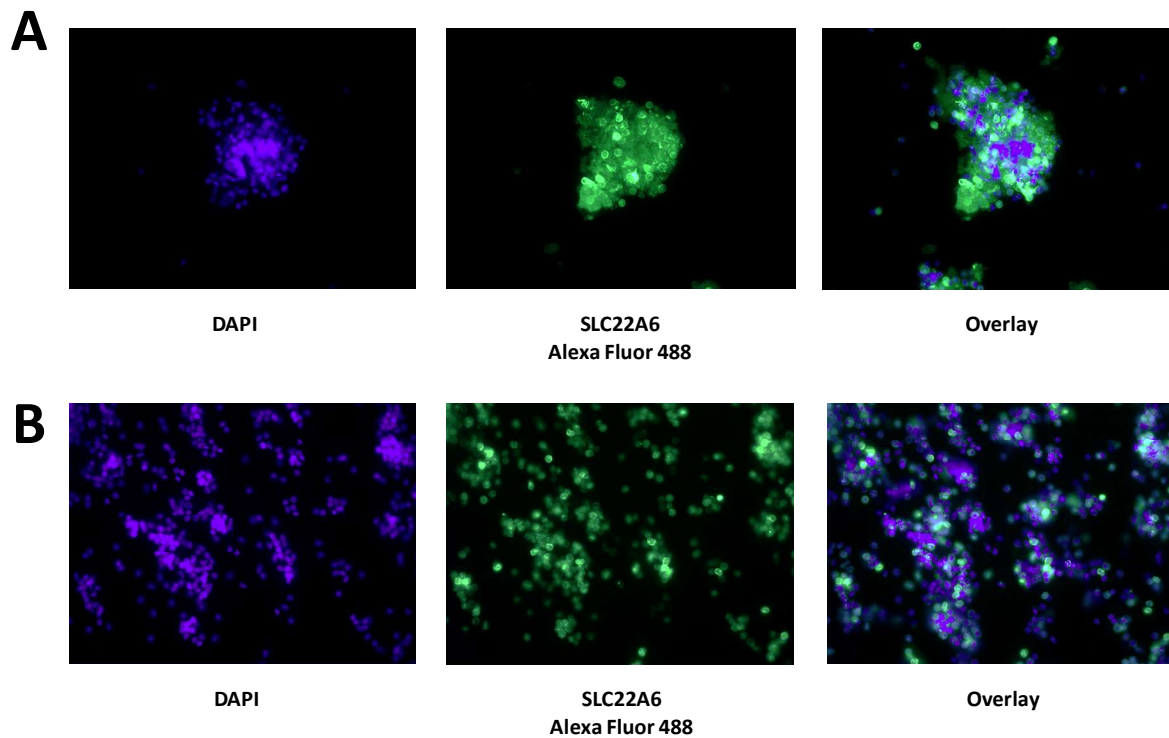


Figure 45 Immunofluorescence staining of *Slc22a6* probe in primary **A.** pig hepatocytes and **B.** pig splenocytes. Immunofluorescence staining using goat anti-rabbit secondary antibody tagged to Alexa Fluor 488 (green), DAPI (blue label acting as a DNA-binding fluorescent probe used to localize the nuclei of a cell) and overlay image to show a merge of both stains. 2592x1944 pixels; Wavelength 470 nm (blue stain), 527 nm (green stain). The leica upright microscope 5000 and x45 objective was used to capture the images.

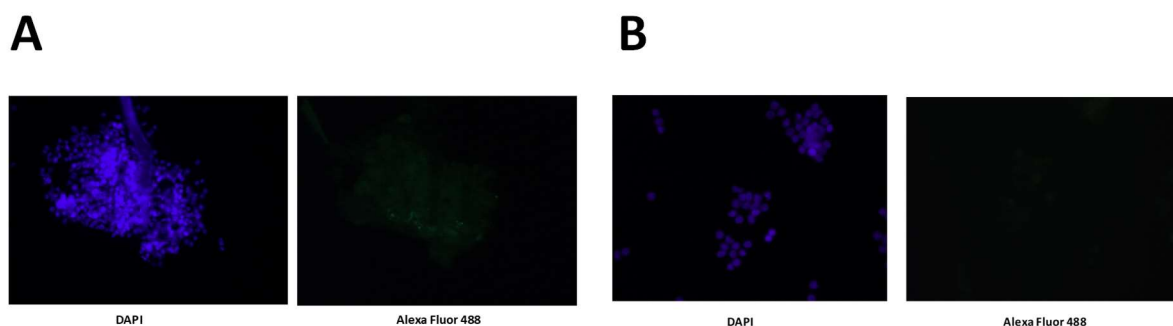


Figure 46 Immunofluorescence staining of a (no primary antibody) negative control in primary **A.** pig hepatocytes and **B.** pig splenocytes. Immunofluorescence staining using goat anti-rabbit secondary antibody tagged to Alexa Fluor 488 absence of primary antibody (green), DAPI (blue label acting as a DNA-binding fluorescent probe used to localize the nuclei of a cell) and overlay image to show a merge of both stains. 2592x1944 pixels; Wavelength 470 nm (blue stain), 527 nm (green stain). The leica upright microscope 5000 and x45 objective was used to capture the images.

4.2.4 Co-immunoprecipitation analysis in rat and pig for protein identification

This experiment involved the precipitation of selected antibodies (see Table 24) to probe for seven protein targets in rat, and six protein targets in pig. The immunoblot images show both bands for the targets and their co-interacting proteins. In rat, there is differences in band intensities for the expressed targets with CYP450 generic showing the highest expression and *Abcc10/Mrp7* for the spleen tissue lysate showing the least expression (see Figure 47). Strong expression was also observed for *Cyp2e1*, *Abcb1/P-gp*, *Cyp27a1* and *Abcc10/Mrp7*, particularly in the liver tissue lysate samples. No expression was determined for *Slc25a6* in rat liver and spleen tissue lysates. The blot images for pig showed a strong expression for the targets β -actin and *Cyp20a1*. Low to moderate expression was observed for CYP450 generic, *Ugt8* and *Cyp20a1* in pig, including a very low expression for *Cyp2e1*.

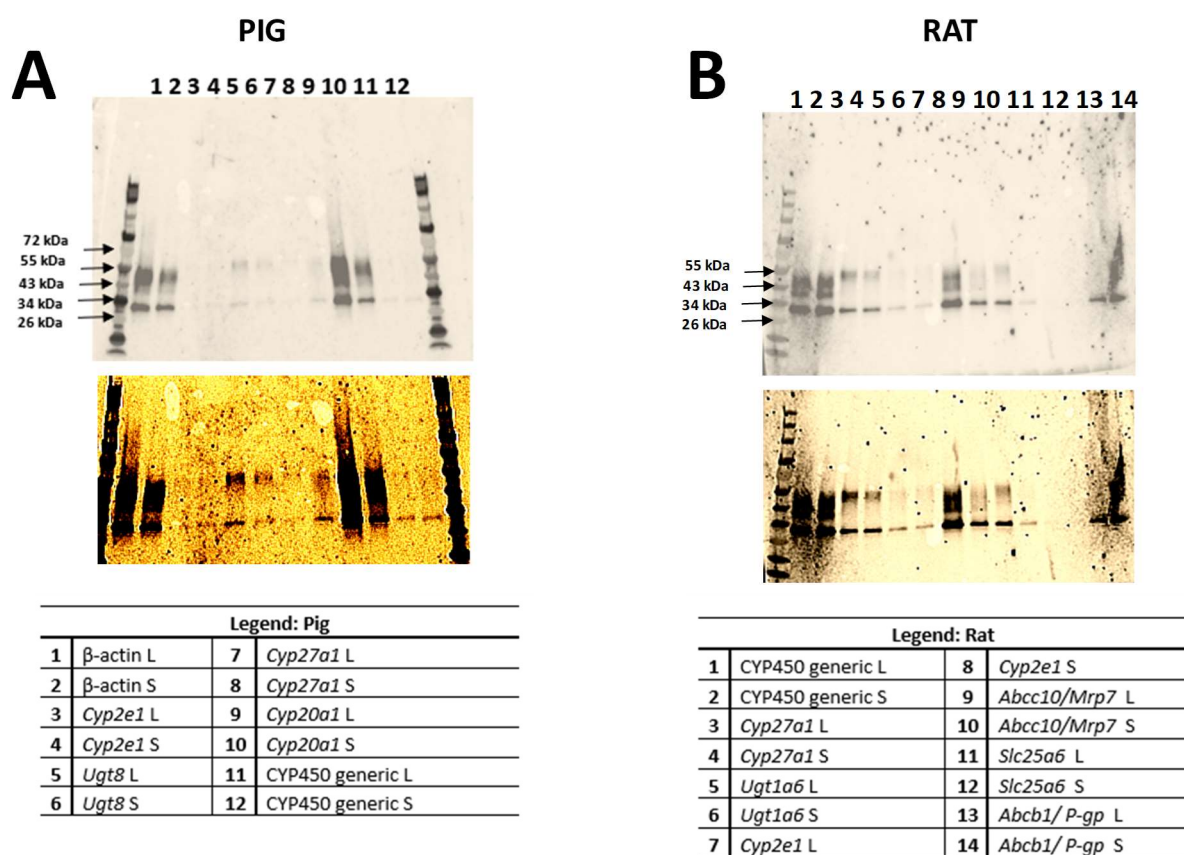


Figure 47 Co-immunoprecipitation analysis via SDS-PAGE of selected CYP450, UGT and transporters target probes in **A.** pig and **B.** rat (L) liver and (S) spleen protein lysates, including a legend to indicate location of the probed target proteins loaded onto the gel. A second exposure of the same blot emphasizes the location of the faint bands from the original using the Image J software (<https://ij.imjoy.io/>).

4.3 Discussion

Although western blotting is arguably considered a 'semi-quantitative' method, it is apparently a very sensitive technique as demonstrated from these results. The data produced with a western blot enables a relative comparison of protein levels to be achieved using a protein standard or marker, but it cannot provide an absolute measure of quantity (Mahmood & Yang, 2012). All the protein targets tested in the western blot assay indicated a positive protein expression in rat (three individual animals) and in pig (four individual animals) from the liver tissue lysate samples. The corresponding expressed bands were more intensified in pig compared to the rat. The liver microsomes samples showed protein expression for all the tested targets, apart from *Cyp20a1* and *Ugt8* in rat and *Cyp20a1* in pig. On the other hand, varying protein levels were determined in rat and pig spleen tissue lysates. No protein expression was observed in the spleen microsomes samples for any of the protein targets apart from CYP450 generic. Only two animals in pig spleen tissue lysates expressed the CYP450 generic target and in rat too including *Ugt8* (which has been shown to be lowly expressed based from the NGS analysis). Genetic variation or differences observed in different breeds of swine or within the same breeds of pigs has been realised and studied for many years, with current studies noting the varying degrees of resistance or susceptibility to diseases like the porcine reproductive and respiratory syndrome (PRRS) and the African swine fever (ASF) resulting from deletions in their nucleotide sequences (Wang et al., 2022; You et al., 2023). In respect to this, performing biological replicates is of great importance and should be a key factor considered in the determination of protein expression studies.

The addition of a loading control (e.g. β -actin) in the western blot assay was to demonstrate stable protein expression across different organism and organ tissue. Such genes like β -actin

are also known as reference genes or referred to as housekeeping genes and are described as essential for cellular existence regardless of its specific function in tissue or organism (Joshi et al., 2022). β -actin was identified as a highly expressed gene in both rat and pig spleen from the NGS analysis and hence was deemed a good indicator of a 'housekeeping gene' control. Across the tested protein targets, β -actin was carried out simultaneously with each of the targets and showed a consistent level of protein expression in all liver and spleen tissue lysates (including the microsomes samples) from both rat and pig animals. In particular, the bands indicating protein expression for β -actin in pig, has a difference in bands density between the liver and spleen tissue lysates to suggest the differences in β -actin expression between liver and the spleen though the reason remains unknown.

The results from this IHC study has showed mostly high to moderate expression of the targets on the tested liver tissue sections from both rat and pig, as expected. The spleen tissue sections (from rat and pig) showed low to moderate protein expression of the targets, and in some cases was difficult to identify and differentiate the low expression from negative expression. This method appears to work well for the tissue section(s) in question showing at least a moderate expression. Overall, the rat spleen tissue sections fared better in protein expression of the targets compared to the pig spleen tissue sections. The immunofluorescence tests in pig hepatocytes and splenocytes provided a clear indication of protein expression of the selected targets. A defined cell shape was not achievable from the images stained with *Cyp2e1* and CYP450 generic in pig hepatocytes but the (combination of the fluorescent dye, Fluoromount-G™, with a non-antibody) DAPI stain brought reassurance that the indicated protein expression was within the same area. Furthermore, the images showed that the selected target proteins were not necessarily only expressed within splenocytes but also in the surrounding areas of the cells. It was noticed however, some

fluorescent background in the negative control images and this may be related to antigenic similarities among mammalian immunoglobulins (Esteves & Binaghi, 1972). The corresponding blocking solution used was bovine serum albumin (BSA) with a goat secondary antibody, and there is a strong possibility of a cross-reactivity between cow and goat that may have occurred. BSA is a typical standard protein used for such experiments but to improve specificity and reduce background signals, (and without resource constraints) a normal goat serum would be a better choice for a blocking solution in this context.

Nonetheless, the results obtained from IHC or IHC-F studies can provide false-positive and false-negative results (particularly with the spleen IHC images representing CYP450 protein expression in both rat and pig due to the low expression observed); therefore, technical considerations must be born in mind or an analysis that requires multiple validation steps should be implemented (Cell Signaling Technology, 2024; Hawes et al., 2009). First of all, having a strong and specific primary antibody is paramount for validation of IHC or IHC-F studies (also as observed from the western blot results), and using recommended dilutions and reagents for an antibody-specific protocol is prerequisite because variables such as sample storage or reagent quality can have a profound impact on signal strength and background (Cell Signaling Technology, 2024; Hawes et al., 2009).

The Co-IP analysis was intended for the eluted bound protein of interest and its interacting protein in solution to be further analysed by mass spectrometry. The identification of the proteins in the sample would indicate the specificity of the used antibodies. Due to limitations in resources, the protein identification could not be performed, but protein expression was determined in both rat and pig for the selected targets representing CYP450, UGT and transporter proteins using this method.

CHAPTER 5:

**Investigating the functional expression
of CYP450 and UGT enzymes associated
with drug metabolism in pig spleen**

5.0

5.1 Introduction

Enzymes are proteins and are essential in the general operation and health of the body. Their function serves to increase the speed or rate of nearly all the chemical reactions in the body (Cooper GM, 2000). The consequences of a slow biochemical reaction or absence of catalysis could mean that the reaction may never occur, or it could take many years to be fulfilled (Cooper GM, 2000). Enzymes accelerates this process without being consumed in the reaction and thus, determines which chemical reactions take place within a cell (Cooper GM, 2000; Nagar et al., 2014). Hence, enzymes are the most important type of proteins and play a central role as biological catalysts in the human body. Enzymatic catalysis follows the principle that a molecule (the substrate, S) which acts on the enzyme, is converted into a product (P) as the result of the reaction (Cooper GM, 2000). The reaction reaching equilibrium is determined by the energy states of both the substrate and the product without altering the reaction equilibria; as enzymes are specific and only facilitate reactions that are already chemically feasible (Cooper GM, 2000; Nagar et al., 2014). The enzyme-catalysed reaction can only proceed in a transition state, a high-energy intermediate that occurs during the formation of the product (Cooper GM, 2000; Nagar et al., 2014). The energy required to reach the transition state is known as the activation energy (acting as an energy barrier between the reactants and products) which limits the rate of the reaction (Cooper GM, 2000). Enzymes function by lowering the activation energy thereby increasing the rate of reaction (Nagar et al., 2014).

A rate is a constant and is typically expressed as k , which reflects the speed or velocity of a reaction (Nagar et al., 2014). In addition, rates have units of concentration of product formed

or reactant converted per unit time, and so can be defined as the relationship between the substrate concentration and the rate (Nagar et al., 2014). Rate constants can be used to depict the association or dissociation of two molecules, and the dissociation constant K_d (derived from the rate constants) indicate the binding affinity between the two molecules. The reaction order then defines the relationship between the product formation and the rate constant; for example, in a zero-order reaction, the rate is constant regardless of increases in substrate concentration; and a first-order reaction shows a linear relationship between the rate of product formation and the substrate concentration (Nagar et al., 2014).

Therefore, the increase of the rate of product formation is proportional to the increase of the substrate concentration. The half-life is subsequently determined to show the amount of time required for the substrate concentration to decrease by 50 % (of its original concentration) relative to the reaction order (Nagar et al., 2014).

In the context of the drug development processes, estimates of *in-vitro* half-life of new chemical entities (NCEs) are used in the prediction of *in-vitro in-vivo* correlation (IVIVC), thus is also a fundamental parameter in *in-vivo* pharmacokinetics. The half-life is determined by calculating the fraction of substrate remaining at different timepoints and fitting the data to a Michalis Menten equation (Nagar et al., 2014). This equation provides the ‘activity’ of the enzyme, using the parameters (of Michalis Menten kinetics) V_{max} and k_m to describe when the enzyme is saturated with substrate (i.e. maximum rate of reaction) and to measure the affinity of the enzyme for its substrate at half the maximum rate of reaction respectively (Pant, 2022). The study of drug metabolism is based on two principal concepts involving the extent and rate of metabolism; the latter is intrinsically related to the kinetics of depletion of substrates (endogenous or exogenous) and the formation of metabolites (Nagar et al., 2014).

Drug metabolising enzymes (DMEs) are vital to the drug disposition of NCEs and may impact the design of potential new drug candidates. Therefore, this highlights the importance of quantitative evaluation of enzymatic processes in the field of pharmaceutical sciences (Nagar et al., 2014). DMEs generally metabolise a wide variety of substrates with overlapping substrate-specificities, which enables them to efficiently break down a myriad of exogenous compounds (e.g. like drugs) exposed in the body (Nagar et al., 2014). DMEs as protein catalysts are categorised as oxidative, reductive, hydrolytic enzymes (referred to as phase I reactions), or conjugative enzymes (referred to as phase II reactions) (Nagar et al., 2014). Cytochrome P450 enzymes (CYP450s) mainly catalyse the oxidation of drug substrates, whereas conjugative enzymes like the Uridine 5'-diphospho-glucuronosyltransferases (UGTs) catalyse a water-soluble molecule (e.g. glucuronic acid) with the drug substrates; for both to produce a water-soluble product that can be easily excreted from the body.

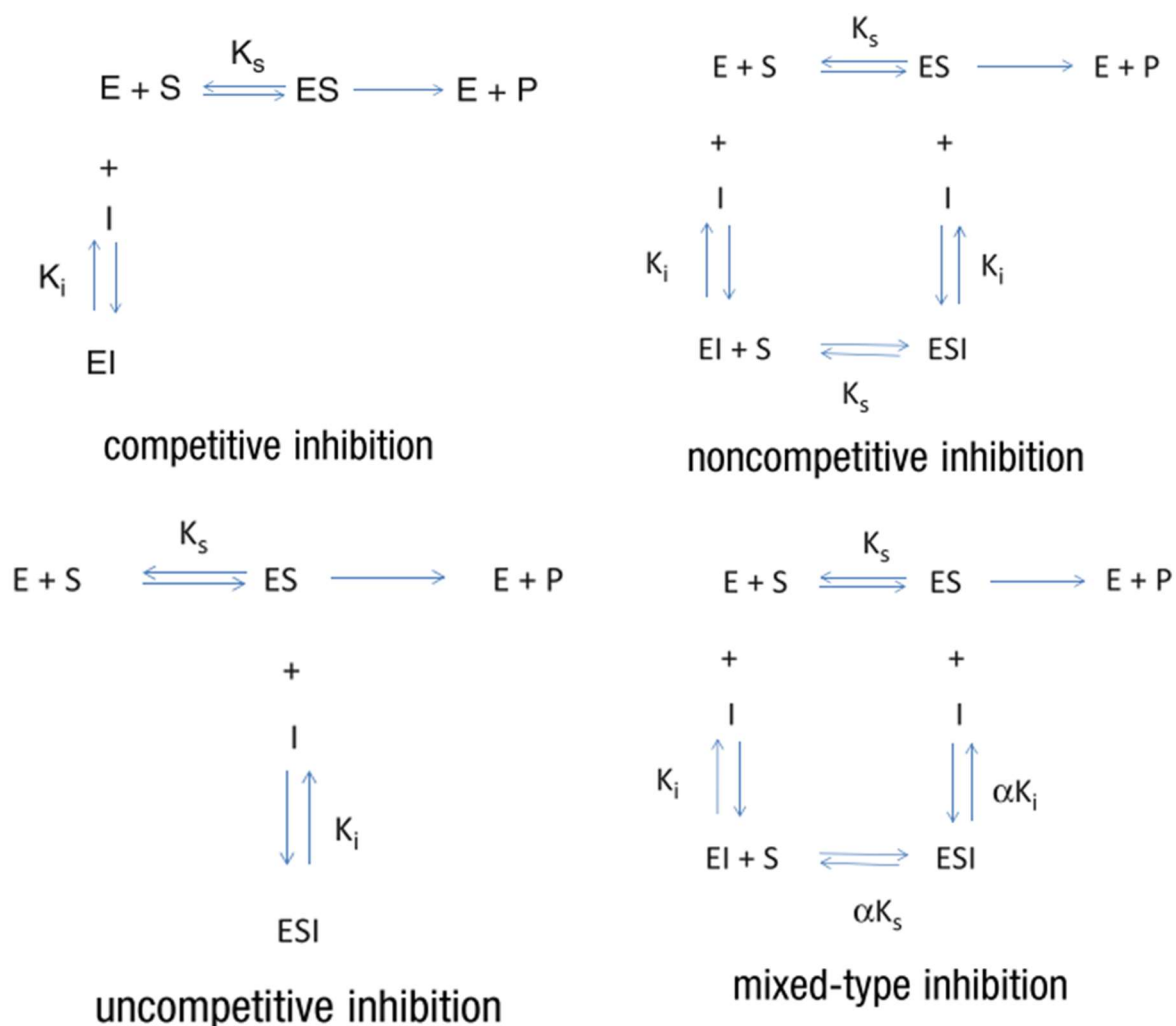


Figure. 48 Mechanisms of enzyme inhibition by drugs (Nagar et al., 2014).

Liabilities in drug-enzyme kinetics is often associated with inhibitory drug-drug interactions (DDIs) with DMEs (Nagar et al., 2014). Such interactions can lead to severe adverse effects or a reduction in therapeutic effects for some drug substances (Cascorbi, 2012). This provides the basis for the clinical significance of DMEs in examining the reversible mechanisms of inhibition exhibited by drugs (Nagar et al., 2014). These mechanisms include competitive, non-competitive, uncompetitive, and mixed competitive/non-competitive modes of

inhibition (see Figure 48). Competitive inhibition involves a substrate or inhibitor mutually competing for a single active or binding site of a DME, which can then alter the active site; in non-competitive inhibition, the substrate binding is unaffected by the binding of the inhibitor to the enzyme; mixed-type inhibitors appear to affect both the binding of the substrate and the rate of the catalysed reaction; un-competitive inhibitors do not bind to available enzyme but rather binds to enzyme-substrate complexes (Nagar et al., 2014).

As a result of this, the levels and function of CYP450s (see Figure 49) and UGTs (e.g. due to their ability to metabolise diverse substrates simultaneously) are considered important factors leading to individual differences in drug disposal and treatment results (Li et al., 2022). In addition, the metabolic enzymes involvement in genetic polymorphism (in combination with other factors e.g. environmental) have led to inter-individual and inter-ethnic differences in their ability to metabolise the same substrate (Li et al., 2022).

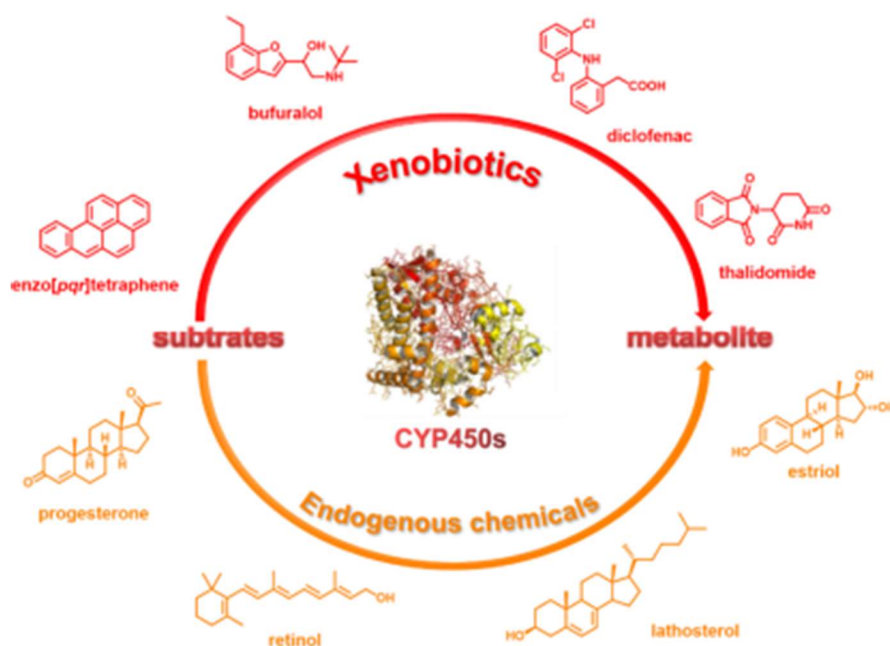


Figure 49 CYP450s involvement in the drug metabolism depicting its diverse capabilities to metabolise range of xenobiotics and endogenous chemicals (Li et al., 2022).

Measurements of CYP450 and/or UGT activities have become important screens in drug discovery and lead optimisation. Moreover, such measurements or studies are typically recommended by e.g. the MHRA and FDA – drug regulatory agencies (Kim et al., 2015). There have been several sensitive methods for determining levels of the expression of individual CYP450 mRNA and proteins such as PCR, bioluminescence, fluorescent probes-based assays, and mass spectrometry (Sinz et al., 2008; Ung et al., 2018). The latter is a commonly used method for CYP450/UGT quantitation and evaluation, though it can be an expensive option. In this study, the total P450 assay was incorporated into the evaluation of the functional expression of CYP450 and UGT enzymes in pig liver and spleen tissues. The principle of the CYP450 spectral assay (to determine the total content of CYP450) involves a reaction between the ferrous form of the heme protein (of CYP450s) with carbon monoxide (CO), to form a complex (Guengerich et al., 2009). This CYP450-CO complex specifically produces a spectrum at a wavelength of 450 nm; a characteristic feature of the cysteine thiolate axial ligand to the heme iron of CYP450 proteins (Guengerich et al., 2009). Nonetheless, advances in bioanalytical sciences with improved mass spectrometry has become vital in the integration of ADME (absorption, distribution, metabolism and excretion) sciences in drug discovery and development (Khojasteh et al., 2011). The bioanalysis process involves a series of steps that includes chemical ionisation of analytes and then, mass analysis and fragmentation whereby ions are separated according to their mass-to-charge ratio in MS/MS mode. Thus, meaning that a specific fragment ion is monitored as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) if multiple transitions (analyte + internal standard, or analyte + metabolite + internal standard) are monitored for quantitative bioanalysis (Khojasteh et al., 2011). Therefore, this study aims to discover the amount of CYP450 content in pig spleen to test for presence of CYP450 enzymes in splenic microsomes and to compare with that in pig

liver microsomes. Furthermore, the goal is to also establish CYP450 and UGT activity (adopting the substrate depletion approach) in pig spleen microsomes including splenocytes by quantitative bioanalysis using UPLC/MSMS.

5.2 Results

5.2.1 CYP450 content in pig liver and spleen tissues

The protein concentration was measured for the prepared pig liver microsomes at 37.8 mg/mL and pig spleen microsomes at 11.2 mg/mL. The homogenates samples (of liver and spleen tissues) was not completely consumed in the microsomes preparation process; instead samples were kept for the CYP450 content analysis and the protein concentration was not determined. The results from CYP450 content analysis was compared between two methods involving an autoclave chamber and cuvettes (Table 25-26).

Table 25 Absorbance readings at 450 nm and 490 nm of pig liver and spleen microsomes including homogenate samples. (autoclave chamber)

Test Sample	Absorbance readings at 450 nm							
	Reduced CYP450 (n=3)			Mean	Reduced CYP450-CO complex (n=3)			Mean
Pig Liver Microsomes	1.166	1.246	1.246	1.219	1.217	1.282	1.244	1.248
Pig Spleen Microsomes	0.984	1.041	1.041	1.022	1.081	1.114	1.084	1.093
Pig Liver Homogenate	0.260	1.491	0.258	0.670	0.277	0.353	0.304	0.311
Pig Spleen Homogenate	0.233	0.267	0.257	0.252	0.232	0.229	0.243	0.235

Test Sample	Absorbance readings at 490 nm							
	Reduced CYP450			Mean	Reduced CYP450-CO complex			Mean
Pig Liver Microsomes	0.939	1.010	1.009	0.986	0.997	1.054	1.019	1.023
Pig Spleen Microsomes	0.795	0.845	0.844	0.828	0.888	0.921	0.890	0.900
Pig Liver Homogenate	0.181	1.458	0.180	0.606	0.205	0.267	0.227	0.233
Pig Spleen Homogenate	0.154	0.183	0.175	0.171	0.174	0.171	0.185	0.177

Table 26 Absorbance readings at 450 nm and 490 nm of pig liver and spleen microsomes including homogenate samples. (cuvette method)

Test Sample	Reduced CYP450-CO complex (n=3)							
	Absorbance readings at 450 nm			Mean	Absorbance readings at 490 nm			Mean
Pig Liver Microsomes	0.498	0.544	0.544	0.529	0.401	0.440	0.439	0.427
Pig Spleen Microsomes	0.381	0.426	0.425	0.411	0.303	0.341	0.341	0.328
Pig Liver Homogenate	0.363	0.408	0.407	0.393	0.301	0.338	0.338	0.326
Pig Spleen Homogenate	0.123	0.168	0.168	0.153	0.095	0.132	0.133	0.120

Table 27 CYP450 content determination in the pig liver and spleen tissues and homogenates

Sample	CYP450 content using autoclave chamber		CYP450 content using cuvette method	
	nMoles/P450/mL	nMoles/P450/mg protein	nMoles/P450/mL	nMoles/P450/mg protein
Pig Liver Microsomes	-0.099	-0.003	1.121	0.030
Pig Spleen Microsomes	-0.007	-0.001	0.905	0.081
Pig Liver Homogenate	0.165	0.004	0.736	0.019
Pig Spleen Homogenate	-0.260	-0.023	0.363	0.032

*protein concentration of microsomes was used for the respective homogenate samples calculations

It appears that the cuvette method to determine the CYP450 content in the selected pig samples was the better option (see Table 27). Both approaches followed the same order which includes, a CO-treatment made to all samples and dithionite added later to the test samples only. The results obtained from using the autoclave chamber route presented almost all negative values including the positive control in pig liver, indicating no CYP450 content was detected. Measured CYP450 content was only apparent for the pig liver homogenate. On the other hand, the cuvette method was a less labour-some method since it was a well-established assay, and the analysis using the autoclave chamber required significant optimisation to validate the assay. Though the protein concentration was higher in pig liver microsomes compared to the spleen microsomes, the latter contained more concentrated CYP450 content than the former. Both liver and spleen homogenates appeared to have less CYP450 content than in their microsomes counterparts; but it should be noted that the protein concentrations derived from the microsomes samples were used in the homogenate samples CYP450 content calculations.

5.2.2 CYP450 and UGT-mediated drug clearance in pig liver and spleen microsomes

The CYP450 and UGT-mediated drug elimination rate was determined using the slope (k) derived from a Michalis Menten regression curve to generate calculated intrinsic clearance (Cl_{int}) for each of the tested drug substrates. The Cl_{int} values were subsequently normalised with mg microsomal protein concentration to determine the initial rate profile (see Figure 50-53).

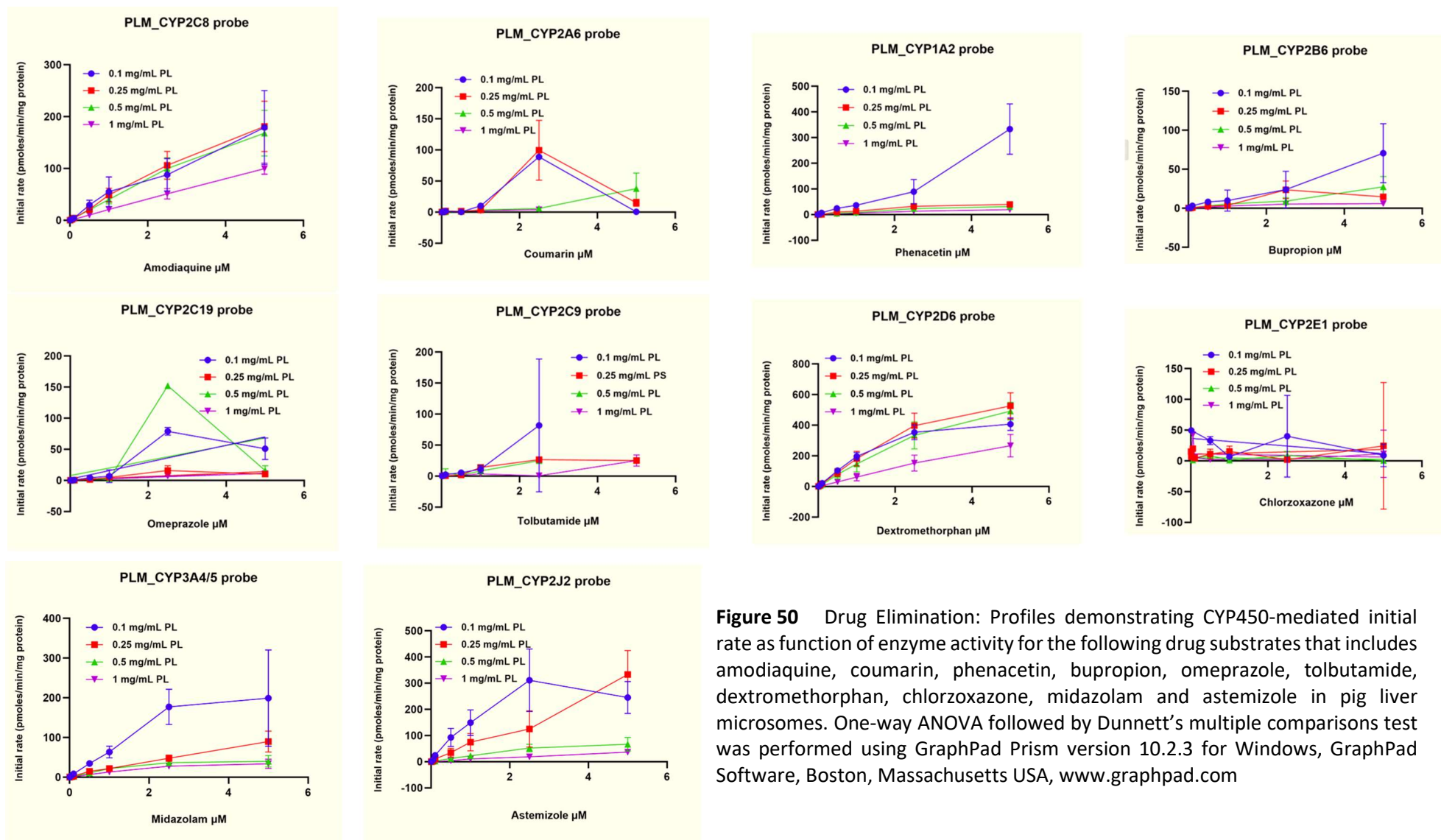


Figure 50 Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity for the following drug substrates that includes amodiaquine, coumarin, phenacetin, bupropion, omeprazole, tolbutamide, dextromethorphan, chlorzoxazone, midazolam and astemizole in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

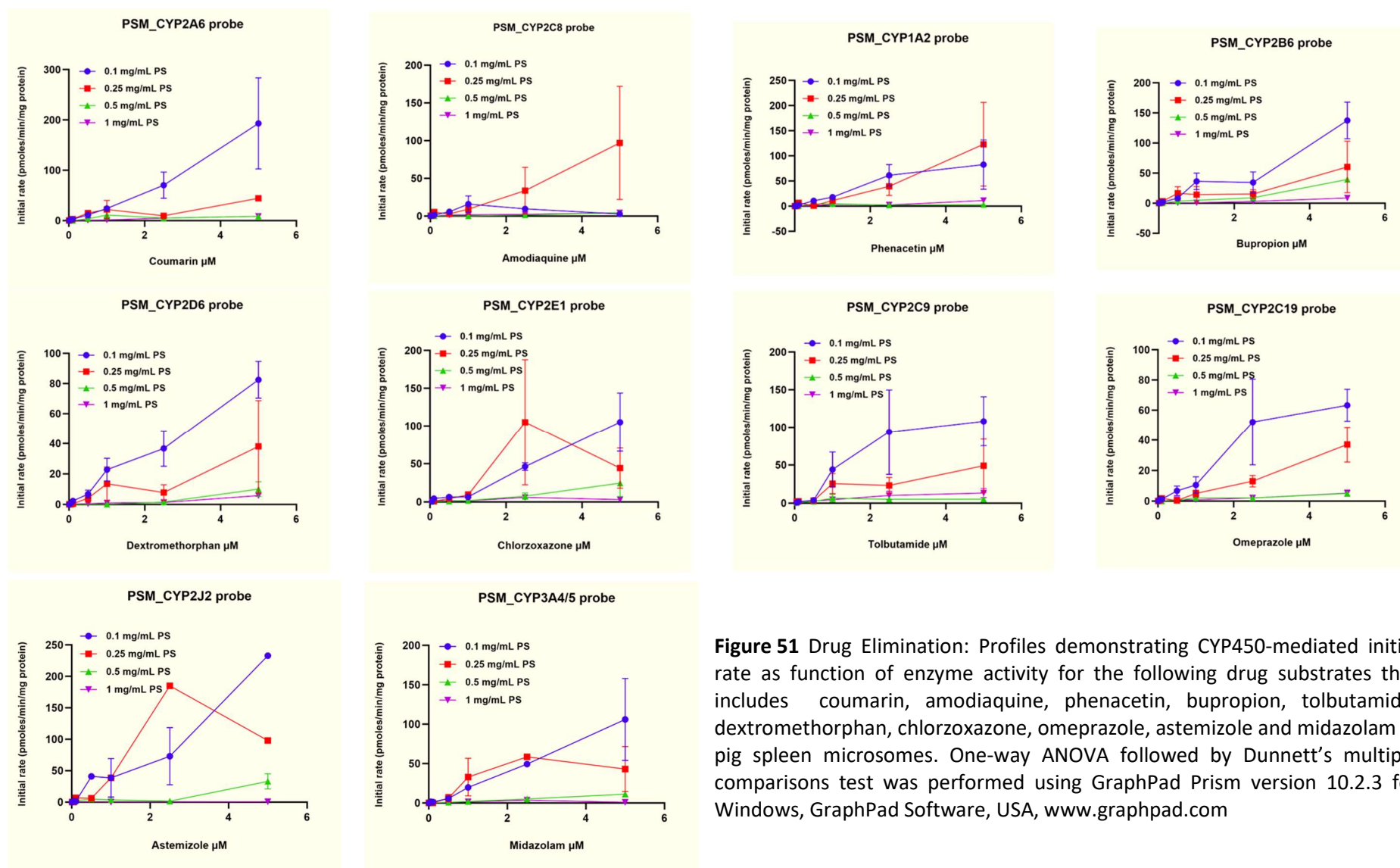
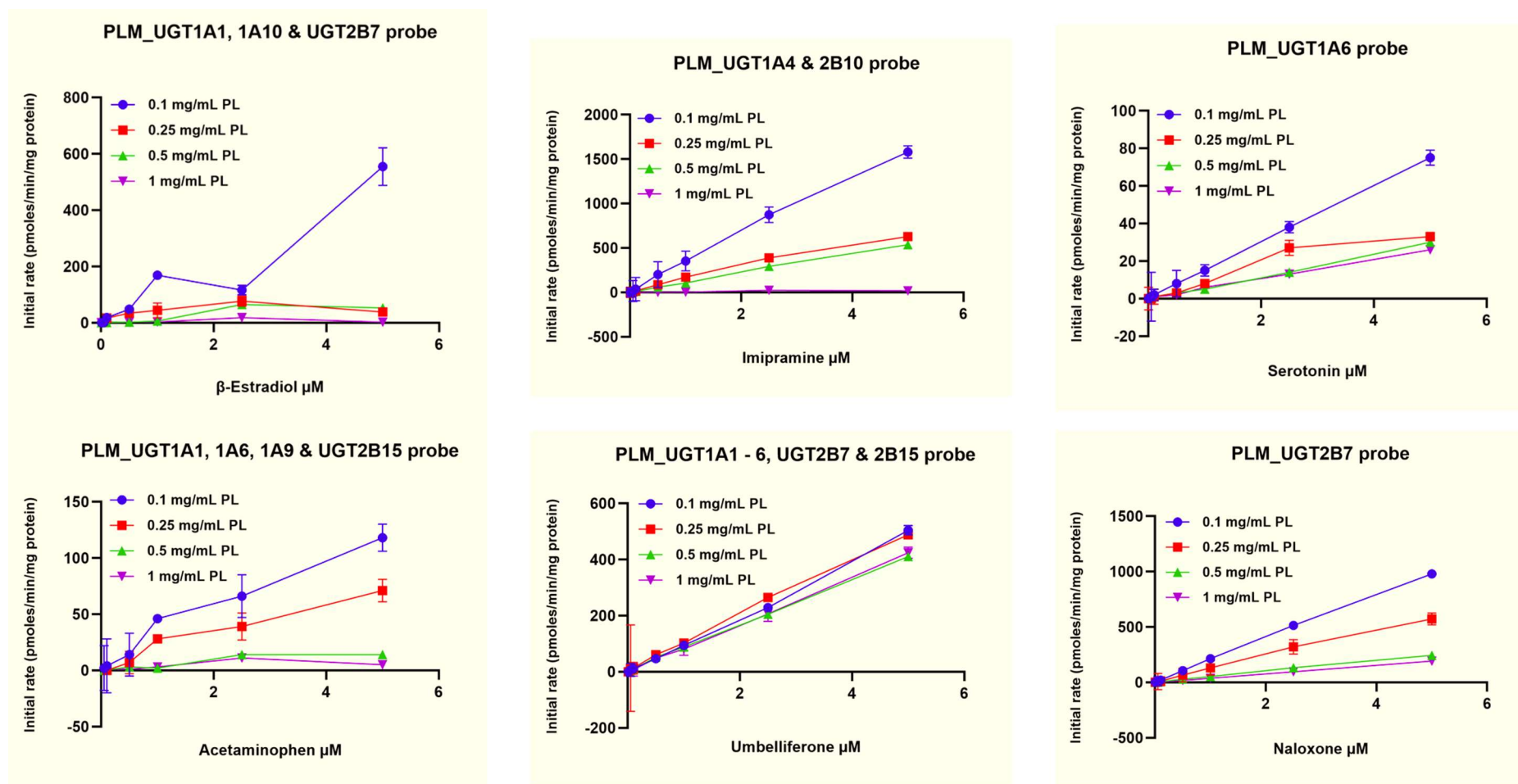


Figure 51 Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity for the following drug substrates that includes coumarin, amodiaquine, phenacetin, bupropion, tolbutamide, dextromethorphan, chlorzoxazone, omeprazole, astemizole and midazolam in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, USA, www.graphpad.com

**Figure 52**

Drug Elimination: Profiles demonstrating UGT-mediated initial rate as a function of enzyme activity for the following drug substrates that includes β -Estradiol, imipramine, serotonin, acetaminophen, umbelliferone and naloxone in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

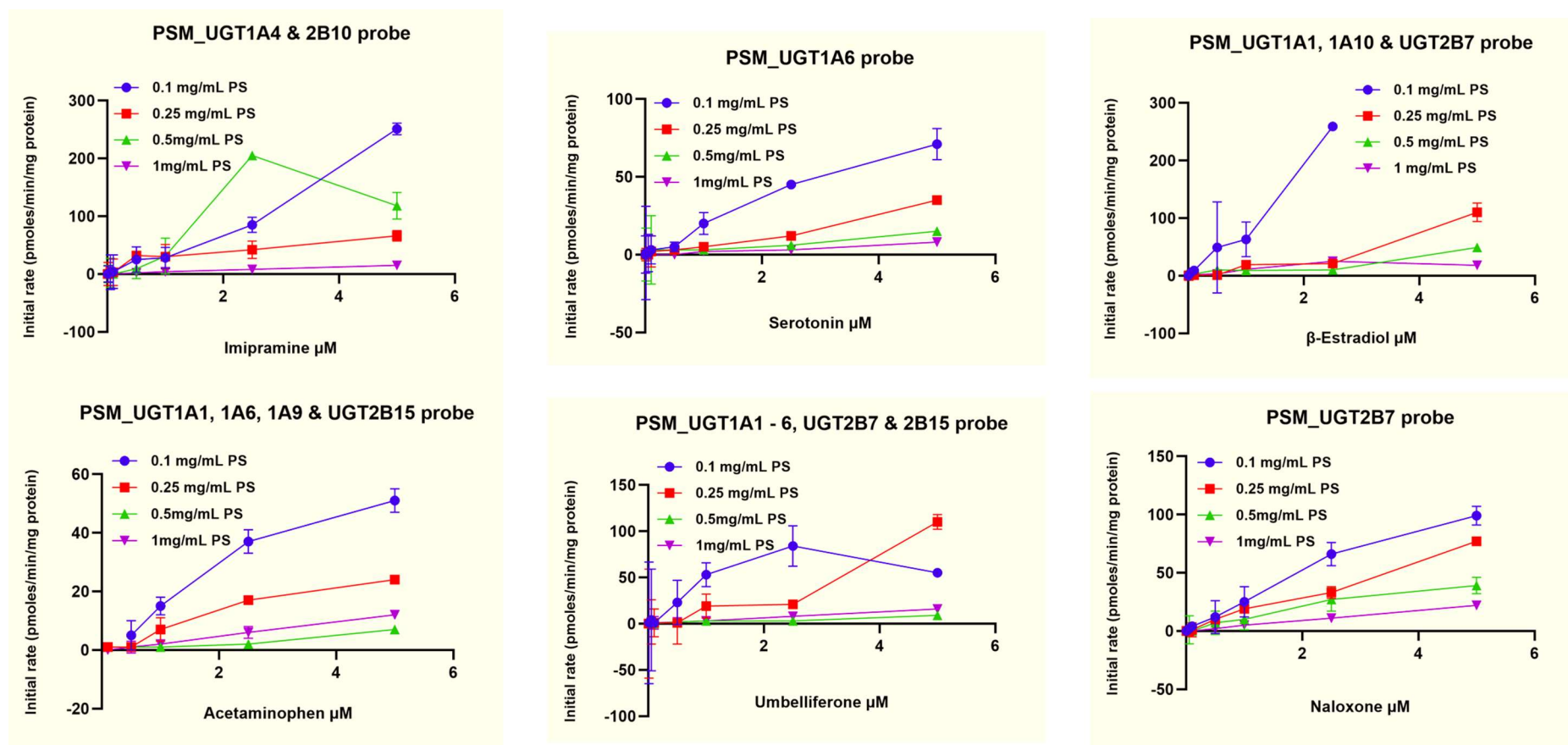


Figure 53 Drug Elimination: Profiles demonstrating UGT-mediated initial rate as a function of enzyme activity for the following drug substrates that includes imipramine, serotonin, β -Estradiol, acetaminophen, umbelliferone and naloxone in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

There appears to be a recurring theme across the selected drug substrates of CYP450 probes in pig liver and spleen microsomes, however the drug elimination rate appears higher in pig liver compared to pig spleen. With increasing rate of drug clearance, the drug substrate concentration increases, but the rate of clearance decreases when the microsomal protein concentration is increased (see Figure 40-51). Generally, the profiles also suggest that the rate of clearance drops between 2.5 and 5 μ M substrate concentration. Likewise, the rate of drug clearance for UGT probes in pig liver and spleen microsomes conveys an almost identical theme as observed with the CYP450-mediated drug elimination profiles (see Figure 52-53). It is worth mentioning that the variation observed across the results overall are higher for some of the drug substrate profiles, but this has not affected the conditions of the assay.

5.2.3 CYP450-mediated metabolite formation in pig liver and spleen microsomes I

Specific MS/MS methods was used to monitor corresponding metabolite levels from the drug elimination analysis in pig liver and spleen microsomes. The following metabolite formation profiles are presented both as a function of microsomal protein concentration and drug substrate concentration (see Figure 54-68). The peak area response is the analytical output obtained from the UPLC/MS/MS analysis. Measurement of the drug levels is relative to the peak area response detected for each of the drug substrate concentration. This would reflect that as the drug substrate concentration is increased, the response will proportionally increase. Selected probes monitoring CYP450 and UGT enzyme activity over time should describe a higher drug level at 5 minutes and a drop-in drop levels by 45 minutes; thereby indicating that the enzymes are responsible for the decrease in the drug levels. In addition,

this observation should also coincide with the production of metabolites being formed, lower at 5 minutes but an increase in metabolite formation levels at 45 minutes.

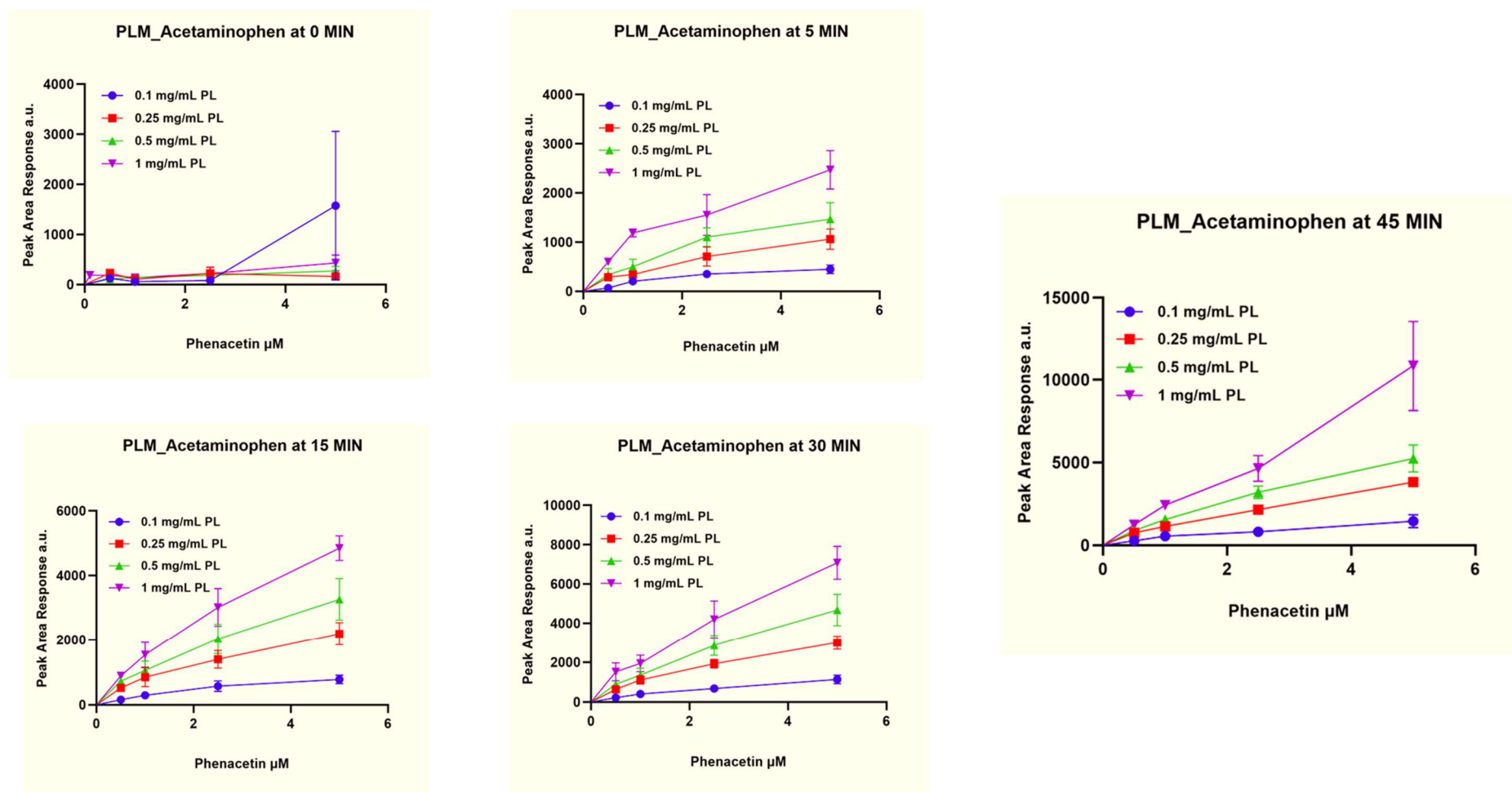


Figure 54 Metabolite Formation: CYP450-mediated metabolite profile of acetaminophen across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

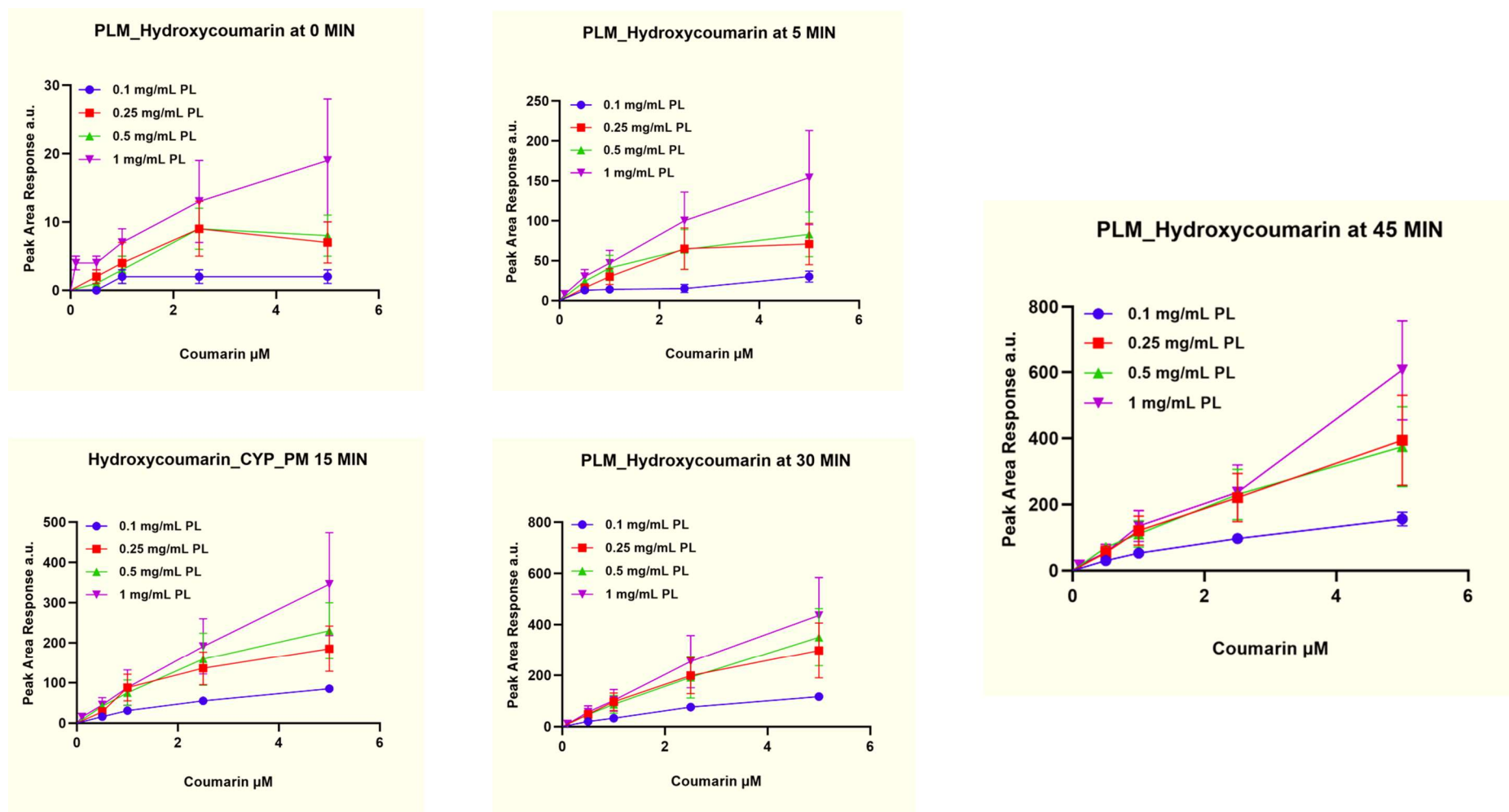


Figure 55 Metabolite Formation: CYP450-mediated metabolite profile of hydroxycoumarin across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

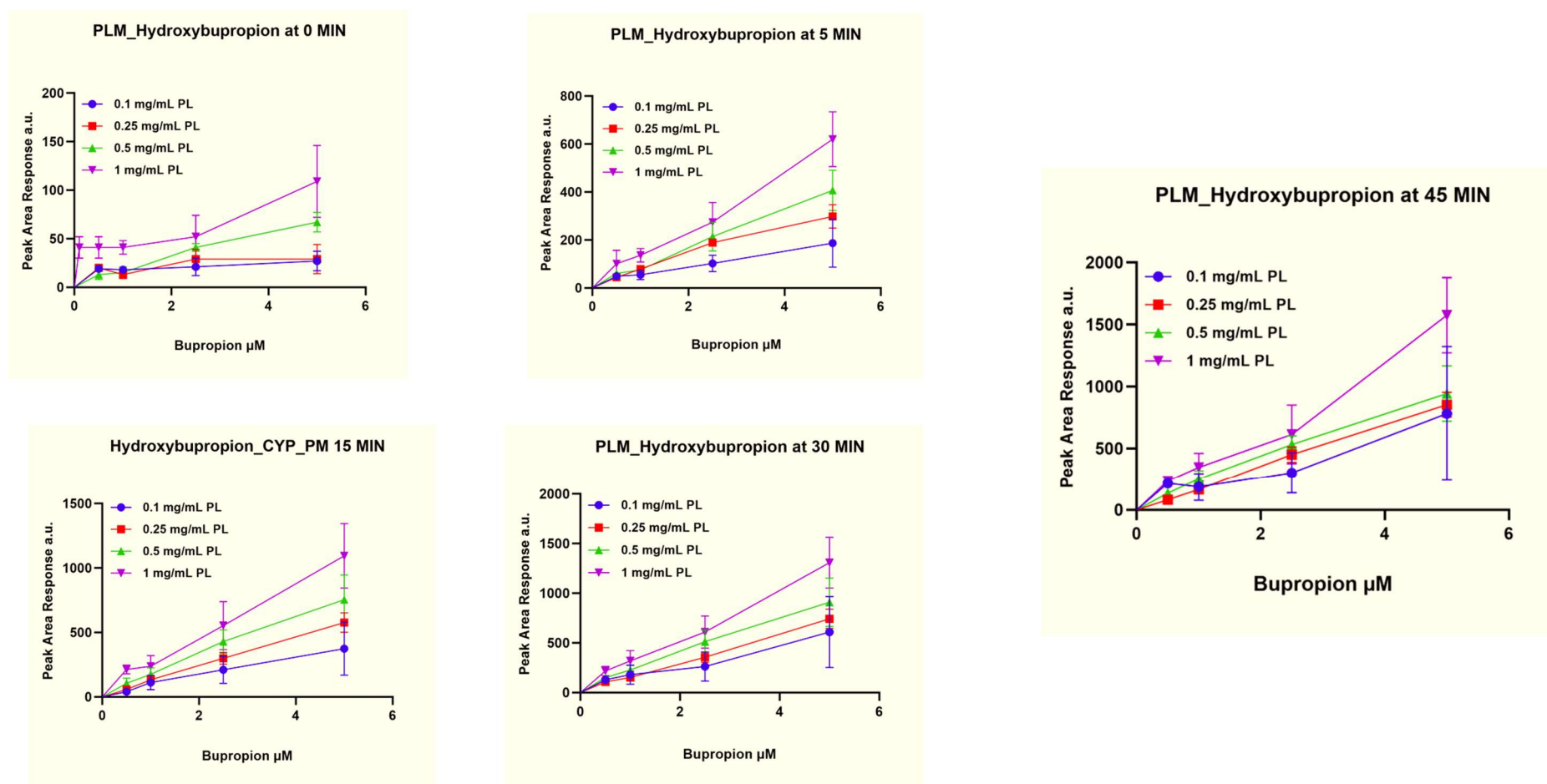


Figure 56 Metabolite Formation: CYP450-mediated metabolite profile of hydroxybupropion across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

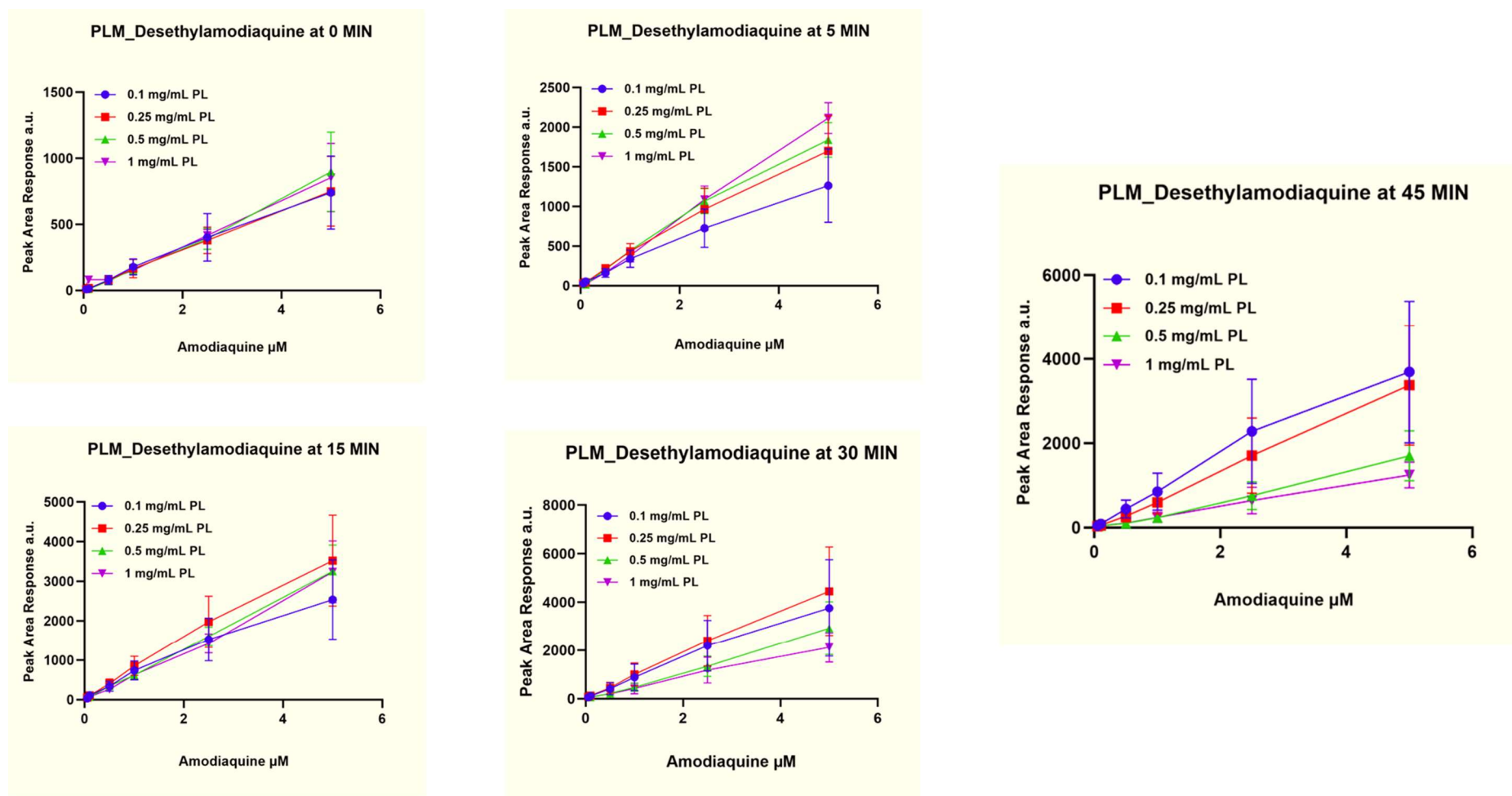


Figure 57 Metabolite Formation: CYP450-mediated metabolite profile of desethylamodiaquine across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

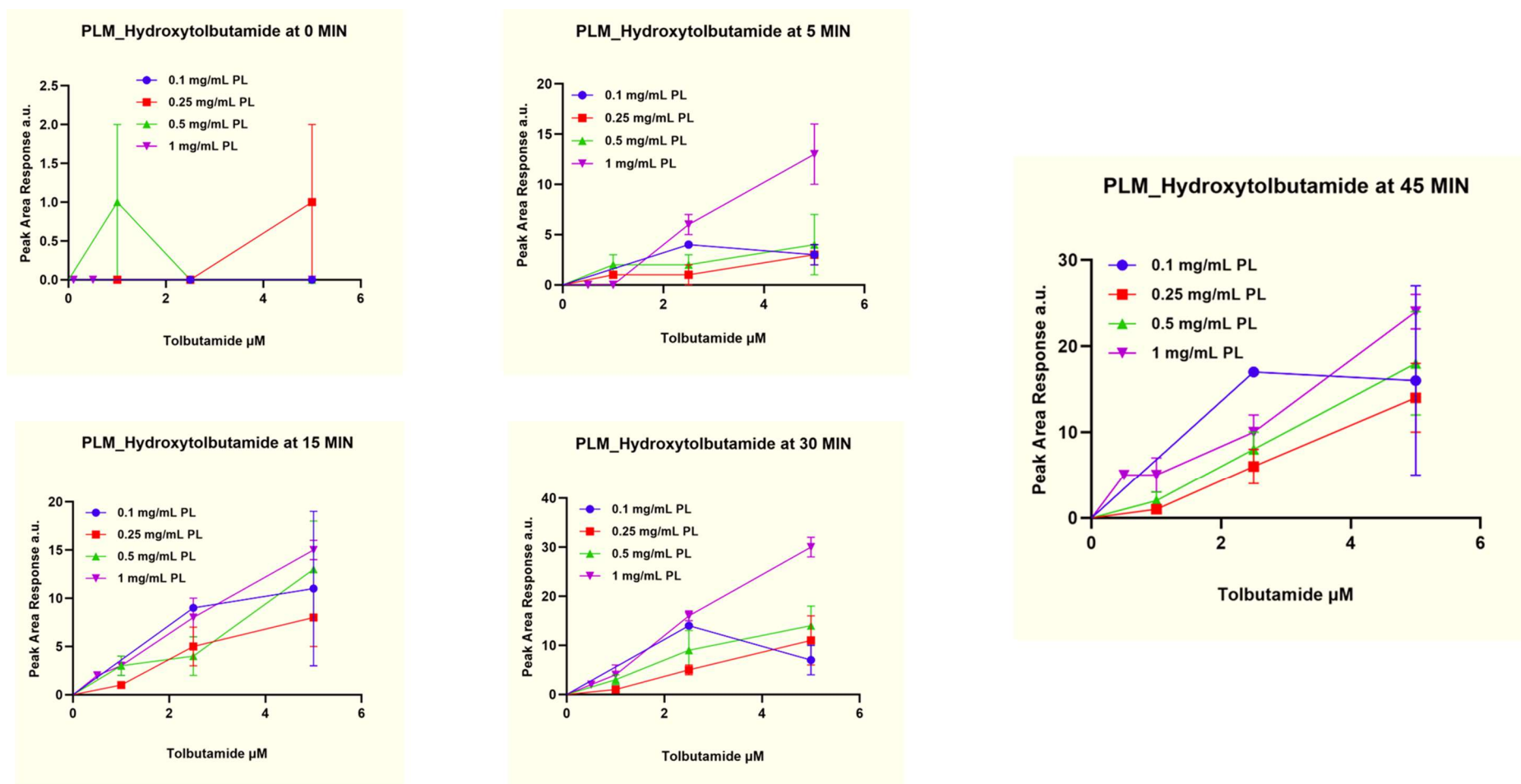


Figure 58 Metabolite Formation: CYP450-mediated metabolite profile of hydroxytolbutamide across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

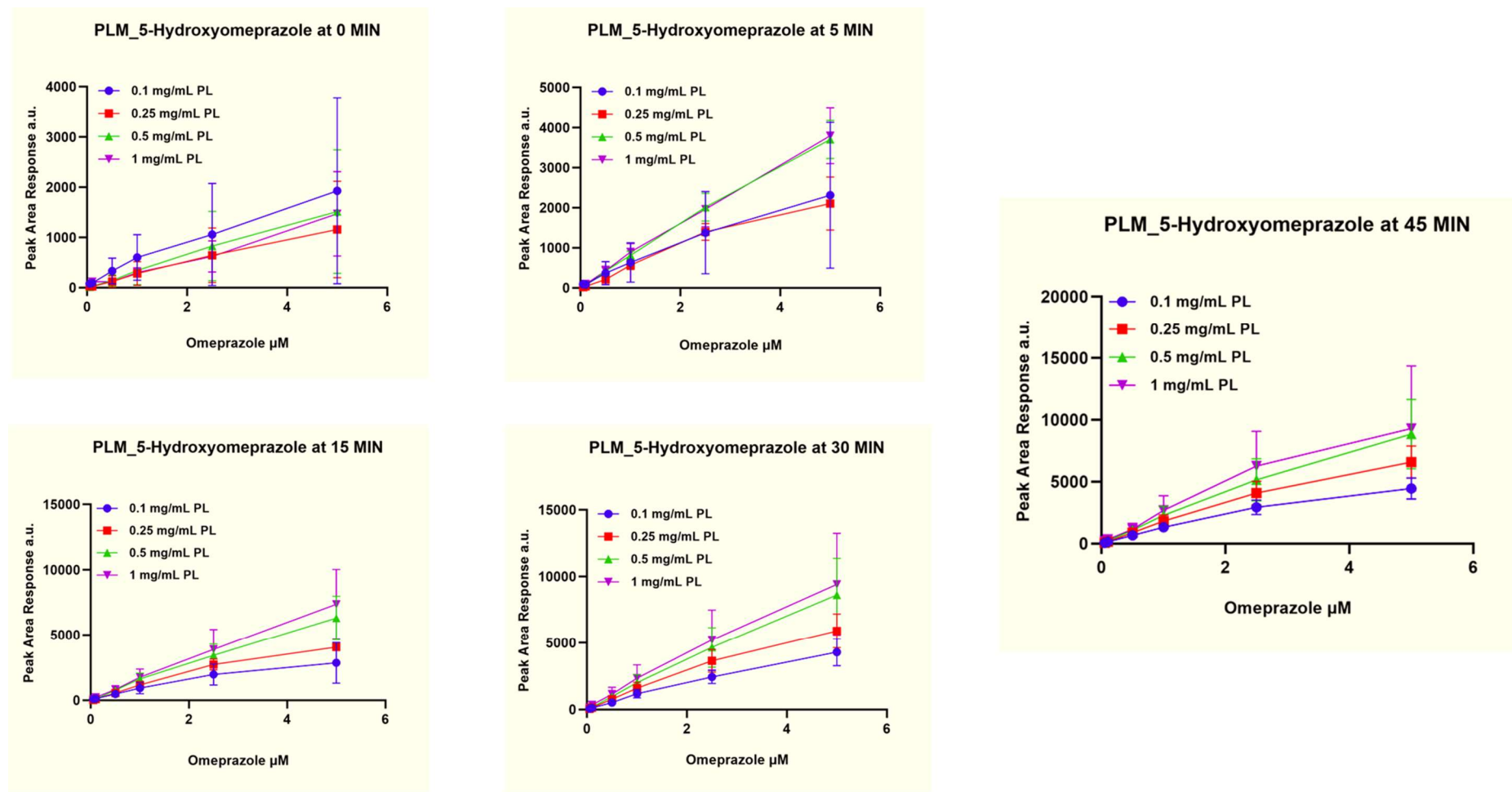


Figure 59 Metabolite Formation: CYP450-mediated metabolite profile of 5-hydroxyomeprazole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

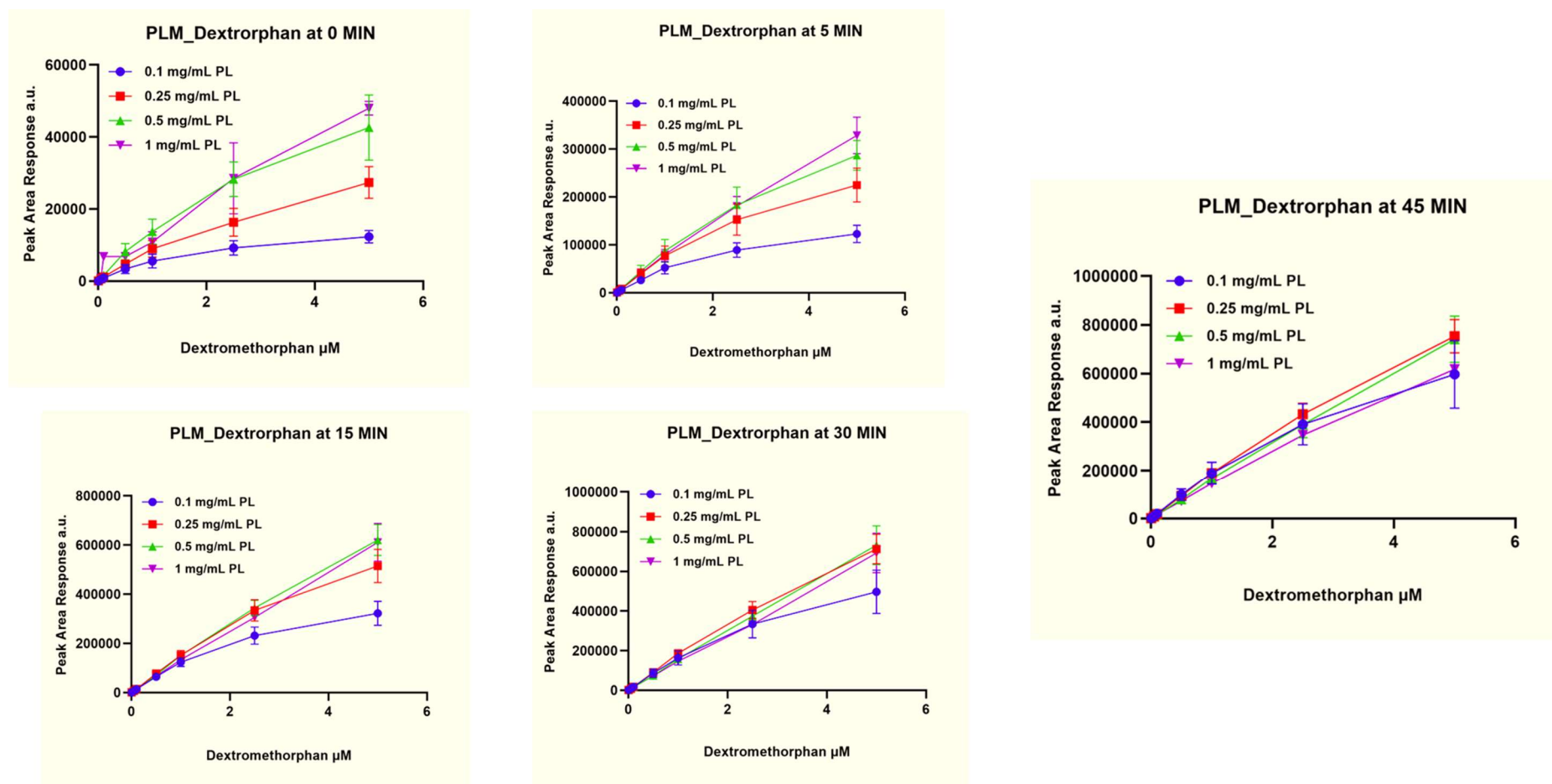


Figure 60 Metabolite Formation: CYP450-mediated metabolite profile of dextrophan across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

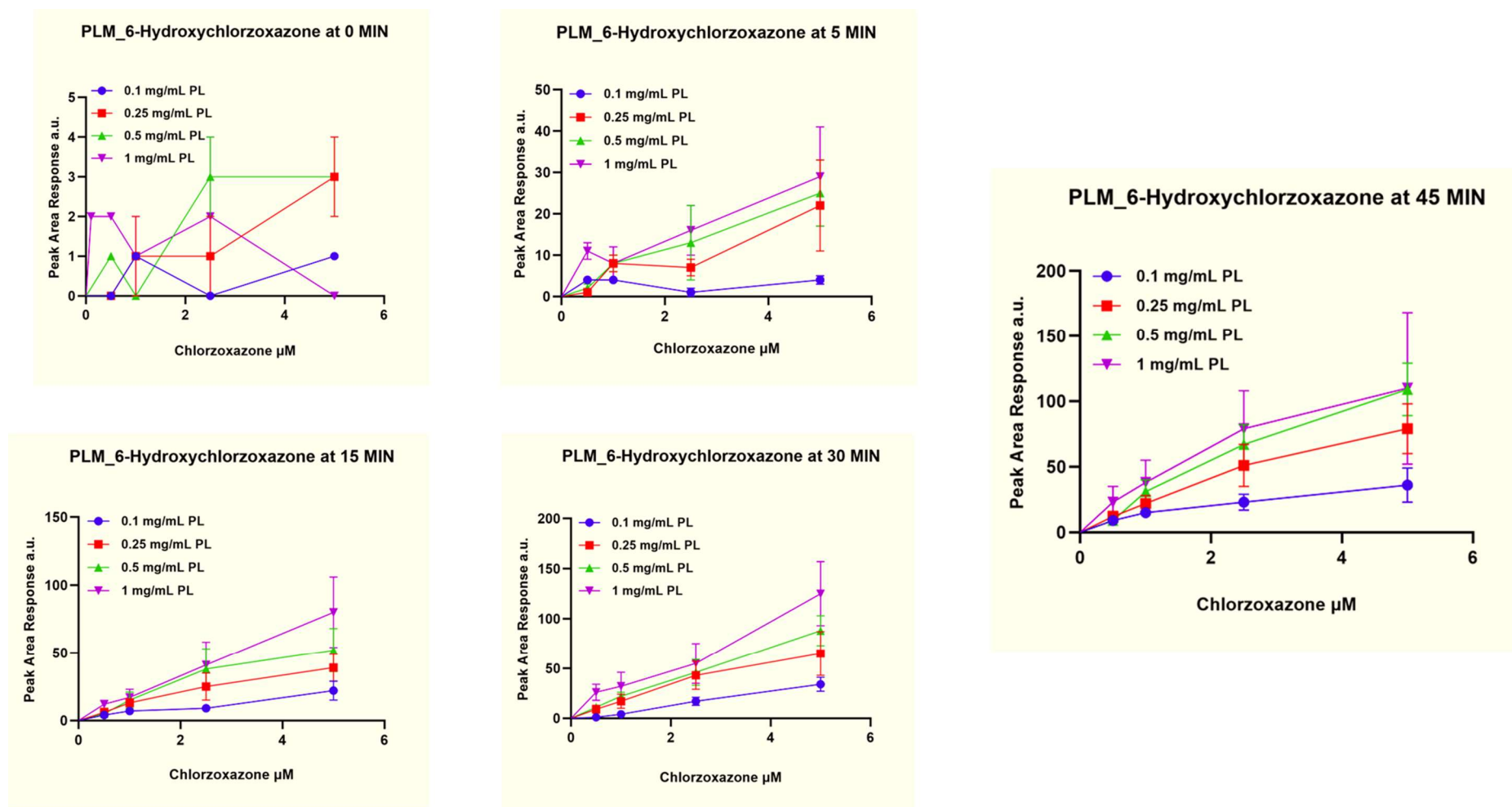


Figure 61 Metabolite Formation: CYP450-mediated metabolite profile of 6-hydroxychlorzoxazone across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

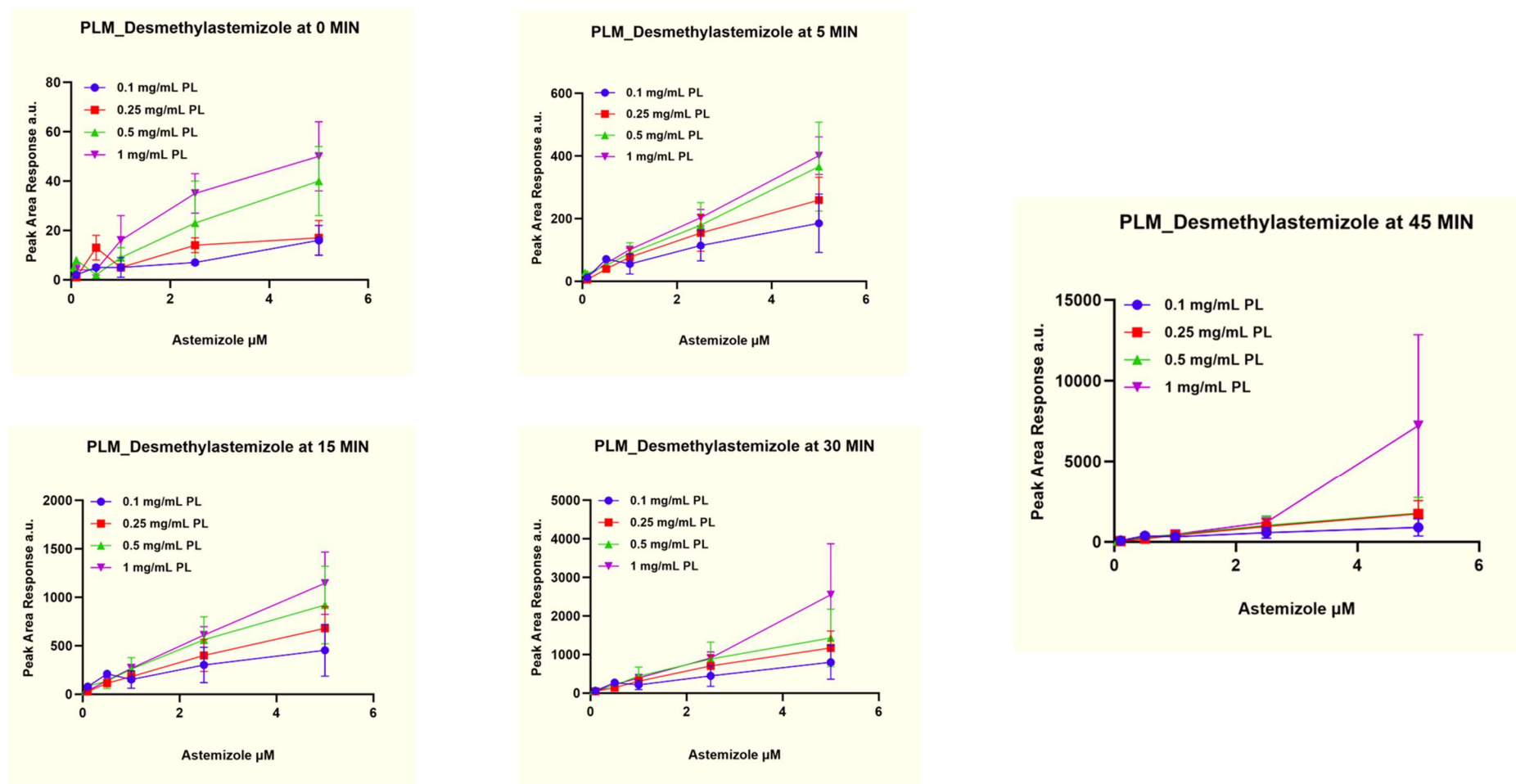


Figure 62 Metabolite Formation: CYP450-mediated metabolite profile of desmethylastemizole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

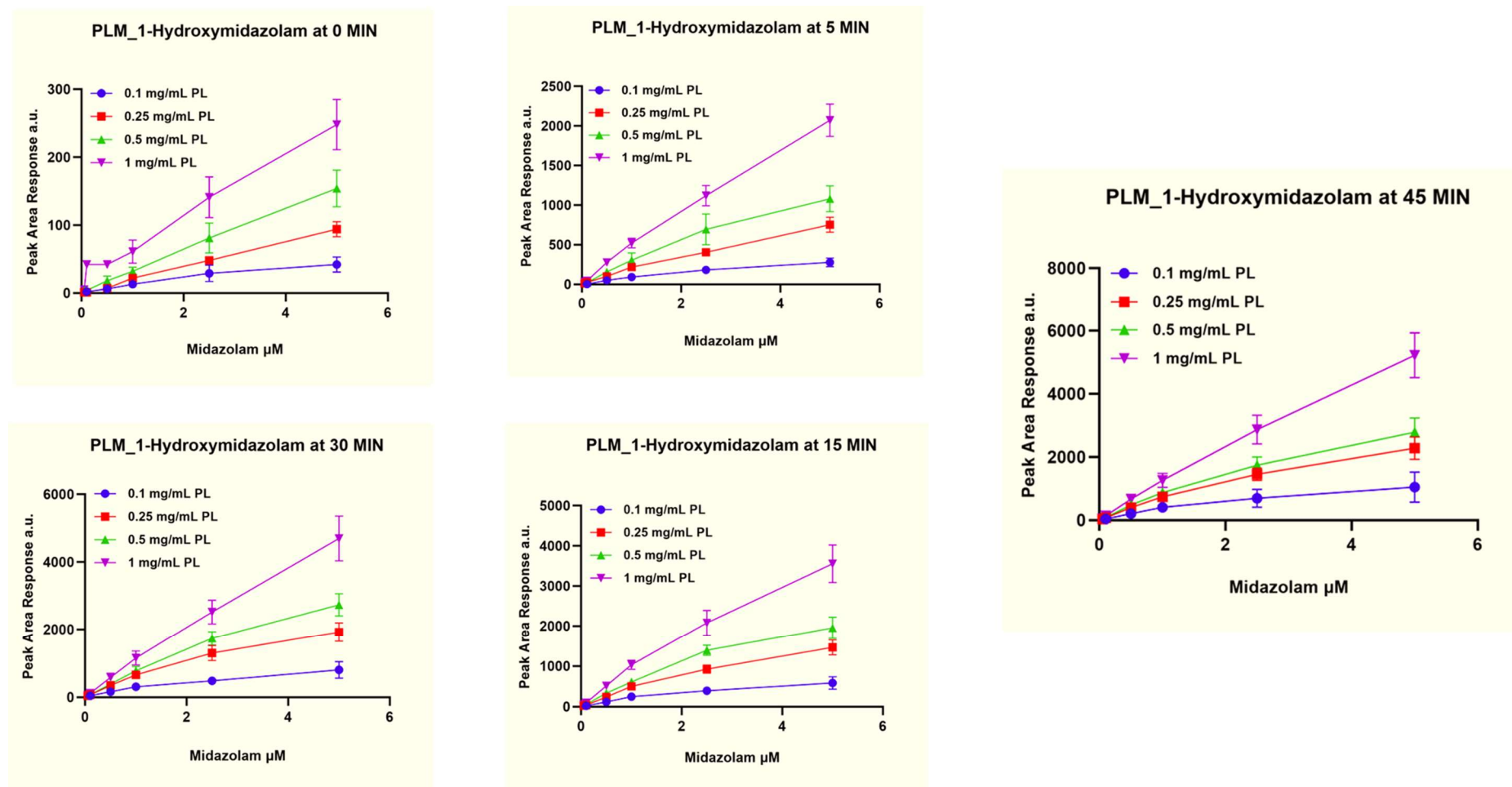


Figure 63 Metabolite Formation: CYP450-mediated metabolite profile of 1-hydroxymidazolam across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

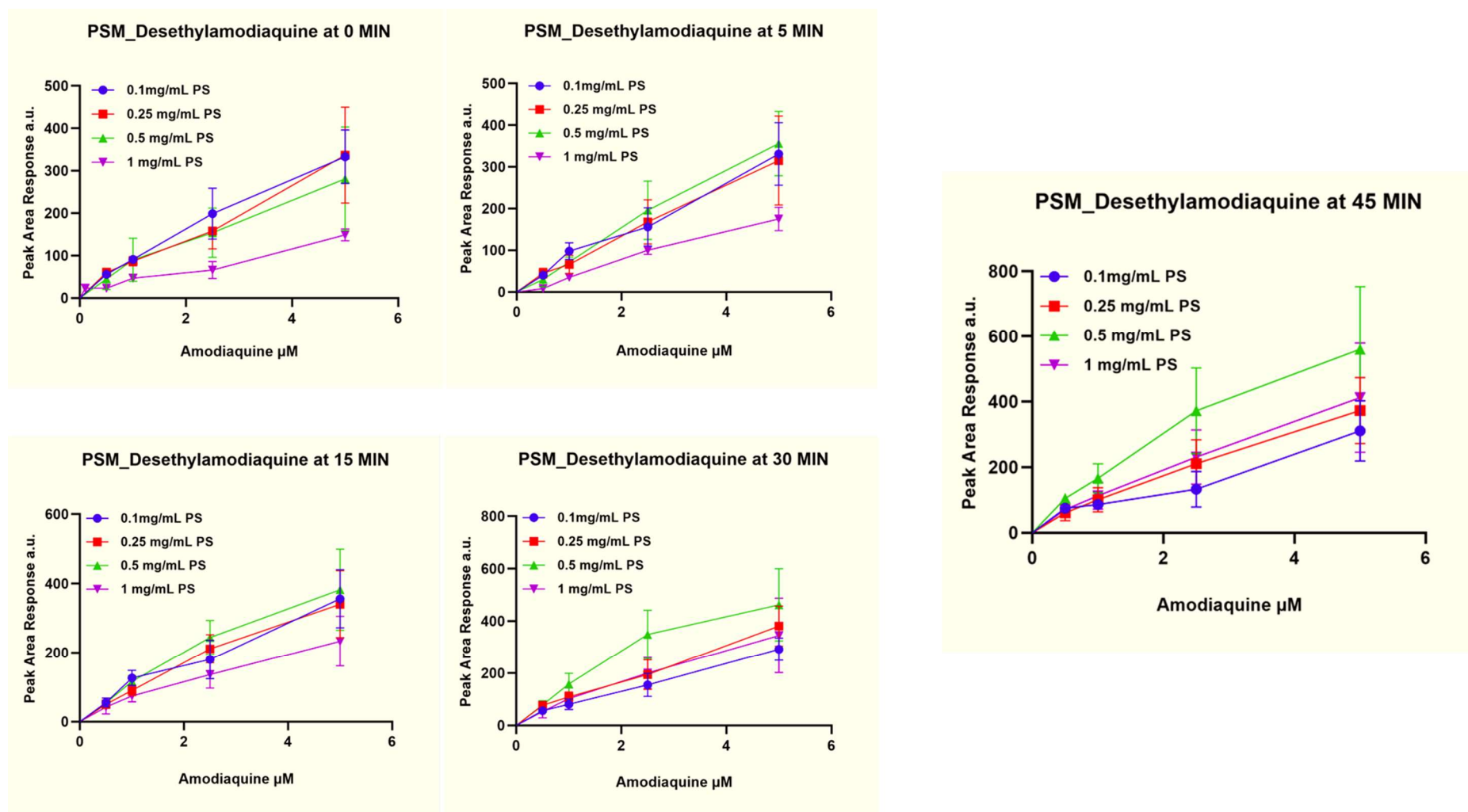


Figure 64 Metabolite Formation: CYP450-mediated metabolite profile of desethylamodiaquine across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

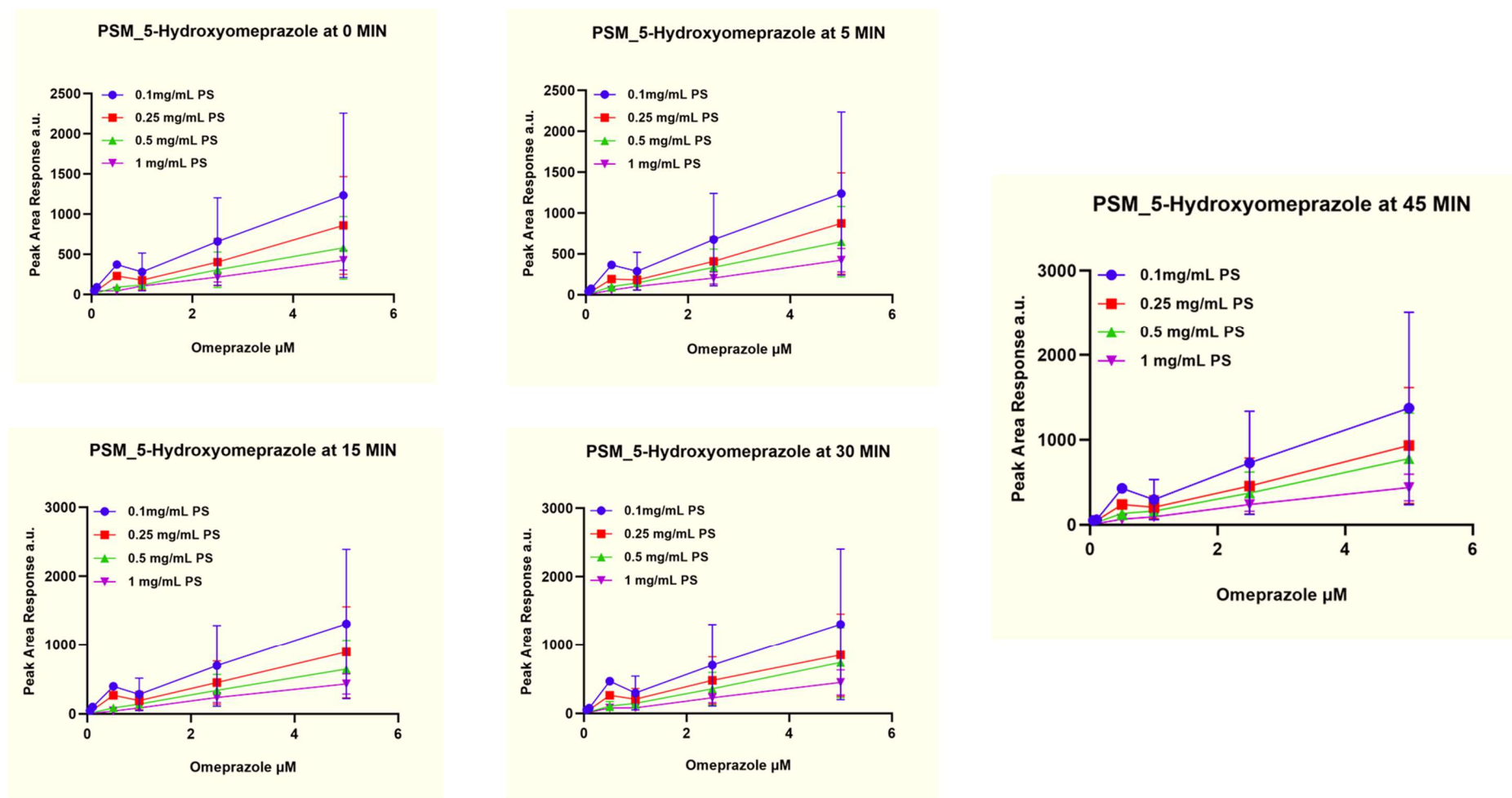


Figure 65 Metabolite Formation: CYP450-mediated metabolite profile of 5-hydroxyomeprazole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

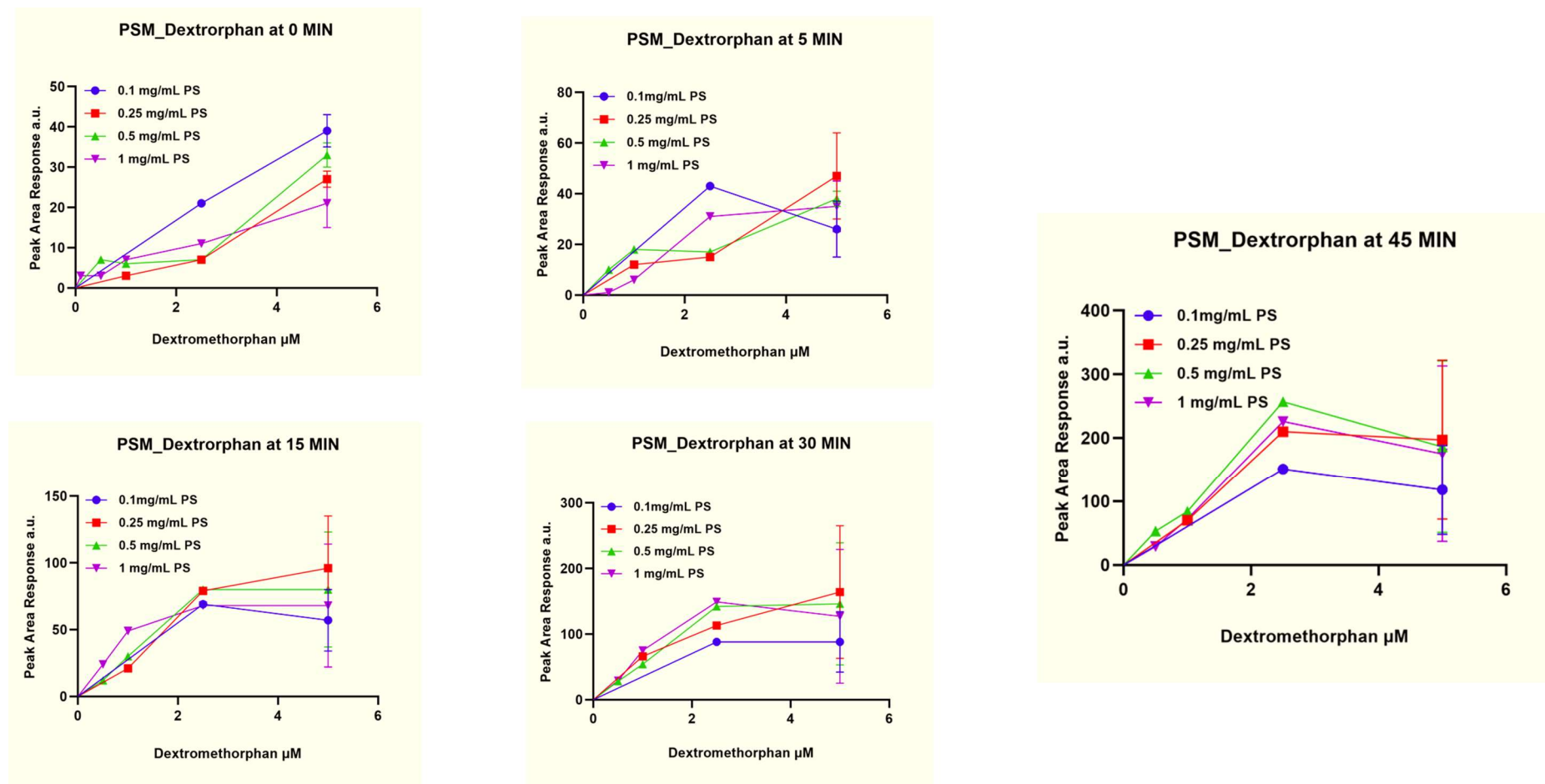


Figure 66 Metabolite Formation: CYP450-mediated metabolite profile of dextrophan across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

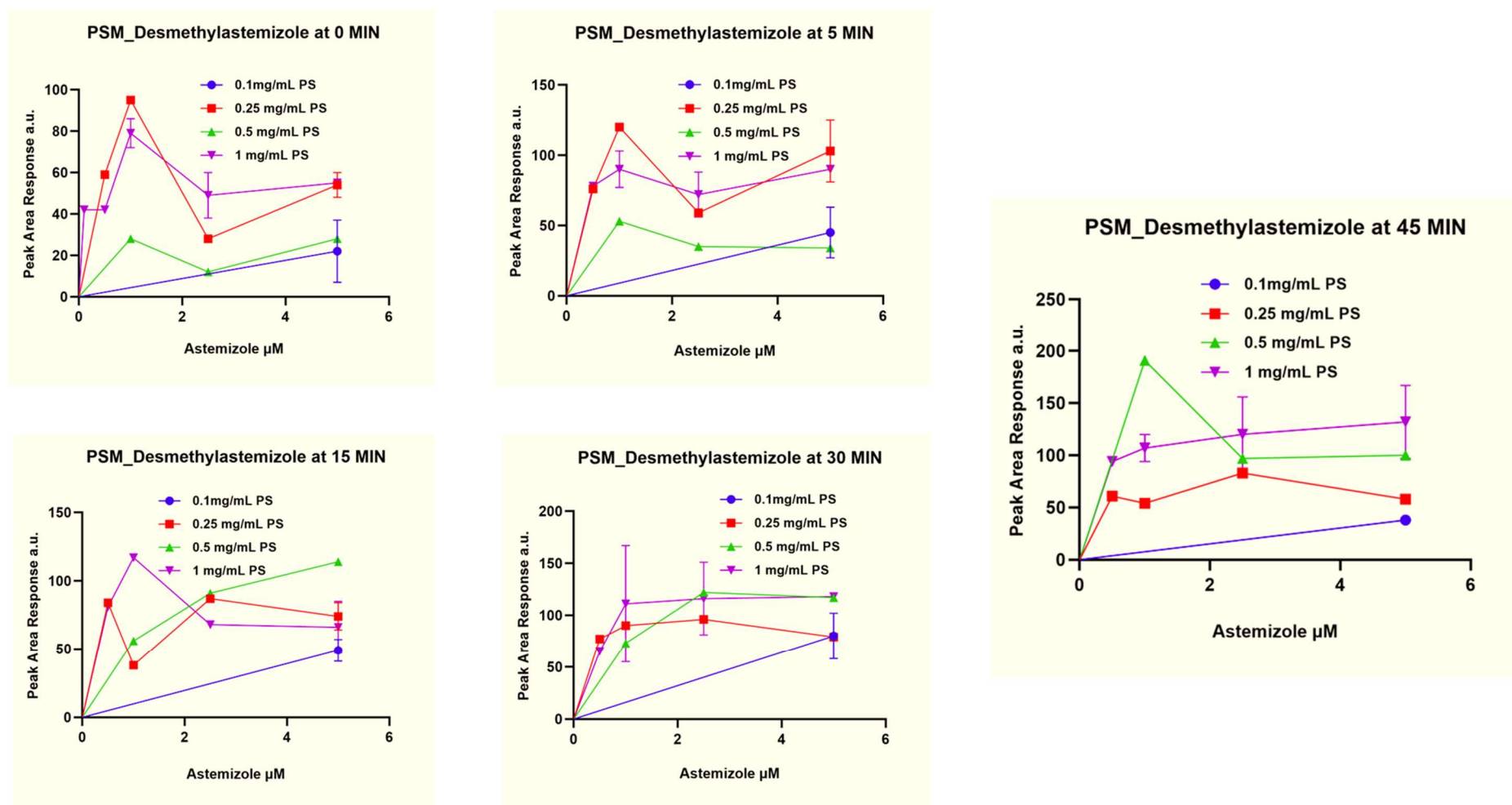


Figure 67 Metabolite Formation: CYP450-mediated metabolite profile of desmethylastemizole across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

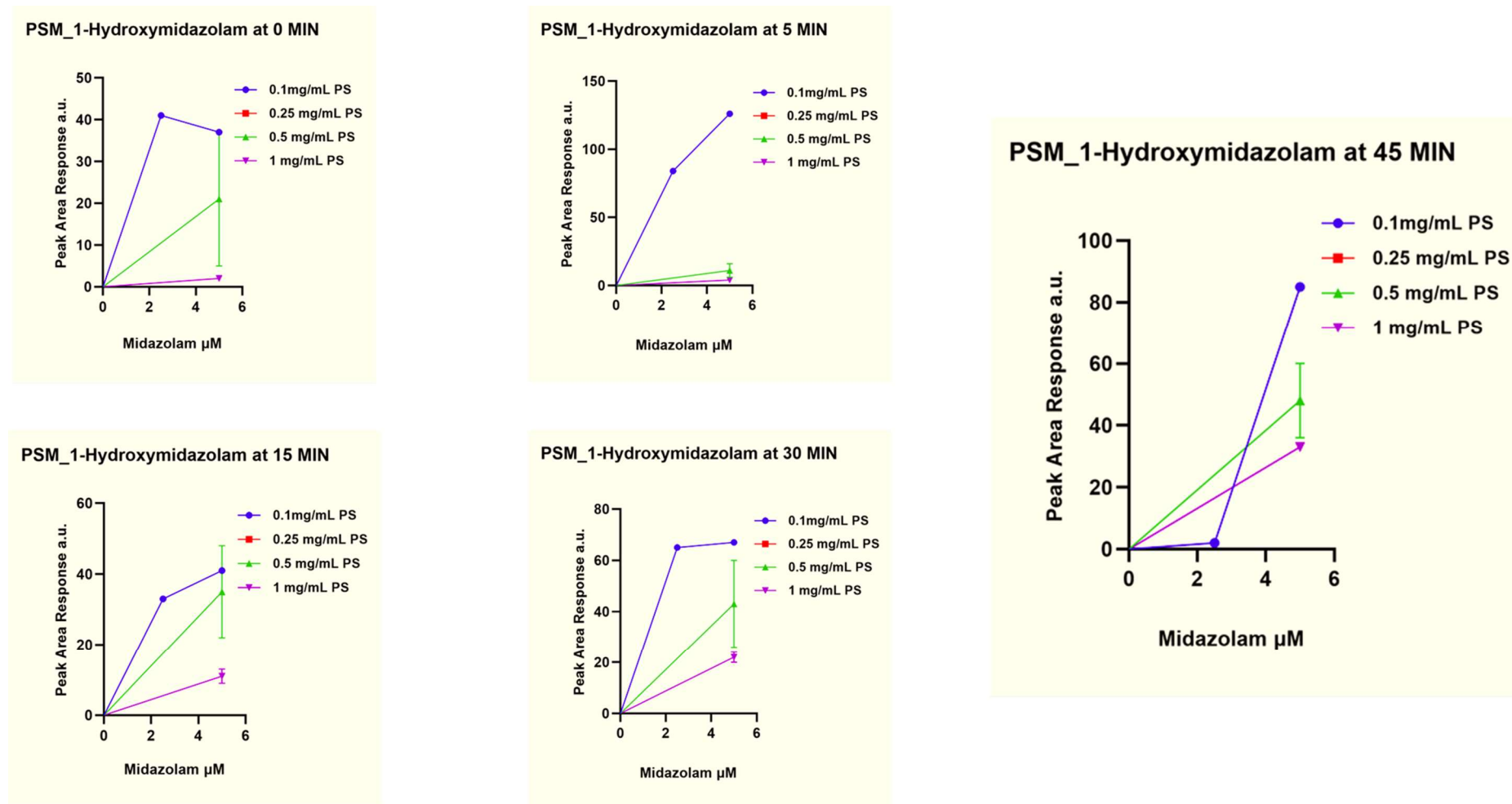


Figure 68 Metabolite Formation: CYP450-mediated metabolite profile of 1-hydroxymidazolam across the selected microsomal protein concentration at individual timepoints (0, 5, 15, 30 and 45 minutes) in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

Metabolite formation levels were observed for all the drug substrates in pig liver microsomes, and overall the metabolite levels decreased with increasing protein concentration although appeared to increase with increasing drug substrate concentration. Similarly, in pig spleen microsomes, observations of the metabolite formation profile showed the same pattern, but the metabolite levels appear to decrease at top concentrations, 5 μ M (drug substrate concentration) and 1 mg/mL (microsomal protein concentration).

5.2.4 CYP450-mediated metabolite formation in pig liver and spleen microsomes II

The following metabolite formation profiles are presented both as a function of drug substrate concentration and time (see Figure 69-73).

In pig liver microsomes, metabolites were detected for all the tested drug substrates, whereas, only five individual metabolite levels were observed in pig spleen microsomes. The metabolites of coumarin, bupropion, omeprazole, chlorzoxazone, astemizole and midazolam showed increasing levels proportional to increasing protein concentration and over time in pig liver. Similarly, also for the metabolite of phenacetin except at 0.1 mg/mL, its levels appear to decrease at 5 μ M; observations for the metabolites of dextromethorphan, amodiaquine and tolbutamide either shows increasing metabolite levels up to 0.5 mg/mL, then drops at 1 mg/mL microsomal protein concentration, or levels are linear up to 2.5 μ M, then decrease at 5 μ M drug substrate concentration, or increased levels up to 30 minutes are observed but declines by 45 minutes in the pig liver microsomes. In contrast, the metabolite of dextromethorphan showed levels increasing with drug substrate concentration up to 2.5 μ M, then the levels drops at 5 μ M drug substrate concentration; and similarly with increasing

protein concentration where metabolite levels increased up to 0.5 mg/mL but decrease at 1 mg/mL, though over time the metabolite levels increased in pig spleen microsomes. The observations for the metabolites of omeprazole and amodiaquine showed almost identical profiles, where the metabolite levels increased with increasing drug substrate concentration; but over time, the metabolite levels appeared constant except at 0.5 mg/ mL protein concentration or constant up to 0.25 mg/ mL protein concentration then increased from 0.5 mg/ mL protein concentration respectively. In addition, the metabolite levels increased with increasing protein concentration up to 0.5 mg/mL then decreases at 1 mg/ mL from observations with amodiaquine; whereas with omeprazole, the metabolite levels decreased with increasing protein concentration in pig spleen microsomes. Low metabolite levels of midazolam were detected but showed to decrease with increasing protein concentration even though nothing was detected at 0.25 mg/mL. Nonetheless, the metabolite levels observed for midazolam were only detected at 5 μ M at 0.5 and 1 mg/mL protein concentration and showed to increase over time in pig spleen microsomes.

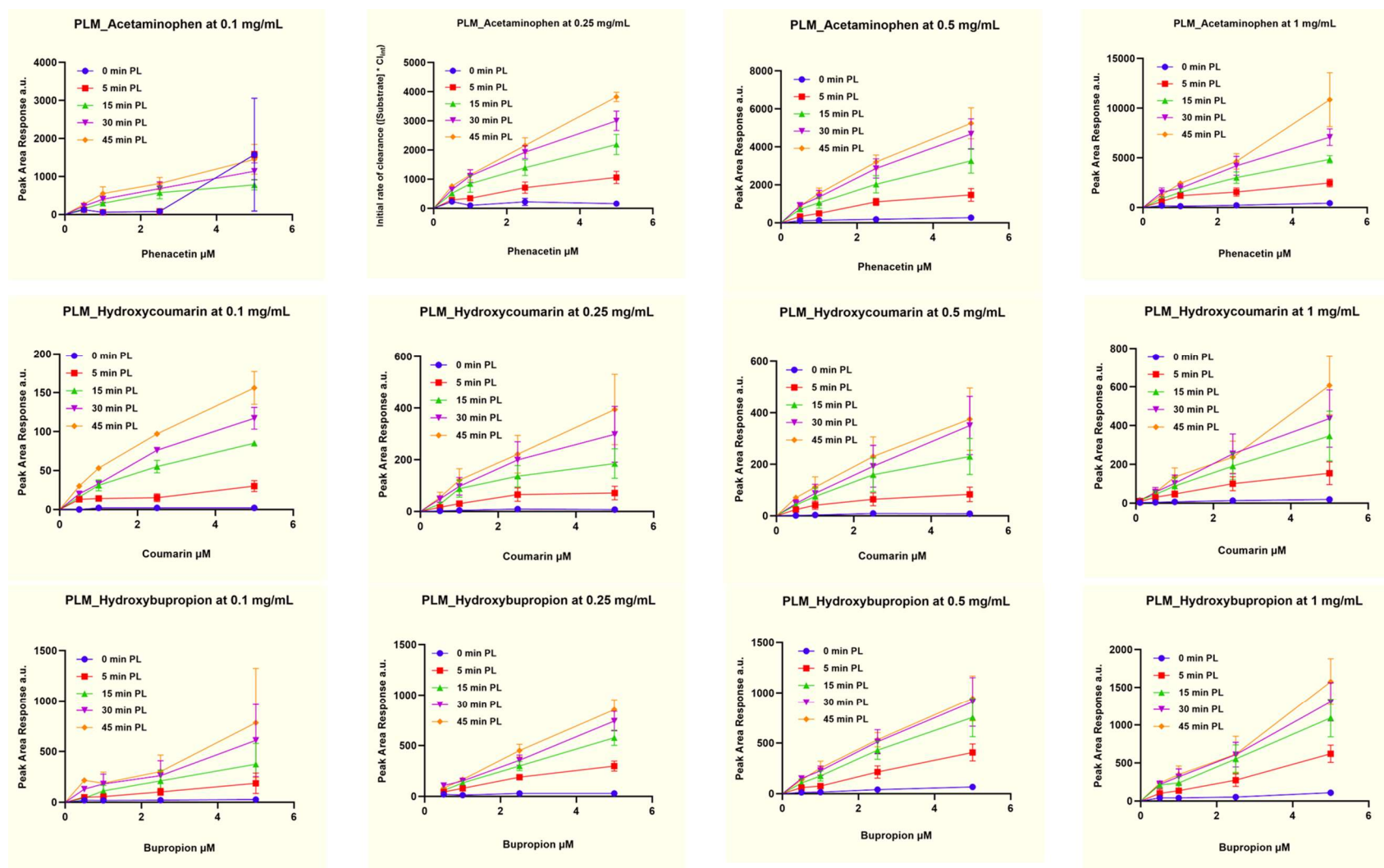


Figure 69 CYP450-mediated metabolite profiles of acetaminophen, hydroxycoumarin and hydroxybupropion over time at the selected protein concentration in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

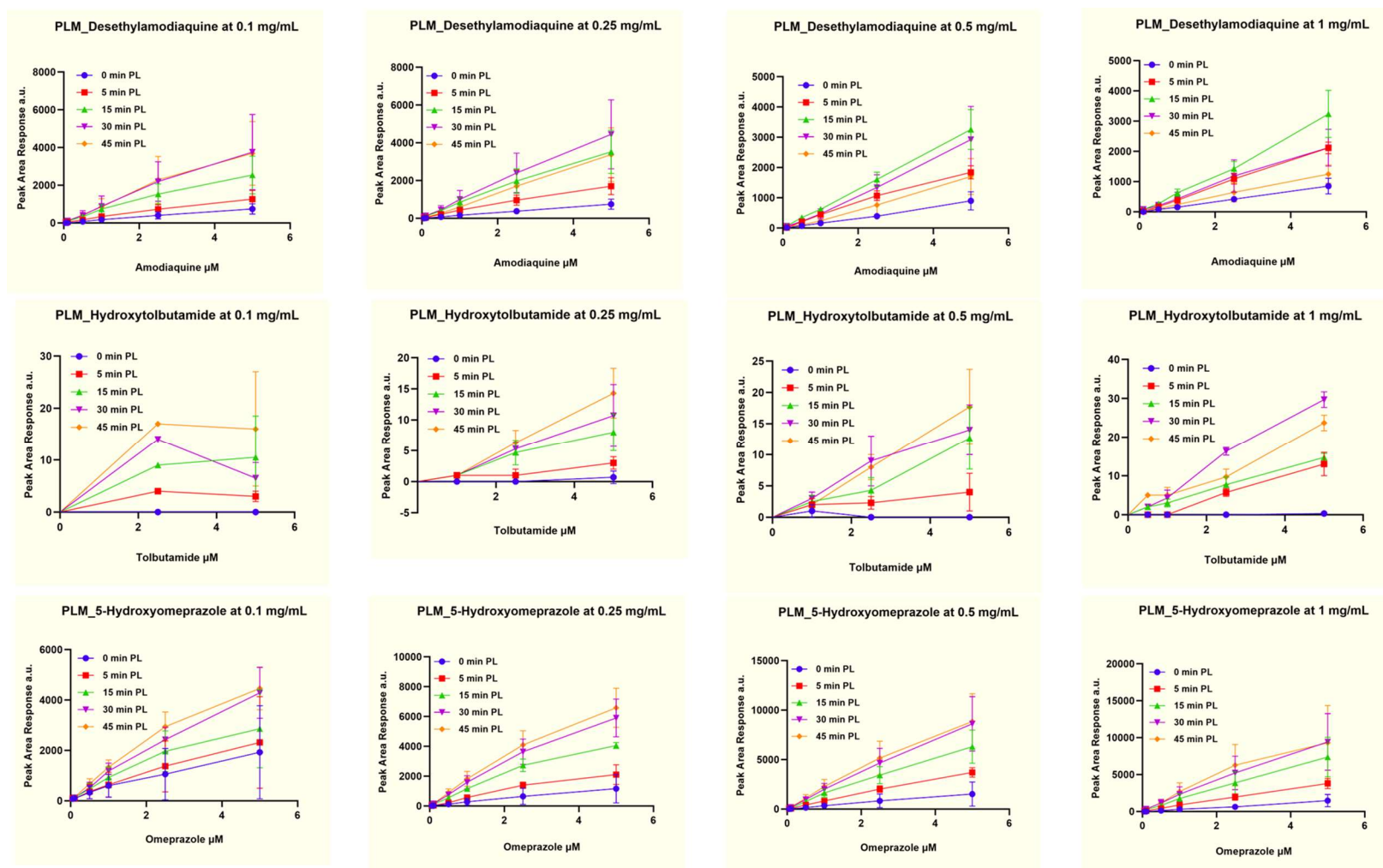


Figure 70 CYP450-mediated metabolite profiles of desethylamodiaquine, hydroxytolbutamide and 5-hydroxyomeprazole over time at the selected protein concentration in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

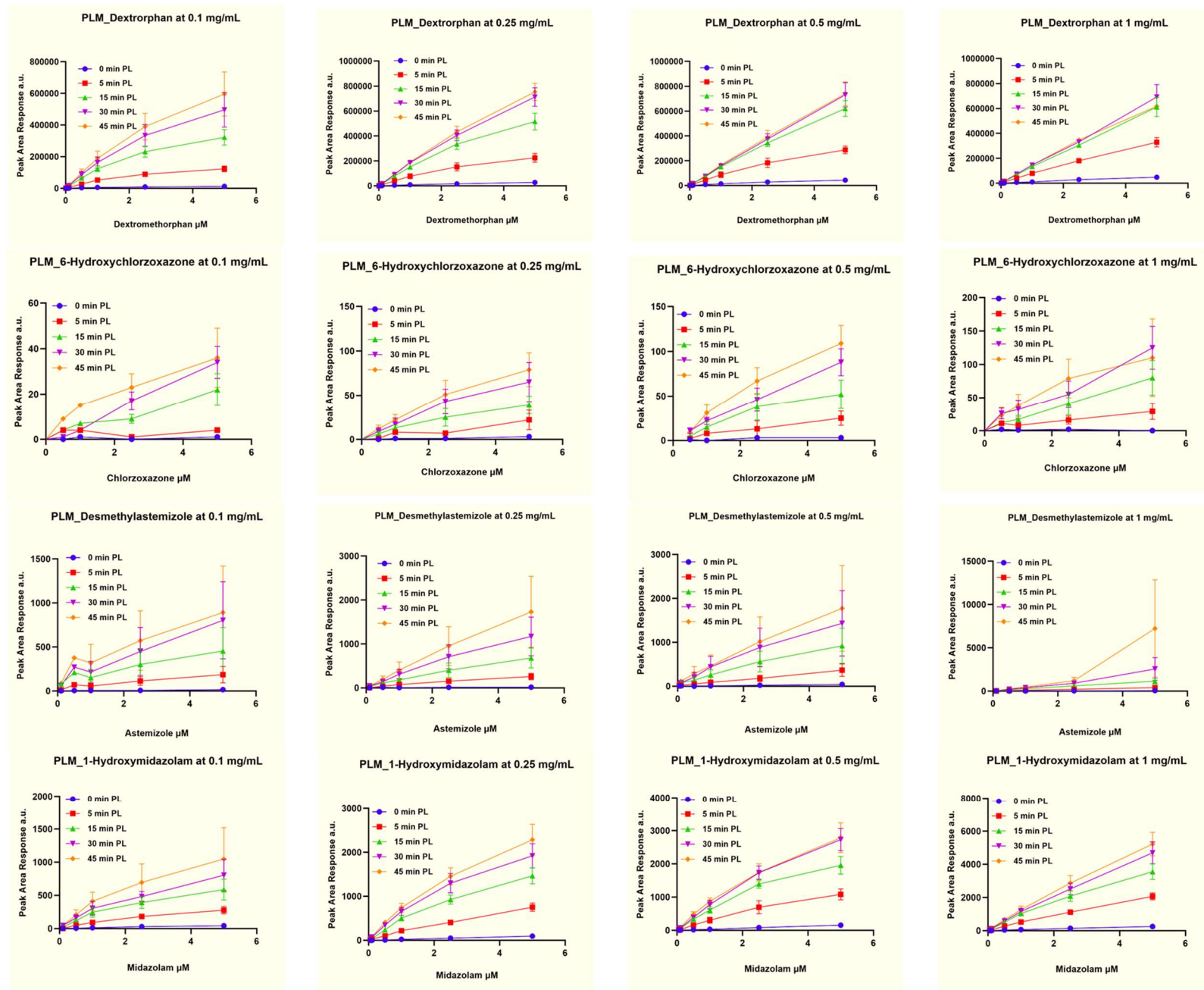


Figure 71

CYP450-mediated metabolite profiles of dextrophan, 6-hydroxychlorzoxazone, desmethylastemizole and 1-hydroxymidazolam over time at the selected protein concentration in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

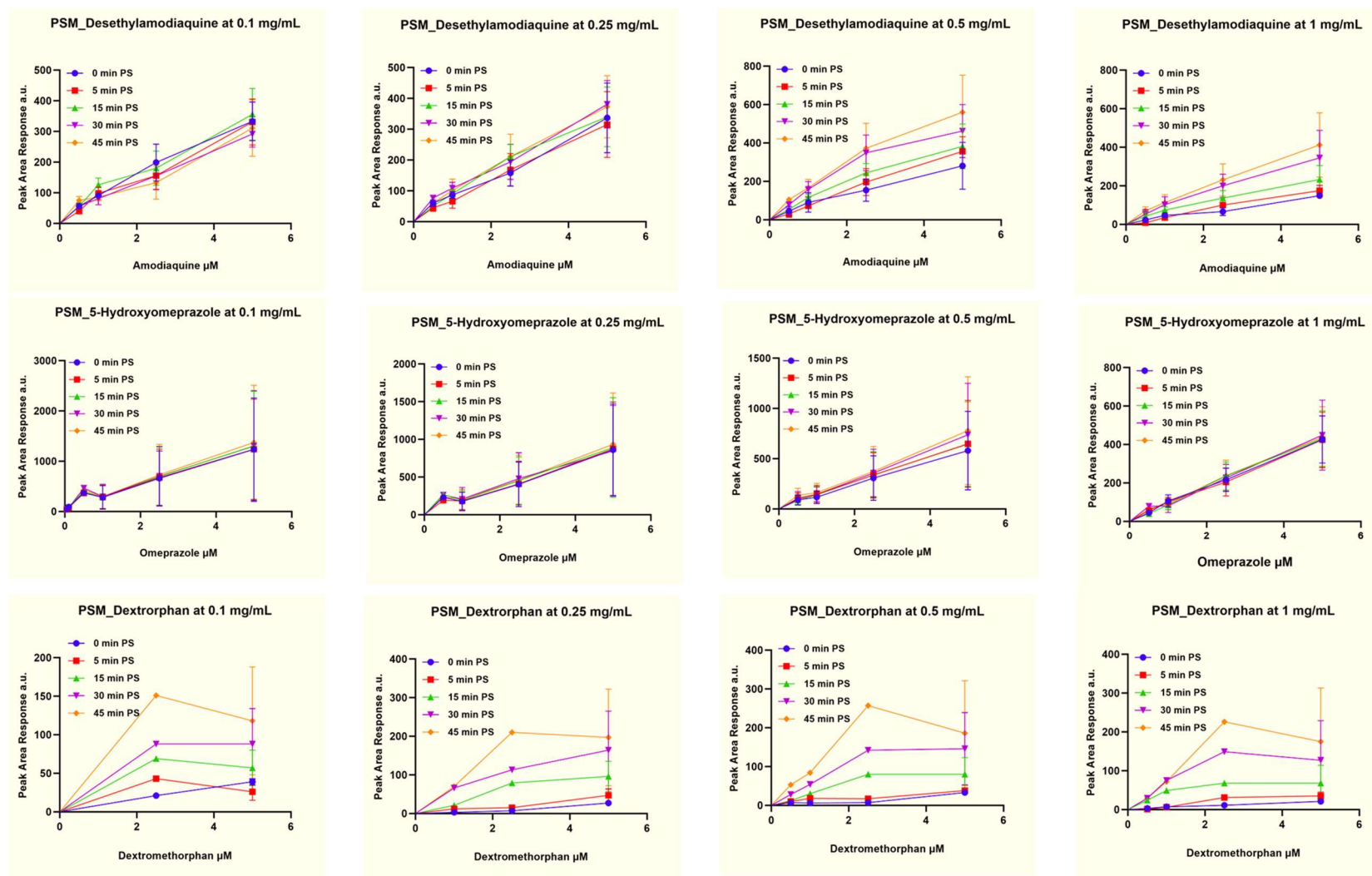


Figure 72 CYP450-mediated metabolite profiles of desethylamodiaquine, 5-hydroxyomeprazole and dextromethorphan over time at the selected protein concentration in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, USA, www.graphpad.com

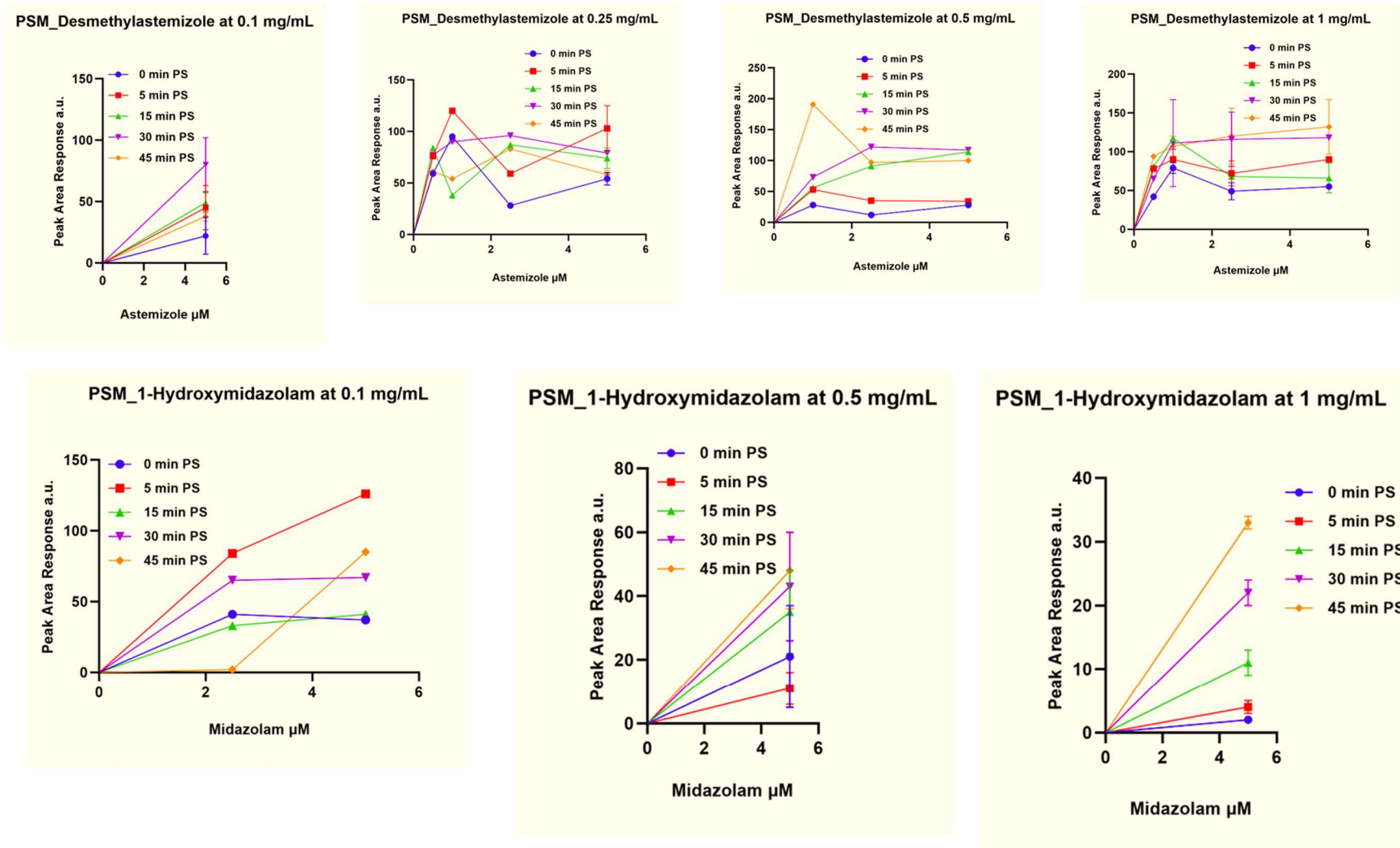


Figure 73 CYP450-mediated metabolite profiles of desmethylastemizole and 1-hydroxymidazolam over time at the selected protein concentration in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

5.2.5 UGT-mediated metabolite formation in pig liver and spleen microsomes I

Metabolite formation levels were monitored for the selected UGT drug substrates in pig liver and spleen microsomes. However, the creation of the MS/MS methods for both 2-hydroxyimipramine glucuronide and acetaminophen glucuronide was not achieved, as it failed both optimisation and the acceptance criteria checks. Therefore, the samples were not analysed for the metabolites of imipramine and acetaminophen. The following metabolite formation profiles are presented both as a function of microsomal protein concentration and drug substrate concentration (see Figure 74-80).

The results from UGT-mediated metabolite formation showed varying profiles in both pig liver and spleen microsomes. Firstly, metabolite levels of all the tested UGT drug substrates were determined in pig liver microsomes. Generally, the metabolite levels increased with increasing protein concentration and drug concentration. On the other hand, only three detectable metabolites were observed in pig spleen microsomes, and only Naloxone-3-glucuronide showed a similar pattern observed with the metabolites in pig liver microsomes. Serotonin- β -D-glucuronide and 7-hydroxycoumarin glucuronide showed a decrease in the metabolite levels with increasing protein concentration, though the former showed constant levels with increasing drug concentration, while the metabolite levels for the latter increased up to 2.5 μ M drug substrate concentration. 17- β -Estradiol-3-glucuronide was not detected in pig spleen microsomes.

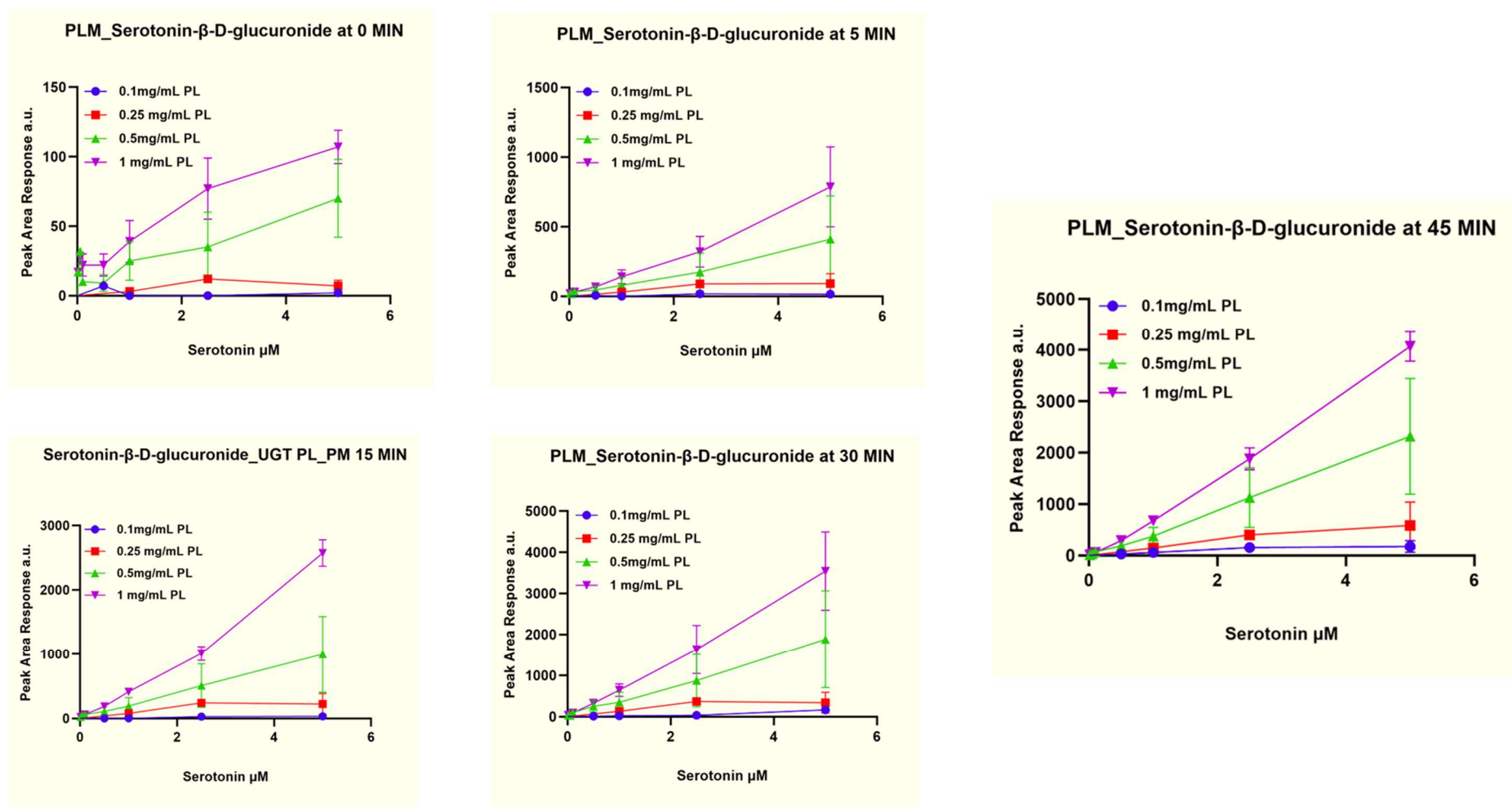


Figure 74 UGT-mediated metabolite profile of serotonin-β-D-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

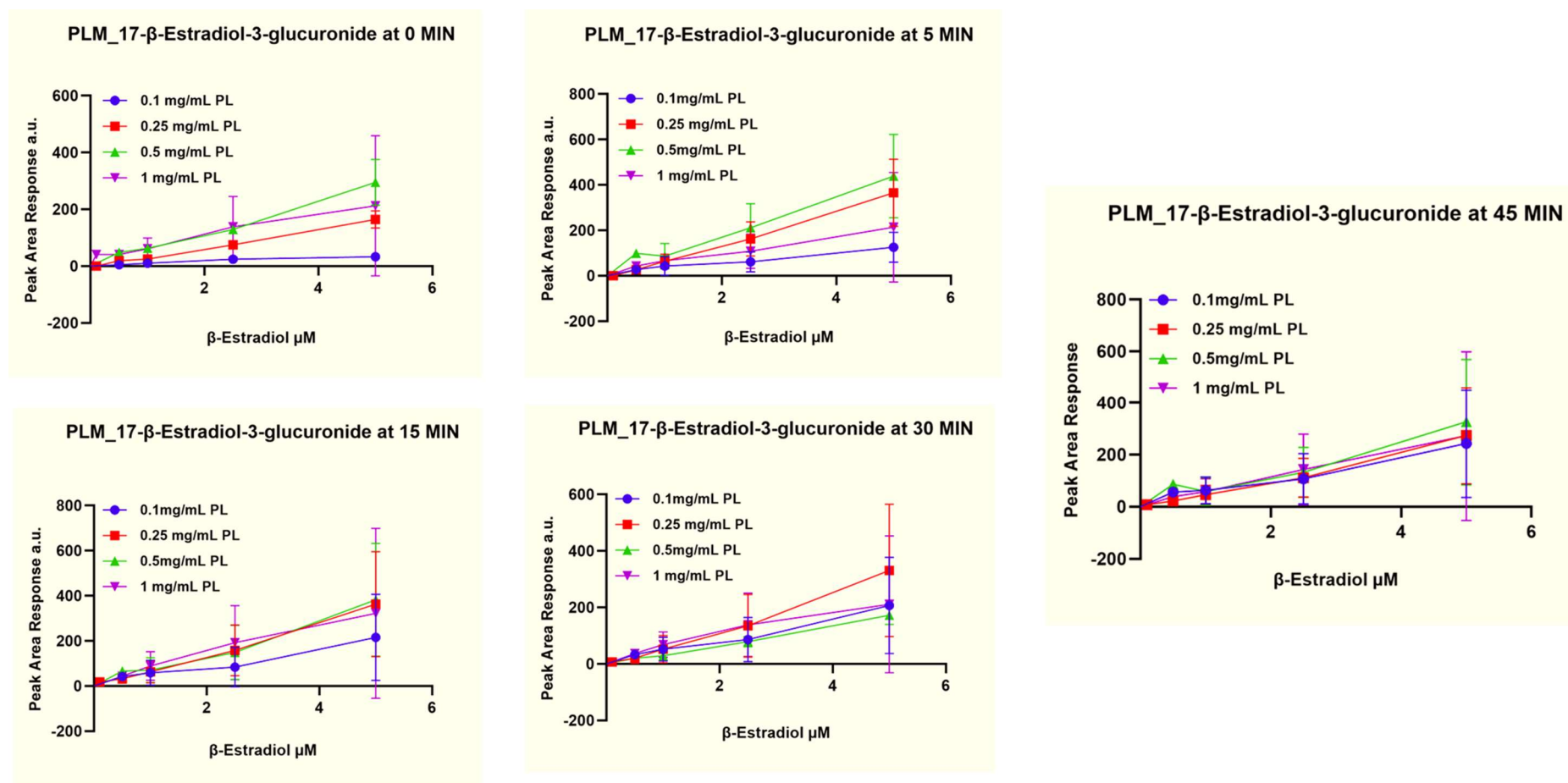


Figure 75 UGT-mediated metabolite profile of 17-β-Estradiol-3-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

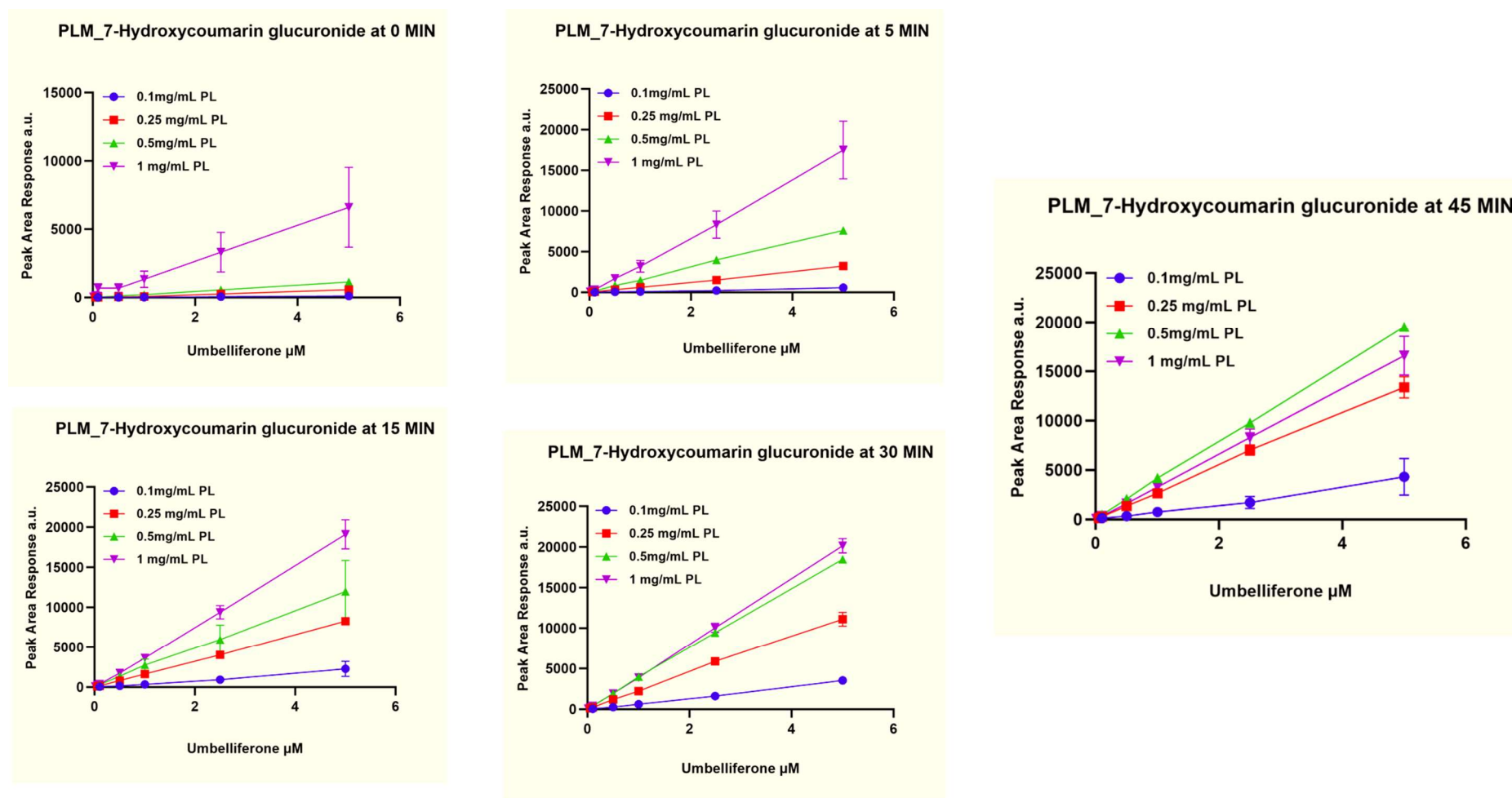


Figure 76 UGT-mediated metabolite profile of 7-hydroxycoumarin glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

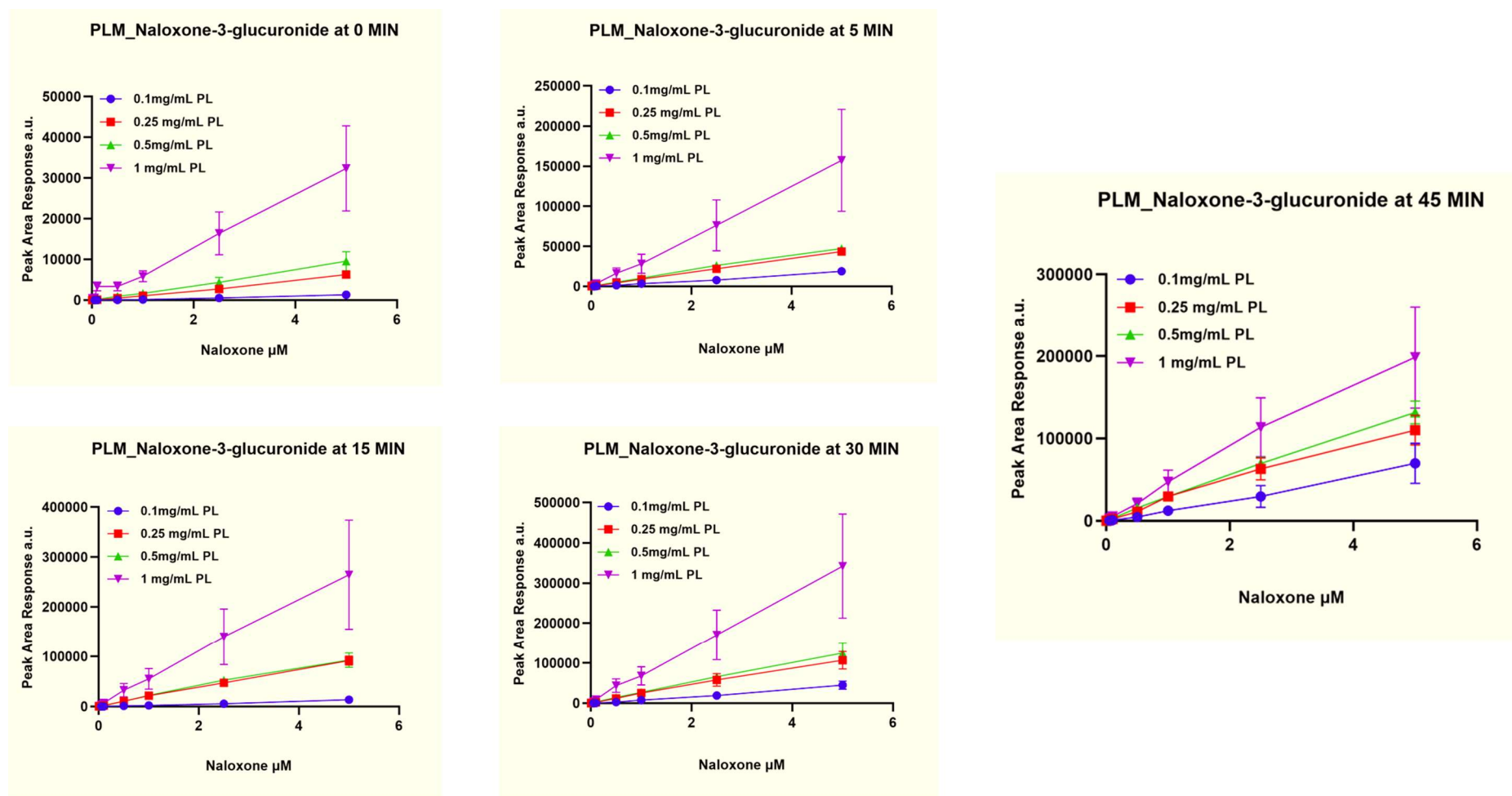


Figure 77 UGT-mediated metabolite profile of Naloxone-3-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

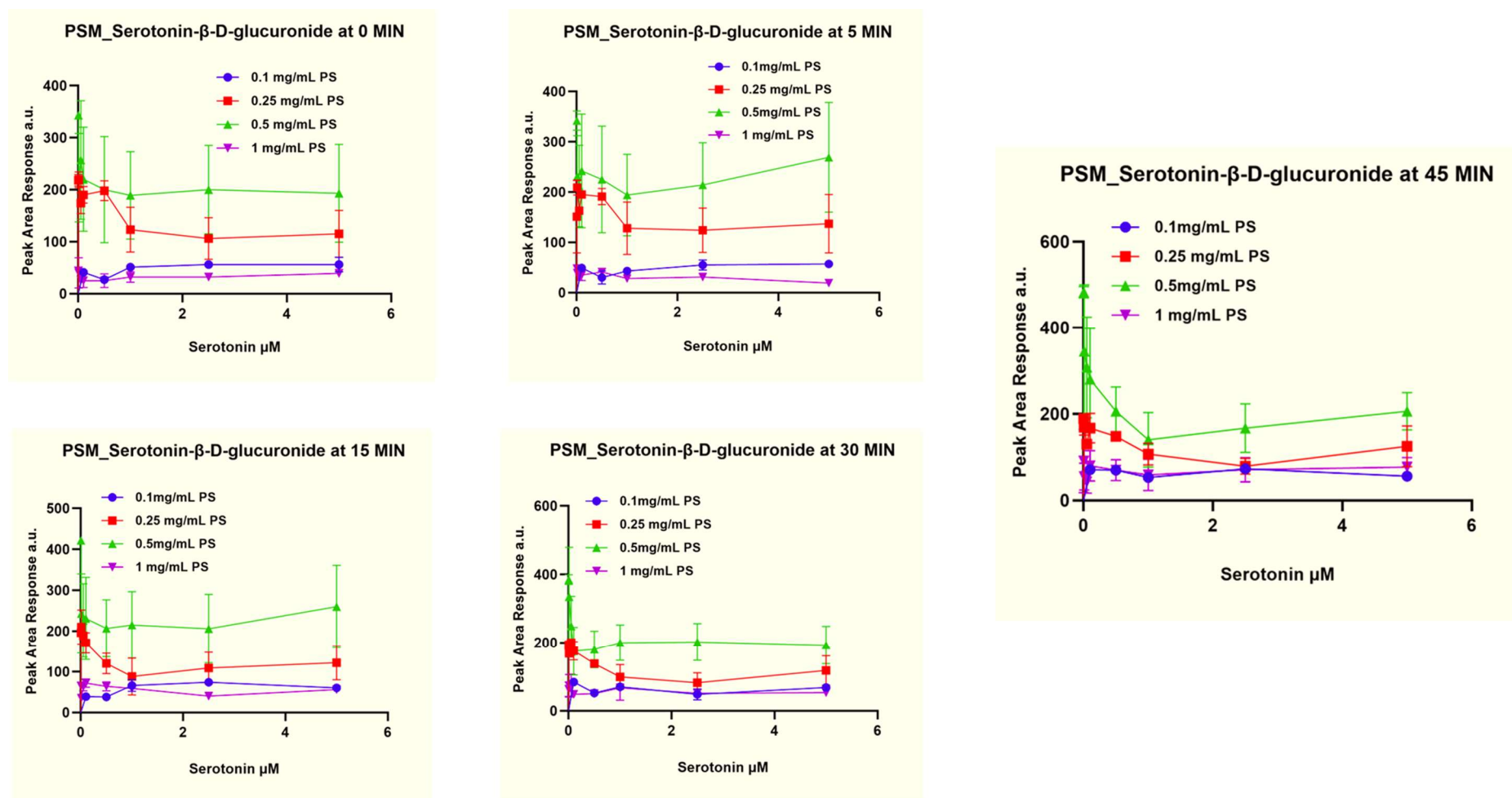
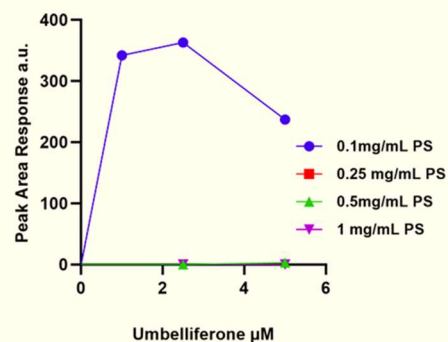
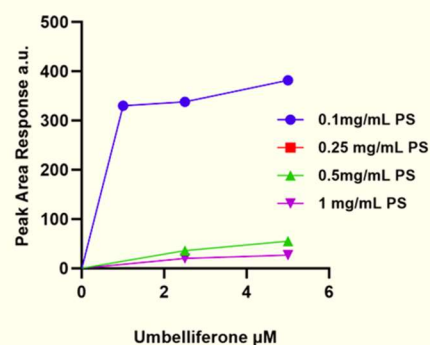


Figure 78 UGT-mediated metabolite profile of serotonin-β-D-glucuronide across the selected microsomal protein concentration at individual timepoints in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

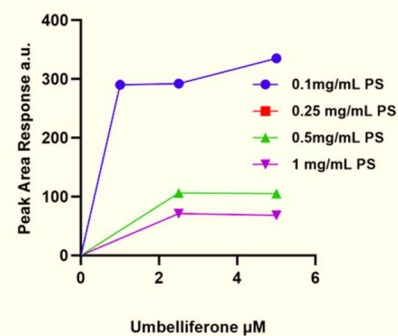
PSM_7-Hydroxycoumarin glucuronide at 0 MIN



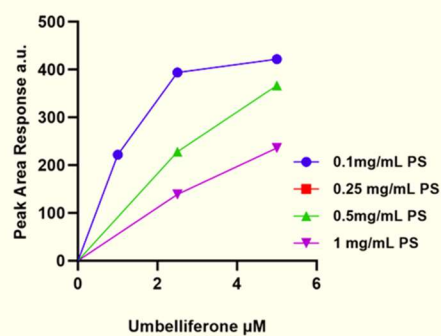
PSM_7-Hydroxycoumarin glucuronide at 5 MIN



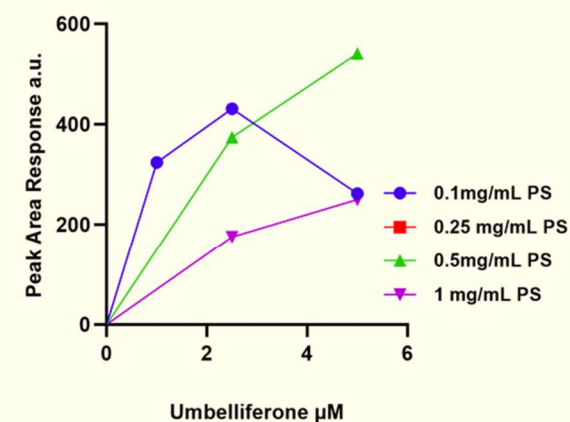
PSM_7-Hydroxycoumarin glucuronide at 15 MIN



PSM_7-Hydroxycoumarin glucuronide at 30 MIN



PSM_7-Hydroxycoumarin glucuronide at 45 MIN

**Figure 79**

UGT-mediated metabolite profile of 7-hydroxycoumarin glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

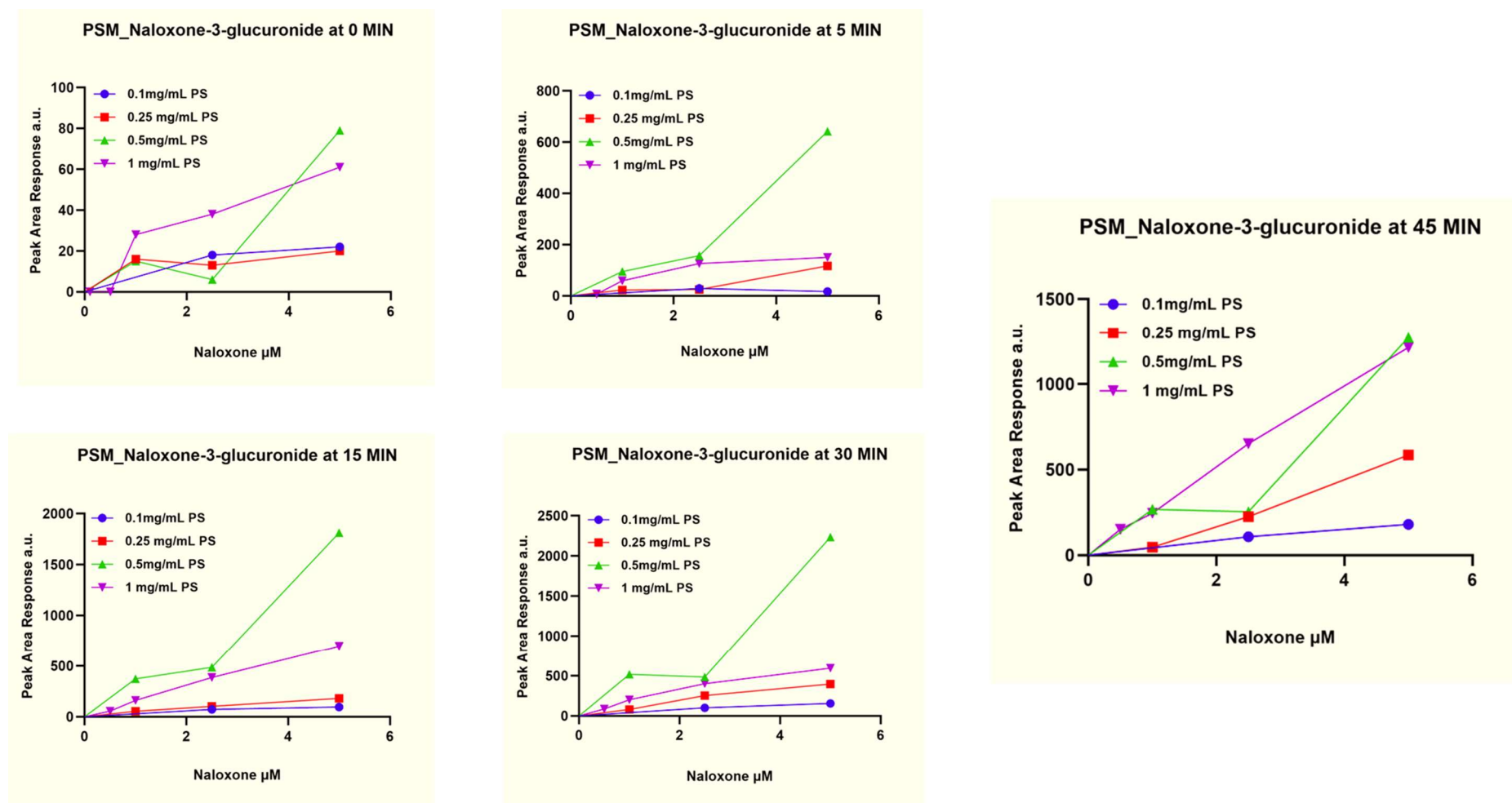


Figure 80 UGT-mediated metabolite profile of Naloxone-3-glucuronide across the selected microsomal protein concentration at individual timepoints in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com

5.2.6 UGT-mediated metabolite formation in pig liver and spleen microsomes II

The following metabolite formation profiles are presented both as a function of drug substrate concentration and time (see Figure 81 and 82).

The metabolite levels of the UGT drug substrates overall increased with increasing drug concentration and over time to an extent in pig liver microsomes with exception for the metabolite 17- β -Estradiol-3-glucuronide where the levels remained constant. In pig spleen microsomes, only Naloxone-3-glucuronide conveyed somewhat an increase in its metabolite levels with increasing drug concentration and over time, while for the other metabolites, their levels were constant over time. Although, glucuronidation was also observed in pig spleen microsomes using Serotonin, the metabolite formation levels showed a peculiar pattern indicating that the levels were unaffected by the increasing drug substrate concentration. The reasons as to why or what is behind this observation remains unknown. *Ugt8* is not known to be a major contributor to drug metabolism in human liver but is primarily the only one of two genes identified in pig spleen by NGS.

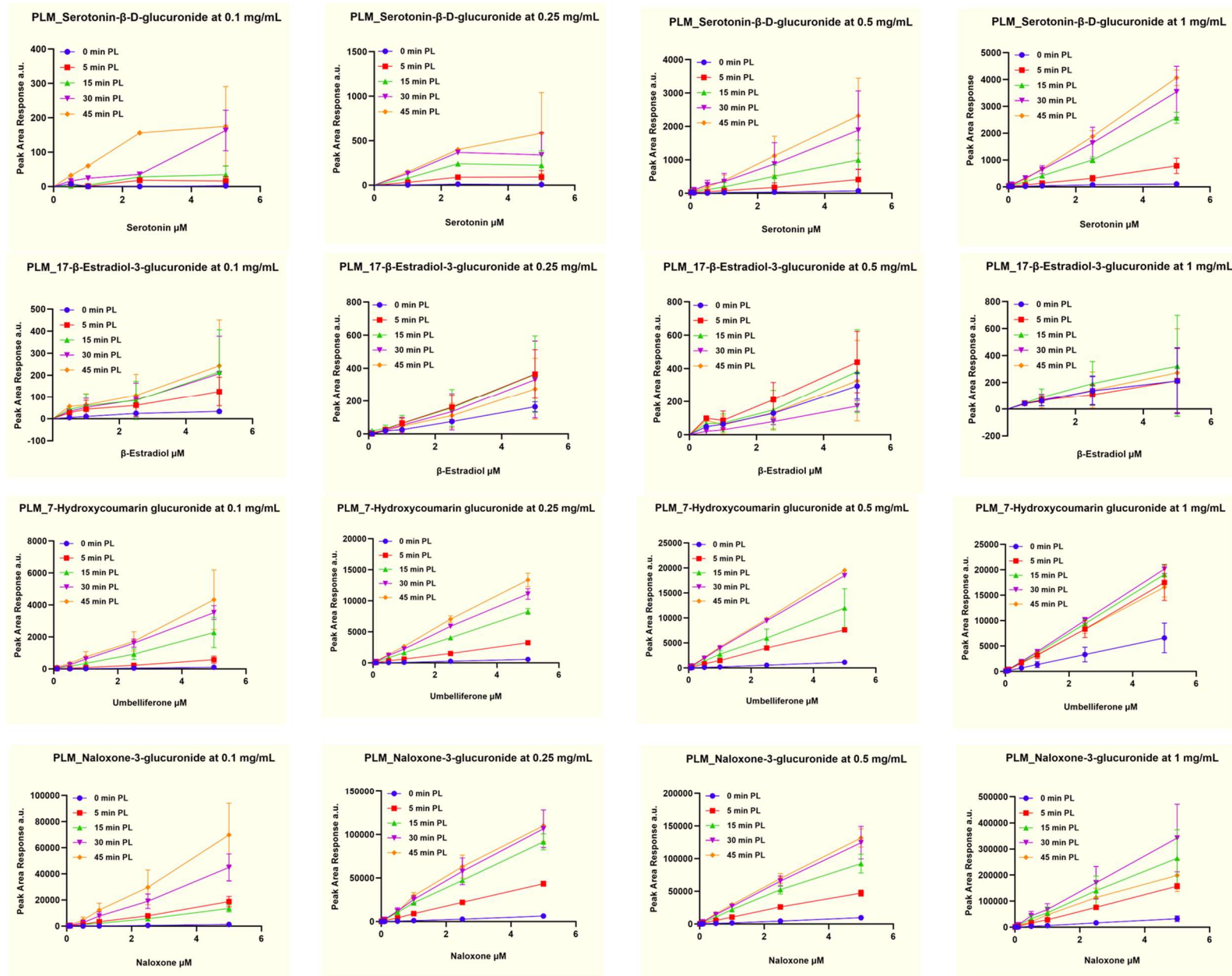


Figure 81

CYP450-mediated metabolite profiles of Serotonin-β-D-glucuronide, 17-β-Estradiol-3-glucuronide, 7-hydroxycoumarin glucuronide and Naloxone-3-glucuronide over time at the selected protein concentration in pig liver microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, USA, www.graphpad.com

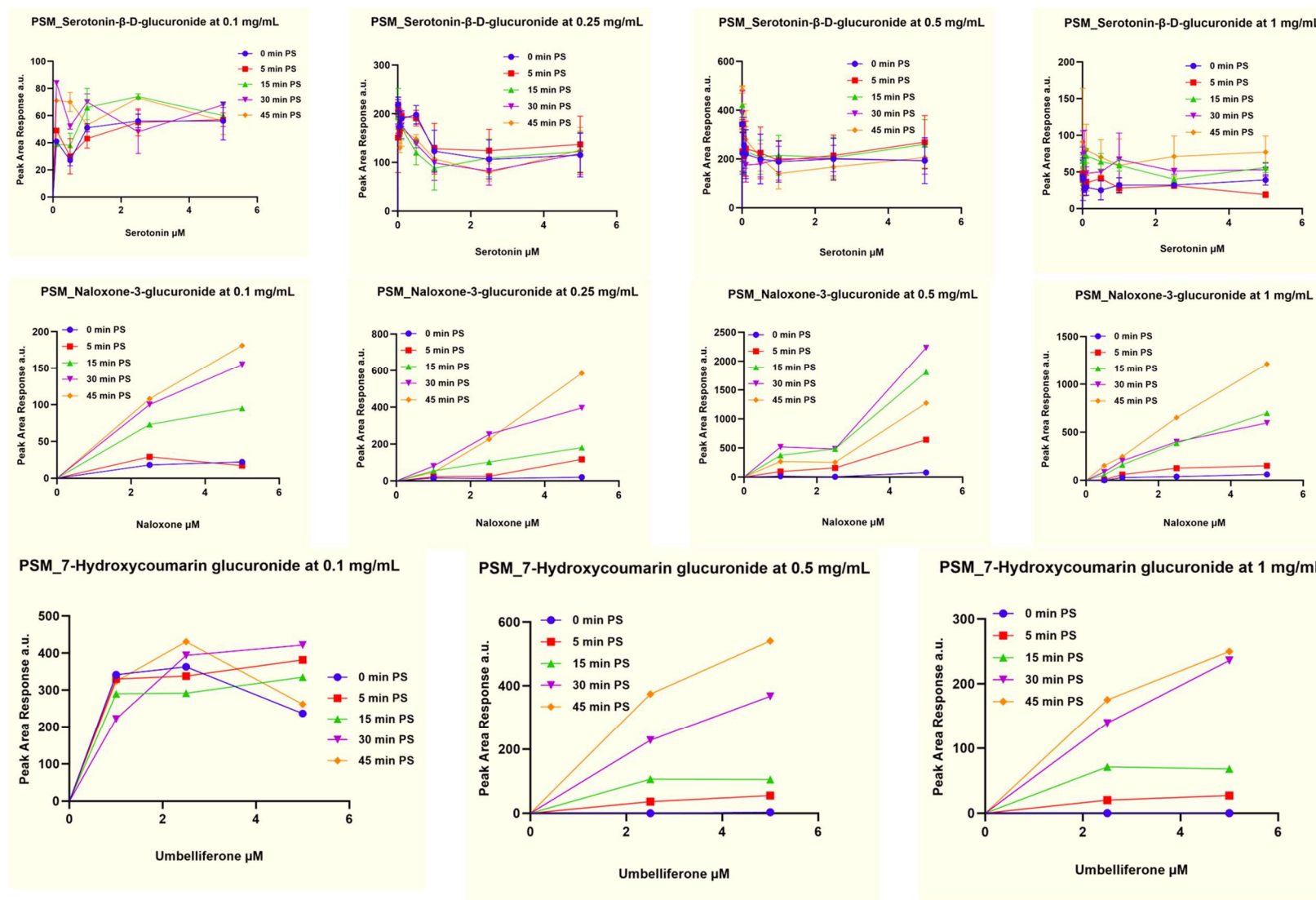


Figure 82

CYP450-mediated metabolite profiles of Serotonin-β-D-glucuronide, 7-hydroxycoumarin glucuronide and Naloxone-3-glucuronide over time at the selected protein concentration in pig spleen microsomes. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.2.3 for Windows, GraphPad Software, USA, www.graphpad.com

5.2.7 CYP450-mediated drug clearance in pig hepatocytes and splenocytes

The CYP450 and UGT-mediated drug elimination rate was also determined in pig hepatocytes and splenocytes. The same CYP450 drug substrates were investigated in a cell-based assay. The following profiles are the initial rate in pmoles/min/number of cells in the incubation (tested at 0.5 million cells).

Drug elimination was observed for almost all the CYP450 drug substrates in both pig hepatocytes and splenocytes (see Figure 83 and 84). The drug clearance observed in pig splenocytes appeared linear compared to that observed in the pig hepatocyte profiles. Astemizole appeared not to be metabolised in pig splenocytes, as the profile almost seems flat. The drug clearance levels across both cell organs were relatively comparable. The substrate depletion approach is a well-established assay particularly in industry to monitor the metabolic stability of new chemical entities or drug candidates both in microsomal fractions and cell-based (i.e. hepatocyte) systems. Observations from these studies would indicate whether the drug candidates would be subjected to extensive metabolism and thereby altering the dose required at the site of action of the intended target which is necessary to produce the desired therapeutic response. In this context, flat profiles would initially indicate no drug metabolism has occurred, but it could also mean the drug substrates are metabolically stable in those tested systems (e.g. microsomes or cells) as observed with astemizole in pig splenocytes (Figure 84).

Moreover, to observe enzyme activity by demonstrating drug disappearance and appearance of metabolite formation to confidently confirm evidence of functional metabolism in the spleen. It was important therefore to ensure the optimal enzyme activity was monitored at

the linear phase of the progress saturation curve (typical of enzyme kinetic studies), hence the intrinsic clearance was determined to generate an initial rate profile illustrating changes in drug levels with increasing microsomal protein concentration, over time and in a cellular matrix observed proportionally to increasing drug substrate concentration. The initial rate profiles observed in pig hepatocytes and splenocytes are somewhat linear suggesting further that drug metabolism of the tested drug substrates were monitored at optimal CYP450 enzyme activity.

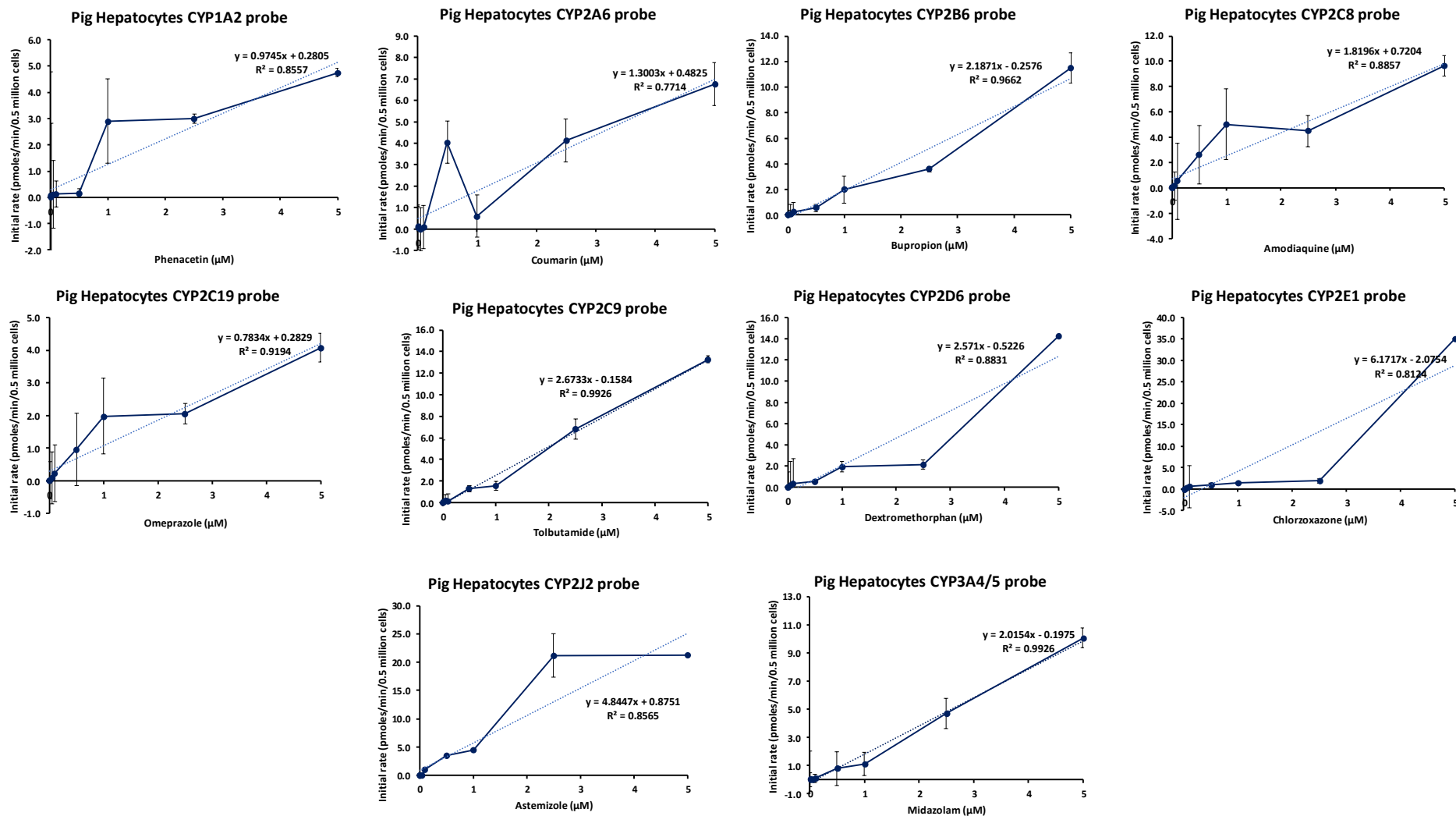


Figure 83 Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity in pig hepatocytes of the following drug substrates phenacetin, coumarin, bupropion, amodiaquine, omeprazole, tolbutamide, dextromethorphan, chlorzoxazone, astemizole and midazolam.

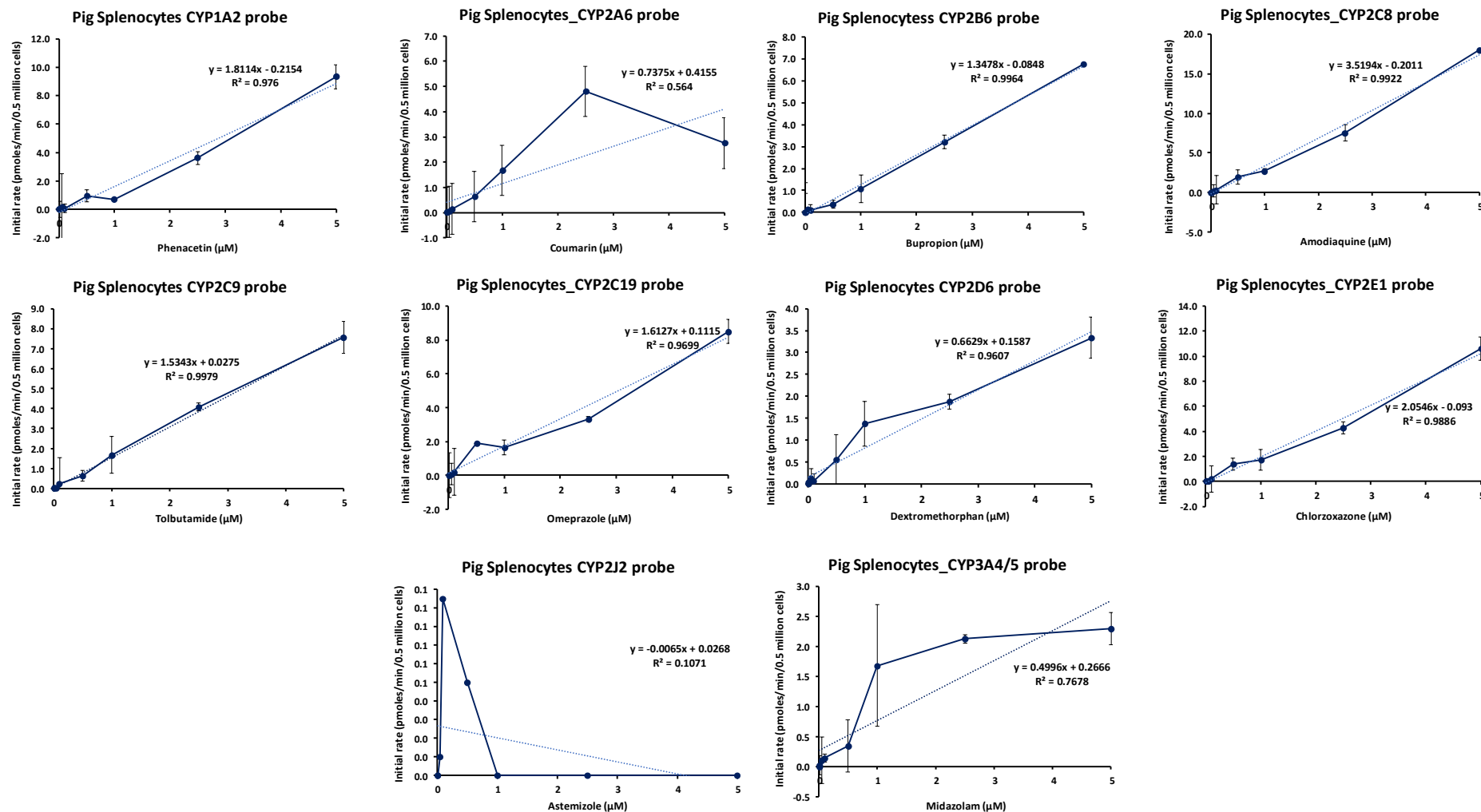


Figure 84 Drug Elimination: Profiles demonstrating CYP450-mediated initial rate as function of enzyme activity in pig splenocytes of the following drug substrates phenacetin, coumarin, bupropion, amodiaquine, tolbutamide, omeprazole, dextromethorphan, chlorzoxazone, astemizole and midazolam.

5.2.8 CYP450-mediated metabolite formation in pig hepatocytes and splenocytes

The metabolites of the CYP450 drug substrates were monitored in pig hepatocytes and splenocytes. However, only four metabolites were detected in both pig cell organs, with the same substrates observed (see Figure 85 and 86). The metabolite formation levels in pig hepatocytes appear comparable with pig splenocytes with exception to dextrorphan (Figure 85), where the drug substrate (dextromethorphan) is highly metabolised in the liver compared to the spleen. There is a high probability that the selected drug substrates will be subjected to extensive drug metabolism in the cellular matrix by phase I and II enzymes and other cells expressing drug metabolizing enzymes e.g. Macrophages (Cho et al., 2023). It is not surprising therefore to observe low levels of CYP450-mediated drug elimination observed in both pig hepatocytes and splenocytes, but the appearance of metabolites at those levels observed in pig splenocytes is significant (Figure 86). Moreover, the metabolite 1-hydroxymidazolam was not detected in either pig hepatocytes and splenocytes but was detected in both liver and spleen microsomes further emphasising the involvement of other drug metabolising enzymes.

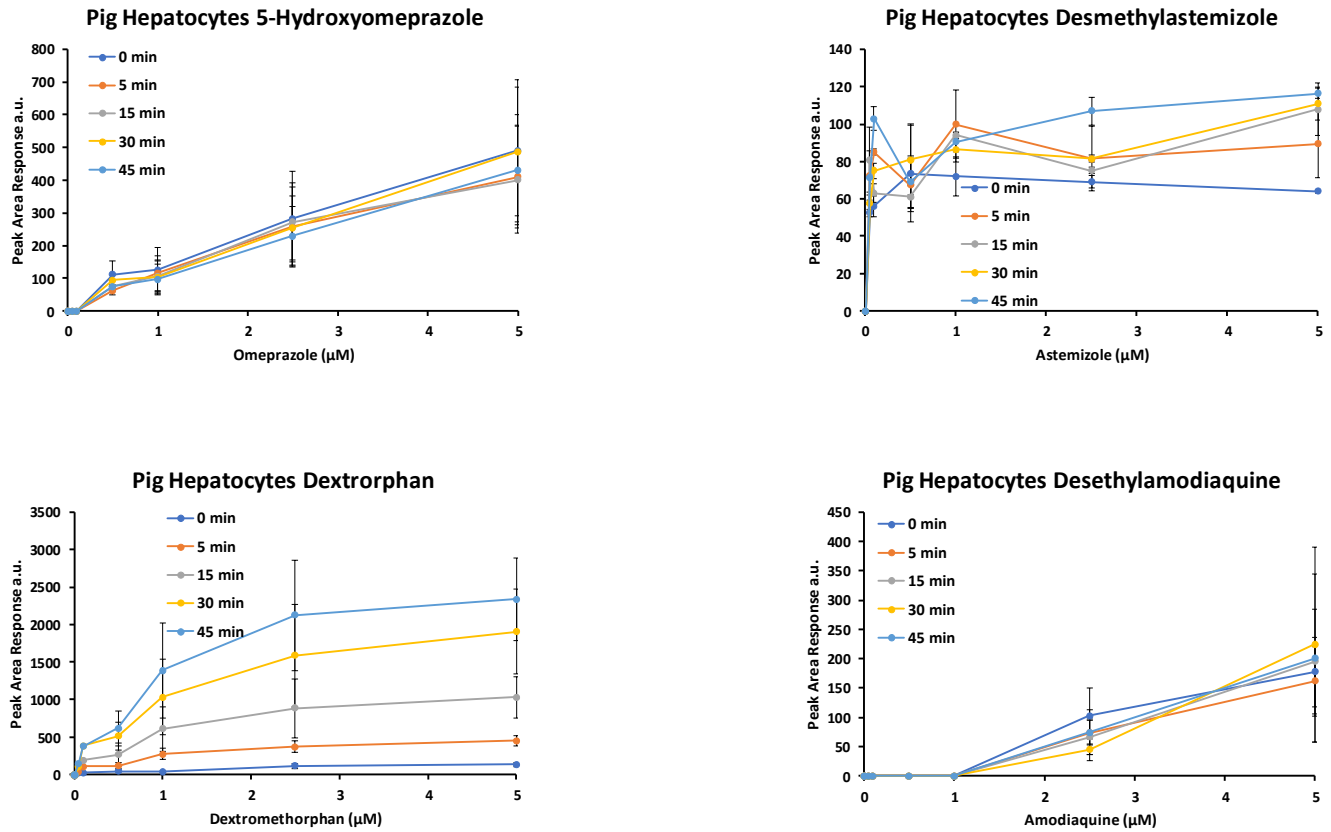


Figure 85 CYP450-mediated metabolite profiles of desethylamodiaquine, 5-hydroxyomeprazole, desethylastemizole and dextromethorphan over time in pig hepatocytes.

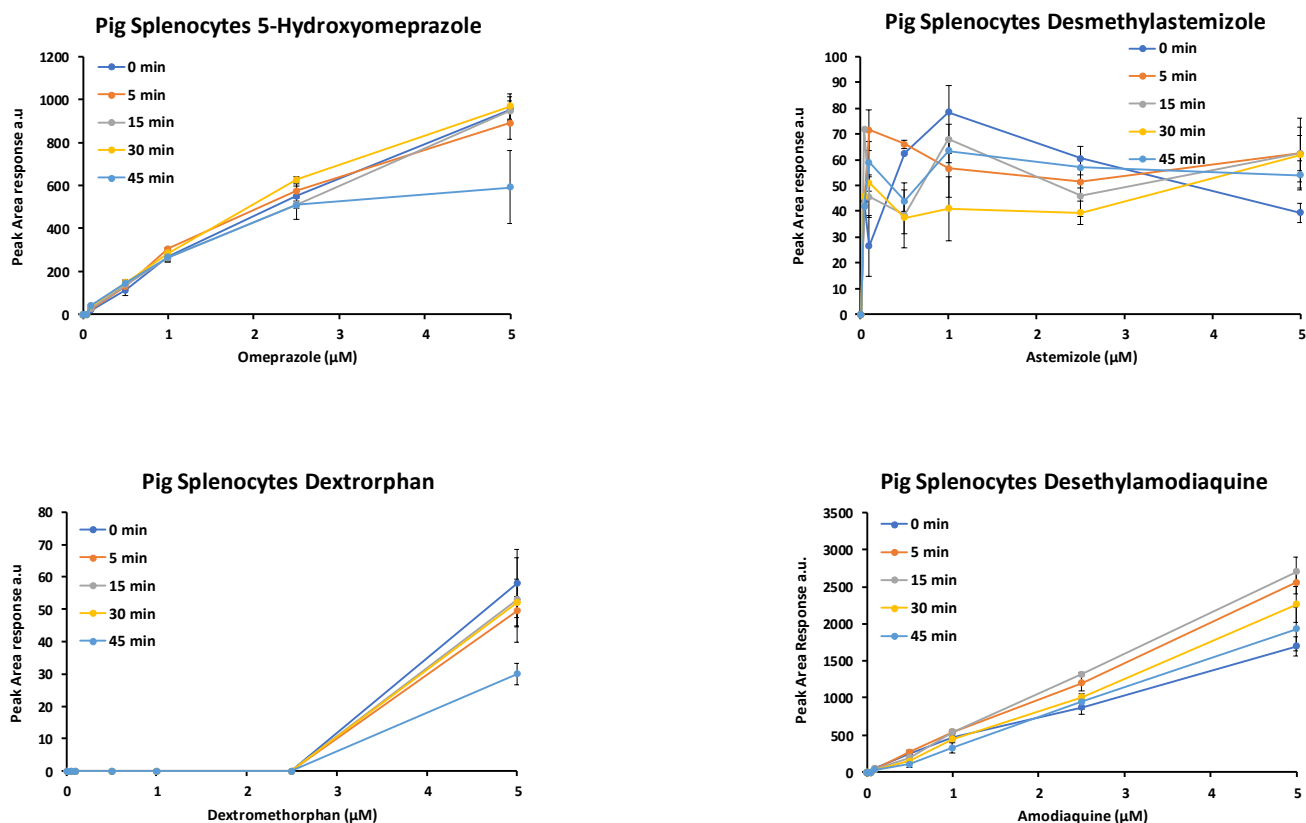


Figure 86 CYP450-mediated metabolite profiles of desethylamodiaquine, 5-hydroxyomeprazole, desethylastemizole and dextromethorphan over time in pig splenocytes.

5.3 Discussion

The CYP450 and UGT drug substrates were selected based on the major contributing drug metabolising CYP450 and UGT enzymes known in human liver (see Table 28). Their contribution including pharmacological significance in relation to drugs and carcinogens has been well-documented (Greener, 2009; Khojasteh et al., 2011). Measuring the activity of these enzymes has been the forefront of early drug discovery research with experimental methods predominantly using microsomes from different animal models to measure total CYP450 content as shown in Table 29 (Pasanen, 2009). The targeted CYP450/UGT enzymes were cross-linked with the equivalent genes identified via the NGS analysis, and it is confirmed that the pig spleen consists of such genes.

Table 28 Literature-based CYP450 enzymes involved in drug metabolism (Greener, 2009).

Enzyme	Proportion of drug metabolism accounted for by the enzyme (%)
CYP1A2	5
CYP2A6	2
CYP2B6	2–4
CYP2CB	1
CYP2C9	10
CYP2C19	5
CYP2D6	20–30
CYP2E1	2–4
CYP3A4	40–45

Table 29 Literature-based hepatic microsomal CYP450 content in different species (Pasanen, 2009).

Species	P450 Content pmol/mg protein
Human (Our Lab)	400 up to 1000
Human	307 ± 160
Monkey	1030 ± 106
Dog	386 ± 36
Pig	488 ± 83 (f)
Minipig	821 ± 183 (f)
Guinea pig	1235 ± 95
Rabbit	681 ± 36
Rat	673 ± 50
Mouse	719 ± 41

Although it has been conveyed from this study that pig spleen does have some CYP450 content, the yield of microsomes per gram of liver was not determined. The proceeding kinetic analysis using microsomes and cells to examine the functional expression of CYP450 and UGT enzyme activity was achieved. The proportion or abundance of CYP450s as detailed in the RNA-seq transcriptome appear to match with drug elimination observations compared between pig liver and spleen microsomes. The drug clearance appears higher in pig liver microsomes compared to pig spleen microsomes; but this contrasted with the CYP450 content analysis that showed the splenic microsomes contained more CYP450 concentration than liver microsomes per mg of protein.

Since the drug substrates used in these experiments were marketed therapeutic drugs, estimates of their V_{max} and k_m values were used as reference (see Table 30) to select the test

drug substrate concentrations required in this *in-vitro* study. Considerations were made with regards to the size of the spleen tissue, the CYP450 content and the basis of the experimental aim (to observe CYP450/UGT activity and not to produce an enzyme progress or saturation curve) in selecting a lower range substrate concentration.

Table 30 Literature-based Michalis Menten parameters for porcine and reference human hepatic CYP450 enzymes (Schelstraete et al., 2019).

	Mean Porcine k_m (μM)	Porcine V_{max} (pmol/min/mg protein)	Mean Human k_m (μM)	Human V_{max} (pmol/min/mg protein)
Phenacetin	20.0 ± 14.88	1404 ± 403.6	10–50	241–2173
Coumarin	1.3 ± 0.47	303 ± 119.2	0.5–2	259–1275
Midazolam	15.3 ± 4.4	1848 ± 637	3–9	190–4380
Tolbutamide	2079 ± 1821	269 ± 182.1	60–400	66–434
Dextromethorphan	6.7 ± 5.70	1592 ± 480.7	2.2–8.5	18–233
Chlorzoxazone	53.6 ± 18.39	764 ± 259.1	39–152	575–2301

In pig spleen microsomes, drug elimination (albeit small for some) was observed for all tested CYP450 and UGT drug substrates. However, the metabolite levels observed were not consistent to expectations. Since true drug metabolism is reflected by the production of metabolite formed, this may suggest at face value that potentially false-positive results were obtained for the cleared drug substrates that showed no metabolites formed.

However, it is important to note that CYP450s are known to display kinetics outside of the standard Michalis Menten model and this may be due to the nature of their diverse substrate specificities (Nagar et al., 2014). This type of ‘atypical’ kinetics can be described as resulting from the CYP450 enzymes’ ability to bind to multiple substrates simultaneously (with different binding affinities) at an active site (Nagar et al., 2014).

What stands out generally from these functional observations in pig liver and spleen microsomes is that drug elimination appears to be higher at lower microsomal protein concentration (0.1 mg/mL) compared to a greater microsomal protein concentration at 1 mg/mL. It may seem logical to rationalize that increasing the protein concentration, increases the volume of available CYP450 or UGT enzymes for the metabolizing reactions. However, these observations appear contrary to that understanding and may likely be a result of two things: firstly, the reaction has reached saturation given that CYP450 enzymes have multiple binding sites (to account for its diverse substrate specificities capability) and the drug substrate amount has been used up at 0.1 mg/mL microsomal protein concentration. Increasing the microsomal protein concentration will not cause any changes as there is limited substrate available to occupy all the binding or active sites of the enzyme and this can be demonstrated from the profiles curving by 5 μ M drug substrate concentration at 0.1 mg/mL; secondly, since the Cl_{int} values were normalized with mg of microsomal protein, this may indicate that there was less drug metabolism that took place initially and this profile also accounts for the non-specific binding of the drug substrates to other proteins in the microsome incubation.

Although the initial rate across the drug substrates in the cell-based assay were significantly lower compared to that in microsomes, it is worth mentioning that the drug substrates are exposed to phase I and II enzymatic reactions. In addition, the low drug elimination observed indicates that CYP450-mediated metabolism is not the major route of drug metabolism for these substrates in the cellular matrix. However, the metabolite formation profiles appear significantly higher particularly from the drug substrates amodiaquine and omeprazole in pig splenocytes. This indicates that the CYP450 and possibly UGT enzymes in pig splenocytes are

much more efficient in metabolizing drugs in the cellular matrix compared to splenic microsomes. In addition, the rate or speed which those reactions took place could have occurred faster than the levels were measured. For example, levels for the metabolite of midazolam could not be detected in either pig hepatocytes or splenocytes but can be observed from pig liver and spleen microsomes. This indicates that the metabolites could possibly be substrates for other enzymes (See Figure 87) and if CYP450-mediated reactions target functional groups with present active oxygen then a chemical modification will happen regardless it is a substrate or metabolite. This notion confirms with the observations seen particularly with the metabolites' levels of amodiaquine and omeprazole in the pig spleen matrices, as these metabolites are not known substrates for other enzymes.

Other reasons not observing metabolites for all the drug substrates in either of the pig spleen matrices, could be that the k_m for some of the tested drug substrates was not reached as shown in Table. 30 thereby impacting the kinetics. For example, *Cyp2e1* pig gene is a direct orthologue to human and with other species like rat and mouse (according to the Ensemble genome database); but its activity between the liver and spleen appears to be somewhat quite different. Drug elimination observations in pig spleen was significantly lower and no metabolite formation levels were detected compared to in pig liver from this study. On closer inspection, the *Cyp2e1* gene identified by NGS showed much higher expression in pig liver and a low expression in pig spleen; with Table 30 demonstrating a k_m in excess of 50 μM drug substrate concentration for chlorzoxazone in pig liver, suggesting that *Cyp2e1* may not be as efficient in metabolising the drug chlorzoxazone and would require a higher drug substrate concentration to observe a reasonable functional expression for the enzyme in pig spleen.

Moreover, it is worth mentioning that the metabolites resulting from the UGT-mediated reactions observed in both pig spleen matrices is a significant find as only *Ugt8* was identified in the NGS analysis, suggesting that this enzyme (or potentially other CYP450s) could have a broad substrate specificity to metabolise those UGT drug substrates.

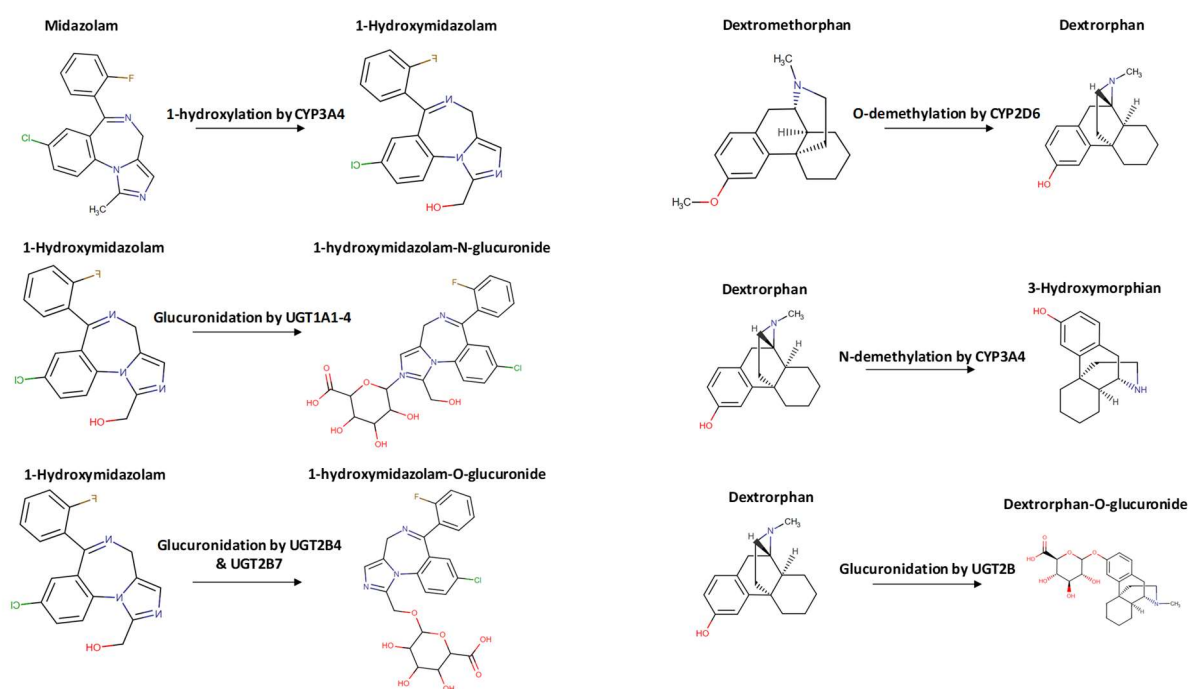


Figure 87 Examples illustrating the metabolites of midazolam and dextromethorphan , 1-hydroxymidazolam and dextrorphan as substrates for UGT and other CYP450 enzymes. Obtained from the drug-bank database (<https://go.drugbank.com/>).

CHAPTER 6:

GENERAL DISCUSSION

6.0 Discussion

Recently, new advances in therapeutics are searching to capitalise on the effective and efficient ways of the immune system. Most diseases including viral or bacterial infections in one way or another show to evade immune cells from recognition as potential antigens; and not necessarily that the immune system is a failure in eradicating these resulting illnesses in patients (Sadeghirad et al., 2023; Sepe et al., 2020). One must also understand the mechanisms behind the activation of immune cells before embarking in its manipulation for therapeutic uses, which is outside the scope of this study. The design of NCEs considers its target, the environment it will be exposed to in reaching the target and treatment indication including possible side effects. Drugs become the therapeutic tools to achieve the therapeutic aims either orally or intravenously, though the latter would increase patient compliance (Jain et al., 2022). ADME studies (or sciences) are pivotal to ensuring such drugs are efficacious, safe and specific in treating that indication, by designing the dosing regimen (from obtained rigorous validated data) within a therapeutic window.

In this respect, the spleen appears to be a very attractive target since administered drugs can access the immune cells area (periarteriolar lymphoid sheath) without facing challenging barriers based on its anatomy. Therefore, the ability the spleen requires to metabolise drugs is dependent on consisting of the main element responsible for the metabolic process. CYP450s and UGTs are well characterised enzymes in the clinical research world and were selected in this study (from the pool of phase I and II enzymes) based on their major contribution to drug metabolism (Zanger et al, 2013; Jetter & Kullak-Ublick, 2020). The clinical implications based on the interactions of drugs with these enzymes has gained widespread

attention, resulting in assessments of enzyme activity incorporated as routine for research in new therapeutics. Drug metabolism studies is key for determining drug efficacy and safety (e.g. toxicity implications resulting from DDIs), though current studies have realised and explored its possible relationship with drug transporters. Distribution of drugs to tissues or targeted site of action were once understood as the primary role of drug transporters, many studies have since established an alliance between them and CYP450s to fulfil effective coverage of drug metabolism (Benet, 2009).

In hindsight, the gene expression analysis revealed the genetic make-up of the spleen tissue and showed the abundance of mRNA sequences (key for making functional gene products). This analysis was performed to support the notion that the spleen should have CYP450 and UGT genes in its DNA to have the ability to metabolise drugs. However, drug research studies have always involved using appropriate animal models for human, and the phylogenetic analysis based on mRNA sequences has shown close homology between rat and pig to human. Such provided the foundation for selecting these models as appropriate for downstream analysis in this study. The equivalent CYP450 and UGT genes were then identified from the RNA-seq transcriptome (via the NGS analysis) in untreated or basal rat and pig spleens. Positive protein expression is a true representation of functional genes as they mainly carry out the biological processes in the body (Alberts et al., 2002). Confirmation of the protein expression of selected CYP450s and UGTs, as identified by NGS, in rat and pig spleens would suggest two things: firstly, those identified genes are not benign or non-coded genes and secondly, the spleen tissue contains functional CYP450 and UGT proteins.

The understanding of CYP450 or UGT activity in the spleen would be advantageous in solidifying the spleen as a potential immunotherapeutic target. Kinetic analysis was

performed to serve this idea and to provide evidence that the CYP450/UGT enzymes in the spleen tissue functions in the metabolism of drugs. The novelty of this research was emphasised following that splenic microsomes were required to perform the bulk of the kinetic analysis. There is limited research into drug metabolism studies in the spleen, which is why the liver tissue was used both as a positive control and as a reference for choosing the assays deemed adequate for CYP450/UGT kinetic analysis. One essential component for this analysis was microsomes - the biological material stated to have concentrated CYP450s in the liver tissue. In addition, CYP450s are resident membrane proteins of the endoplasmic reticulum (Miyauchi et al., 2021), thus preparing splenic microsomes in the same way as the liver microsomes should result in capturing the CYP450s in the spleen. Therefore, total CYP450 content analysis was subsequently performed not only to confirm that indeed splenic microsomes contain CYP450s but to also quantitate the CYP450 concentration in the spleen. This could also possibly unravel the relationship between the CYP450 concentration with the resulting kinetics observed.

The examined drug substrates were also selected based on their target CYP450 probes contribution to drug metabolism, but also, the substrates had to be marketed drugs where the information about their kinetics in the liver was known. This enabled to identify that the working conditions of the assay were running as expected and to validate the results obtained from the spleen tissue. The decision taken to investigate CYP450/UGT activity in microsomes and cells was based on the difference in variables that could impact the kinetic analysis. For the microsome-based assay, accurate preparation and concentration of the co-factors (e.g. NADPH or UDPGA) are required for the metabolic reaction to take place between the substrates and enzymes; but only CYP450 or UGT activity could be monitored in the *in-vitro*

setting. On the other hand, both CYP450 and UGT activity could be monitored simultaneously in the cell-based assay without the need of co-factors and it's a better reflection of the *in-vivo* condition.

Although only five metabolites in the microsome assay and four in the cell-based assay was observed in pig spleen, this is profound as those substrates are not substrates specific to the CYP450s or UGTs in the spleen. Furthermore, this indicates that spleens' CYP450s and UGTs are like the enzymes in the liver where they have overlapping substrate-specificities; and that the spleen could possibly have other CYP450s with drug metabolising properties. Based on the low drug clearance levels compared to the corresponding higher metabolites levels observed in the pig spleen, this observations suggest how efficient the spleen is at metabolising drugs. It also justifies the use of a lower range drug substrate concentration in this study, as the spleen could metabolise drugs as low as 10 nM.

There are drawbacks that have been encountered in this study, for example with the qPCR gene analysis in pig hepatocytes and splenocytes which required qPCR-specific primers, more validation studies in a number of pig animals (considering the gene variation known between pig breeds), not quantifying the possible impact of microsome drug binding to the kinetics observed, not identifying the actual proteins responsible for the protein expression observed, and not using exactly the k_m concentrations of the drug substrates for a better comparability between studies.

Nonetheless, the recommendations for future drug metabolism studies in the spleen would be to discover drug substrates specific to the CYP450 and UGT enzymes in the spleen. It would also be beneficial to subsequently perform an enzyme progress (saturation) curve to

determine the Michaelis-Menten parameters (V_{max} and k_m) for those substrates in the spleen. In addition, it appears that a cell-based assay is a better option for determining enzyme kinetics in the spleen than using splenic microsomes. An *in-vivo* PK assay is necessary for splenic therapeutic intervention in a pre-clinical setting and should also be implemented to correlate with the data obtained from *in-vitro* studies.

REFERENCES

References

- Abdelwahab, N. S., Hassan, H. M., & Magd, A. M. A. (2020). Rapid microwave-assisted hydrolytic degradation of colchicine: In silico ADME/Tox profile, molecular docking, and development of innovative RP-Chromatographic methods. *Microchemical Journal*, 152(August). <https://doi.org/10.1016/j.microc.2019.104419>
- A-Gonzalez, N., & Castrillo, A. (2018). Origin and specialization of splenic macrophages _ Elsevier Enhanced Reader. *Cellular Immunology*, 330, 151–158.
- Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., & Walter, P. (2002a). *The Molecular Biology of the Cell* (M. Singer, A. Dilemnia, B. Dilemnia, & B. Goatly, Eds.). Garland Science.
- Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., & Walter, P. (2002b). *The Molecular Biology of the Cell* (M. Singer, A. Dilemnia, B. Dilemnia, & B. Goatly, Eds.). Garland Science.
- Allain, E. P., Rouleau, M., Lévesque, E., & Guillemette, C. (2020). Emerging roles for UDP-glucuronosyltransferases in drug resistance and cancer progression. *British Journal of Cancer*, 122(9), 1277–1287. <https://doi.org/10.1038/s41416-019-0722-0>
- Al-Naimi, M., Rasheed, H., Hussien, N., Al-Kuraishy, H., & Al-Gareeb, A. (2019). Nephrotoxicity: Role and significance of renal biomarkers in the early detection of acute renal injury. In *Journal of Advanced Pharmaceutical Technology and Research* (Vol. 10, Issue 3, pp. 95–99). Wolters Kluwer Medknow Publications. https://doi.org/10.4103/japtr.JAPTR_336_18
- Amin, S., Baine, M., Meza, J., Alam, M., & Lin, C. (2020). The impact of immunotherapy on the survival of pancreatic adenocarcinoma patients who do not receive definitive surgery of the tumor. *Clinical and Translational Radiation Oncology*, 24, 34–40. <https://doi.org/10.1016/j.ctro.2020.06.003>
- Arora, P. K., Saroj, R. S., Mishra, R., Omar, R. A., Kumari, P., Srivastava, A., Garg, S. K., & Singh, V. P. (2021). Draft genome sequence data of a 4-nitrophenol- degrading bacterium, *Pseudomonas allopuntida* strain PNP. *Data in Brief*, 38. <https://doi.org/10.1016/j.dib.2021.107390>
- Athel Cornish-Bowden. (2013). *Fundamentals of Enzyme Kinetics* (4th ed.). John Wiley & Sons. https://books.google.co.uk/books?id=_3fqaYnrDOSC&dq=enzyme+kinetics&lr=&source=gbs_navlinks_s
- Aungst, B. J. (2017). Optimizing Oral Bioavailability in Drug Discovery: An Overview of Design and Testing Strategies and Formulation Options. *Journal of Pharmaceutical Sciences*, 106(4), 921–929. <https://doi.org/10.1016/j.xphs.2016.12.002>
- Ayrton, A., & Morgan, P. (2001). Role of transport proteins in drug absorption, distribution and excretion. *Xenobiotica*, 31(8–9), 469–497. <https://doi.org/10.1080/004982501100>

- Ayuso, M., Buyssens, L., Stroe, M., Valenzuela, A., Allegaert, K., Smits, A., Annaert, P., Mulder, A., Carpentier, S., Van Ginneken, C., & Van Cruchten, S. (2021). The neonatal and juvenile pig in pediatric drug discovery and development. In *Pharmaceutics* (Vol. 13, Issue 1, pp. 1–36). MDPI AG. <https://doi.org/10.3390/pharmaceutics13010044>
- Bachmann, K. (2009). Drug Metabolism. *Pharmacology: Principles and Practice, March*, 131–173. <https://doi.org/10.1016/B978-0-12-369521-5.00008-7>
- Benet, L. Z. (2009). The drug transporter-metabolism alliance: Uncovering and defining the interplay. In *Molecular Pharmaceutics* (Vol. 6, Issue 6, pp. 1631–1643). <https://doi.org/10.1021/mp900253n>
- Benet, L. Z., Cummins, C. L., & Wu, C. Y. (2004). Unmasking the dynamic interplay between efflux transporters and metabolic enzymes. *International Journal of Pharmaceutics*, 277(1–2), 3–9. <https://doi.org/10.1016/j.ijpharm.2002.12.002>
- Boobis, A., Gundert-Remy, U., Kremers, P., Macheras, P., & Pelkonen, O. (2002). In silico prediction of ADME and pharmacokinetics q Report of an expert meeting organised by COST B15. *European Journal of Pharmaceutical Sciences*, 17, 183–193. www.elsevier.nl/locate/ejps
- Borah, P. (2011). *Primer designing for PCR*. www.sciencevision.in
- Bronte, V., & Pittet, M. J. (2013). The spleen in local and systemic regulation of immunity. *Immunity*, 39(5), 806–818. <https://doi.org/10.1016/j.immuni.2013.10.010>
- Buccitelli, C., & Selbach, M. (2020). mRNAs, proteins and the emerging principles of gene expression control. In *Nature Reviews Genetics* (Vol. 21, Issue 10, pp. 630–644). Nature Research. <https://doi.org/10.1038/s41576-020-0258-4>
- Burn, S. F., Boot, M. J., de Angelis, C., Doohan, R., Arques, C. G., Torres, M., & Hill, R. E. (2008). The dynamics of spleen morphogenesis. *Developmental Biology*, 318(2), 303–311. <https://doi.org/10.1016/j.ydbio.2008.03.031>
- Bush, P. A., & Noe, J. F. (2020). Immunotherapy and Targeted Drugs for Common Cancers. *Journal for Nurse Practitioners*, 16(3), 195–200. <https://doi.org/10.1016/j.nurpra.2019.12.004>
- Caldwell, Gary. W. (2000). Compound optimization in early-and late-phase drug discovery: Acceptable pharmacokinetic properties utilizing combined physicochemical, in vitro and in vivo screens Gary W Caldwell. *Current Opinion in Drug Discovery & Development*, 3(1), 30–41.
- Caldwell, J., Gardner, I., & Swales, N. (1995). An introduction to drug disposition: The basic principles of absorption, distribution, metabolism, and excretion. *Toxicologic Pathology*, 23(2), 102–114. <https://doi.org/10.1177/019262339502300202>
- Cascorbi, I. (2012). Drug Interactions—Principles, Examples and Clinical Consequences. *Deutsches Arzteblatt International*, 109(33–34), 546–556. <https://doi.org/10.3238/arztebl.2012.0546>

- Cataldi, M., Vigliotti, C., Mosca, T., Cammarota, M. R., & Capone, D. (2017). Emerging role of the spleen in the pharmacokinetics of monoclonal antibodies, nanoparticles and exosomes. *International Journal of Molecular Sciences*, 18(6), 1–24.
<https://doi.org/10.3390/ijms18061249>
- Cell Signaling Technology, I. (2024a). *Immunohistochemical Analysis*.
<https://www.cellsignal.com/applications/immunohistochemistry/analysis-immunohistochemistry-staining>
- Cell Signaling Technology, I. (2024b). *Understand Protein Expression in Tissue with Immunohistochemistry (IHC)*.
<https://www.cellsignal.com/applications/immunohistochemistry/protein-expression-immunohistochemistry>
- Cesta, M. F. (2006). Normal Structure, Function, and Histology of the Spleen. *Toxicologic Pathology*, 34(5), 455–465. <https://doi.org/10.1080/01926230600867743>
- Chadburn, A. (2000). *The Spleen: Anatomy and Anatomical Function*.
- Chaturvedi, Pravin. R., Decker, Caroline. J., & Odinecs, A. (2001). Prediction of pharmacokinetic properties using experimental approaches during early drug discovery Chaturvedi et al. 453. *Current Opinion in Chemical Biology*, 5, 452–463.
- Chauveau, A., Pirgova, G., Cheng, H. W., De Martin, A., Zhou, F. Y., Wideman, S., Rittscher, J., Ludewig, B., & Arnon, T. I. (2020). Visualization of T Cell Migration in the Spleen Reveals a Network of Perivascular Pathways that Guide Entry into T Zones. *Immunity*, 52(5), 794–807.
<https://doi.org/10.1016/j.immuni.2020.03.010>
- Che Has, A. T. (2023). The applications of the pilocarpine animal model of status epilepticus: 40 years of progress (1983–2023). In *Behavioural Brain Research* (Vol. 452). Elsevier B.V.
<https://doi.org/10.1016/j.bbr.2023.114551>
- Chen, Q., Chen, J., Yang, Z., Xu, J., Xu, L., Liang, C., Han, X., & Liu, Z. (2019a). Nanoparticle-Enhanced Radiotherapy to Trigger Robust Cancer Immunotherapy. *Advanced Materials*, 31(10), 1–12. <https://doi.org/10.1002/adma.201802228>
- Chen, Q., Chen, J., Yang, Z., Xu, J., Xu, L., Liang, C., Han, X., & Liu, Z. (2019b). Nanoparticle-Enhanced Radiotherapy to Trigger Robust Cancer Immunotherapy. *Advanced Materials*, 31(10), 1–12. <https://doi.org/10.1002/adma.201802228>
- Chiba, M., Ishii, Y., & Sugiyama, Y. (2009). Prediction of hepatic clearance in human from in vitro data for successful drug development. In *AAPS Journal* (Vol. 11, Issue 2, pp. 262–276).
<https://doi.org/10.1208/s12248-009-9103-6>
- Chiba, S., Ro, A., Ikawa, T., Oide, Y., & Mukai, T. (2020a). Interactions of human organic anion transporters 1–4 and human organic cation transporters 1–3 with the stimulant drug methamphetamine and amphetamine. *Legal Medicine*, 44, 1–8.
<https://doi.org/10.1016/j.legalmed.2020.101689>

- Chiba, S., Ro, A., Ikawa, T., Oide, Y., & Mukai, T. (2020b). Interactions of human organic anion transporters 1–4 and human organic cation transporters 1–3 with the stimulant drug methamphetamine and amphetamine. *Legal Medicine*, 44(January), 1–8.
<https://doi.org/10.1016/j.legalmed.2020.101689>
- Cho, H. Y., Ahn, S., Cho, Y. S., Seo, S. K., Kim, D. H., Shin, J. G., & Lee, S. J. (2023). CYP2C19 Contributes to THP-1-Cell-Derived M2 Macrophage Polarization by Producing 11,12- and 14,15-Epoxyeicosatrienoic Acid, Agonists of the PPAR γ Receptor. *Pharmaceuticals*, 16(4).
<https://doi.org/10.3390/ph16040593>
- Chotivanich, K., Udomsangpetch, R., McGready, R., Proux, S., Newton, P., Pukrittayakamee, S., Looareesuwan, S., & White, N. J. (2002). Central Role of the Spleen in Malaria Parasite Clearance. *Journal of Infectious Diseases*, 185, 1538–1541.
<https://academic.oup.com/jid/article-abstract/185/10/1538/837204>
- Conn, Y. (2018). Recent Developments in Oncology Immunotherapy, Implications for NPs Part 1. *Journal for Nurse Practitioners*, 14(4), 251-258.e1.
<https://doi.org/10.1016/j.nurpra.2017.12.013>
- Cooper GM. (2000). The Central Role of Enzymes as Biological Catalysts. In *The Cell: A Molecular Approach* (2nd ed.). Sinauer Associates. <https://www.ncbi.nlm.nih.gov/books/NBK9921/>
- Curry, Stephen. H., & Whelpton, R. (2017). *Introduction to Drug Disposition and Pharmacokinetics* (1st ed.). John Wiley & Sons.
- Daniel, L. H. (2017). Polymorphisms of cytochrome P450 are potential candidates that could potentially help clinicians on the treatment of cardiovascular diseases among Asian populations. *Indian Heart Journal*, 69(5), 655–656. <https://doi.org/10.1016/j.ihj.2017.02.016>
- David F.V. Lewis. (2001). *Guide to Cytochrome P450 Structure and Function*. CRC Press.
- Davidson, R. N., & Wall, R. A. (2001). Prevention and management of infections in patients without a spleen. In *Clinical Microbiology & Infection* (Vol. 7, Issue 12, pp. 657–660).
<http://www.medicalert.co.uk>
- den Braver-Sewradj, S. P. (2017). *Inter-individual variation in hepatic drug metabolism : the potential of in vitro assays in unraveling the role of metabolism in drug induced liver toxicity* [AIMMS-Division of Molecular Toxicology]. Vrije Universiteit Amsterdam.
- Dill, K. A., Bromberg, Kaizhi Yue, S., Fiebig, K. M., Yee, D. P., Thomas, P. D., Chan, H. S., & Dill, K. A. (1995). Principles of protein folding-A perspective from simple exact models. In *Protein Science* (Vol. 4). Cambridge University Press.
- Doogue, M. P., & Polasek, T. M. (2013). The ABCD of clinical pharmacokinetics. In *Therapeutic Advances in Drug Safety* (Vol. 4, Issue 1, pp. 5–7).
<https://doi.org/10.1177/2042098612469335>
- Drożdżik, M., Lapczuk-Romanska, J., Wenzel, C., Skalski, Ł., Szelać-Pieniek, S., Post, M., Syczewska, M., Kurzawski, M., & Oswald, S. (2022). Protein Abundance of Drug Transporters

- in Human Hepatitis C Livers. *International Journal of Molecular Sciences*, 23(14).
<https://doi.org/10.3390/ijms23147947>
- Drożdżik, M., Oswald, S., & Drożdżik, A. (2020). Extrahepatic drug transporters in liver failure: Focus on kidney and gastrointestinal tract. In *International Journal of Molecular Sciences* (Vol. 21, Issue 16, pp. 1–17). MDPI AG. <https://doi.org/10.3390/ijms21165737>
- Endres, C. J., Endres, M. G., & Unadkat, J. D. (2009). Interplay of drug metabolism and transport: A real phenomenon or an artifact of the site of measurement? *Molecular Pharmaceutics*, 6(6), 1756–1765. <https://doi.org/10.1021/mp9002392>
- Esteves, M., & Binaghi, R. A. (1972). Antigenic Similarities Among Mammalian Immunoglobulins. In *Immunology* (Vol. 23).
- Fatunde, O. A., & Brown, S. A. (2020). The role of CYP450 drug metabolism in precision Cardio-Oncology. In *International Journal of Molecular Sciences* (Vol. 21, Issue 2). MDPI AG. <https://doi.org/10.3390/ijms21020604>
- Feleder, C., & Blatteis, C. M. (2006). The role of the spleen in the febrile response induced by endotoxin in guinea pigs. *Journal of Thermal Biology*, 31, 220–228.
- Ferguson, R. E., Carroll, H. P., Harris, A., Maher, E. R., Selby, P. J., & Banks, R. E. (2005). Housekeeping proteins: A preliminary study illustrating some limitations as useful references in protein expression studies. *Proteomics*, 5(2), 566–571. <https://doi.org/10.1002/pmic.200400941>
- Ferrer, M., Martin-Jaular, L., De Niz, M., Khan, S. M., Janse, C. J., Calvo, M., Heussler, V., & Del Portillo, H. A. (2014). Imaging of the spleen in malaria. In *Parasitology International* (Vol. 63, Issue 1, pp. 195–205). <https://doi.org/10.1016/j.parint.2013.08.014>
- Freeman, B. B., Yang, L., & Rankovic, Z. (2019). Practical approaches to evaluating and optimizing brain exposure in early drug discovery. *European Journal of Medicinal Chemistry*, 182, 1–14. <https://doi.org/10.1016/j.ejmech.2019.111643>
- Gandhi, V., O'Brien, M. H., & Yadav, S. (2020). High-Quality and High-Yield RNA Extraction Method From Whole Human Saliva. *Biomarker Insights*, 15. <https://doi.org/10.1177/1177271920929705>
- Gerald M. Cohen, J. Brain Houston, John Caldwell, Susan A. Sangster, J. David Sutton, Walter G. Levine, J. C. Connelly, James W. Bridges, & Steven D. Aust. (2020). *Target Organ Toxicity, Volume I: Vol. I* (Gerald M. Cohen, Ed.). CRC Press, Taylor and Francis Group.
- Ghosh, C., Hossain, M., Solanki, J., Dadas, A., Marchi, N., & Janigro, D. (2016). Pathophysiological implications of neurovascular P450 in brain disorders. *Drug Discovery Today*, 21(10), 1609–1616. <https://doi.org/10.1016/j.drudis.2016.06.004>
- Gokhale, S., Nyayanit, D., & Gadgil, C. (2011). A systems view of the protein expression process. In *Systems and Synthetic Biology* (Vol. 5, Issues 3–4, pp. 139–150). <https://doi.org/10.1007/s11693-011-9088-1>

- Gokulnath, D., & Raveendran, B. (2023). *How do you find Km and Vmax*. BYJU'S Learning. <https://byjus.com/question-answer/how-do-you-find-km-and-vmax/>
- Golub, R., Tan, J., Watanabe, T., & Brendolan, A. (2018). Origin and Immunological Functions of Spleen Stromal Cells. In *Trends in Immunology* (Vol. 39, Issue 6, pp. 503–514). Elsevier Ltd. <https://doi.org/10.1016/j.it.2018.02.007>
- Gomes, A., & Korf, B. (2018). Genetic Testing Techniques. In *Pediatric Cancer Genetics* (pp. 47–64). Elsevier. <https://doi.org/10.1016/B978-0-323-48555-5.00005-3>
- Gopinath, A., & Wilson, M. (2020). Factors affecting drug absorption and distribution. *Anaesthesia and Intensive Care Medicine*, 21(5), 223–228.
- Gouliarmou, V., Lostia, A. M., Coecke, S., Bernasconi, C., Bessems, J., Dorne, J. Lou, Ferguson, S., Testai, E., Remy, U. G., Brian Houston, J., Monshouwer, M., Nong, A., Pelkonen, O., Morath, S., Wetmore, B. A., Worth, A., Zanelli, U., Zorzoli, M. C., & Whelan, M. (2018). Establishing a systematic framework to characterise in vitro methods for human hepatic metabolic clearance. *Toxicology in Vitro*, 53, 233–244. <https://doi.org/10.1016/j.tiv.2018.08.004>
- Greener, M. (2009). Understanding the principles of drug distribution and excretion. In *Nurse Prescribing* (Vol. 7, Issue 4).
- Groth, C. G. (2007). The potential advantages of transplanting organs from pig to man: A transplant Surgeon's view. In *Indian Journal of Urology* (Vol. 23, pp. 305–310).
- Groves, J. T. (2015). Cytochrome P450 enzymes: Understanding the biochemical hieroglyphs. *F1000Research*, 4, 1–8. <https://doi.org/10.12688/f1000research.6314.1>
- Guengerich, F. P., Martin, M. V., Sohl, C. D., & Cheng, Q. (2009). Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. *Nature Protocols*, 4(9), 1245–1251. <https://doi.org/10.1038/nprot.2009.121>
- Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G. G., Liu, Y., Chen, X., Li, L., Wu, S., Chen, Y., Jiang, H., Tan, L., Xie, J., Zhu, X., Liang, S., & Deng, H. (2008). How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. *Acta Biochimica et Biophysica Sinica*, 40(5), 426–436. <https://doi.org/10.1111/j.1745-7270.2008.00418.x>
- Han, J., Park, J. C., Hagiwara, A., Park, H. G., & Lee, J. S. (2019). Identification of the full 26 cytochrome P450 (CYP) genes and analysis of their expression in response to benzo[α]pyrene in the marine rotifer *Brachionus rotundiformis*. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics*, 29, 185–192. <https://doi.org/10.1016/j.cbd.2018.12.001>
- Hashemi, V., Farhadi, S., Ghasemi Chaleshtari, M., Seashore-Ludlow, B., Masjedi, A., Hojjat-Farsangi, M., Namdar, A., Ajjoolabady, A., Mohammadi, H., Ghalamfarsa, G., & Jadidi-Niaragh, F. (2020). Nanomedicine for improvement of dendritic cell-based cancer immunotherapy. *International Immunopharmacology*, 83, 1–12. <https://doi.org/10.1016/j.intimp.2020.106446>

- Hawes, D., Shi, S.-R., Dabbs, D. J., Taylor, C. R., & Cote, R. J. (2009). Immunohistochemistry. In *Modern Surgical Pathology* (pp. 48–70). Elsevier. <https://doi.org/10.1016/B978-1-4160-3966-2.00016-3>
- Henriksen, P. A., & Kotelevtsev, Y. (2002). Application of gene expression profiling to cardiovascular disease. In *Cardiovascular Research* (Vol. 54). www.elsevier.com/locate/cardiores
- Howgate, E. M., Rowland Yeo, K., Proctor, N. J., Tucker, G. T., & Rostami-Hodjegan, A. (2006). Prediction of in vivo drug clearance from in vitro data. I: Impact of inter-individual variability. *Xenobiotica*, 36(6), 473–497. <https://doi.org/10.1080/00498250600683197>
- Huang, S., Wen, J., Zhang, Y., Bai, X., & Cui, Z.-K. (2023). Choosing the right animal model for osteomyelitis research: Considerations and challenges. *Journal of Orthopaedic Translation*, 43, 47–65. <https://doi.org/10.1016/j.jot.2023.10.001>
- Ijuin, S., Liu, K., Gill, D., Kyun Ro, S., Vukovic, J., Ishihara, S., Belohlavek, J., Li Bassi, G., Suen, J. Y., & Fraser, J. F. (2023). Current animal models of extracorporeal cardiopulmonary resuscitation: A scoping review. In *Resuscitation Plus* (Vol. 15). Elsevier B.V. <https://doi.org/10.1016/j.resplu.2023.100426>
- Ionescu, Corina., & Caira, M. R. (2005). *Drug metabolism : current concepts: Vol. Chapter 1-3* (1st ed.). Springer Netherlands. <https://doi.org/10.1007/1-4020-4142-X>
- Ito, K., & Houston, J. B. (2005). Prediction of human drug clearance from in vitro and preclinical data using physiologically based and empirical approaches. *Pharmaceutical Research*, 22(1), 103–112. <https://doi.org/10.1007/s11095-004-9015-1>
- Ito, K., Iwatsubo, T., Kanamitsu, S., Nakajima, Y., & Sugiyama, Y. (1998). QUANTITATIVE PREDICTION OF IN VIVO DRUG CLEARANCE AND DRUG INTERACTIONS FROM IN VITRO DATA ON METABOLISM, TOGETHER WITH BINDING AND TRANSPORT. In *Annu. Rev. Pharmacol. Toxicol* (Vol. 38). www.annualreviews.org
- Jain, M., Mishra, A., Singh, M., Shyam, H., Kumar, S., Shankar, P., & Singh, S. (2022). Immunotherapeutic and their immunological aspects: Current treatment strategies and agents. *National Journal of Maxillofacial Surgery*, 13(3), 322. https://doi.org/10.4103/njms.njms_62_22
- Jarrar, Y., & Lee, S. J. (2021). The functionality of udp-glucuronosyltransferase genetic variants and their association with drug responses and human diseases. *Journal of Personalized Medicine*, 11(6). <https://doi.org/10.3390/jpm11060554>
- Jia, L., & Liu, X. (2007). The Conduct of Drug Metabolism Studies Considered Good Practice (II): In Vitro Experiments. *Current Drug Metabolism*, 8(8), 822–829. <https://doi.org/10.2174/138920007782798207>
- Jiang, L., Li, Y., Wei, W., Liu, A., Zhang, Y., & Liu, S. (2016). Confining nanohybrid of CdTe quantum dots and cytochrome P450 2D6 in macroporous ordered siliceous foam for drug

- metabolism. *Journal of Electroanalytical Chemistry*, 781, 345–350.
<https://doi.org/10.1016/j.jelechem.2016.07.026>
- Jin, Y., & Qian, H. (2023). U.PhyloMaker: An R package that can generate large phylogenetic trees for plants and animals. *Plant Diversity*, 45(3), 347–352.
<https://doi.org/10.1016/j.pld.2022.12.007>
- Joshi, C. J., Ke, W., Drangowska-Way, A., O'Rourke, E. J., & Lewis, N. E. (2022). What are housekeeping genes? *PLoS Computational Biology*, 18(7 July).
<https://doi.org/10.1371/journal.pcbi.1010295>
- Kaur, G., Gupta, S. K., Singh, P., Ali, V., Kumar, V., & Verma, M. (2020). Drug-metabolizing enzymes: role in drug resistance in cancer. *Clinical and Translational Oncology*, 22(10), 1667–1680. <https://doi.org/10.1007/s12094-020-02325-7>
- Khojasteh, S. C., Wong, H., & Hop, C. E. C. A. (2011). *Drug Metabolism and Pharmacokinetics Quick Guide: Vol. Chapter 1-6*. Springer New York. <https://doi.org/10.1007/978-1-4419-5629-3>
- Kimura, S., Khalil, I. A., Elewa, Y. H. A., & Harashima, H. (2019). Spleen selective enhancement of transfection activities of plasmid DNA driven by octaarginine and an ionizable lipid and its implications for cancer immunization. *Journal of Controlled Release*, 313, 70–79.
<https://doi.org/10.1016/j.jconrel.2019.09.009>
- Kim, Y. H., Bae, Y. J., Kim, H. S., Cha, H. J., Yun, J. S., Shin, J. S., Seong, W. K., Lee, Y. M., & Han, K. M. (2015). Measurement of human cytochrome P450 enzyme induction based on mesalazine and mosapride citrate treatments using a luminescent assay. *Biomolecules and Therapeutics*, 23(5), 486–492. <https://doi.org/10.4062/biomolther.2015.041>
- Kok-Yong, S., & Lawrence, L. (2015). Drug Distribution and Drug Elimination. In *Basic Pharmacokinetic Concepts and Some Clinical Applications: Vol. Chapter 4* (pp. 99–115). InTech. <https://doi.org/10.5772/59929>
- Koussounadis, A., Langdon, S. P., Um, I. H., Harrison, D. J., & Smith, V. A. (2015). Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Scientific Reports*, 5. <https://doi.org/10.1038/srep10775>
- Kraal, G., & den Haan, J. M. M. (2016). The Spleen. In *Encyclopedia of Immunobiology* (Vol. 3, pp. 407–412). Elsevier Inc. <https://doi.org/10.1016/B978-0-12-374279-7.07003-X>
- Kruger, N. J. (2009). The Bradford Method For Protein Quantitation. In J.M. Walker (Ed.), *The Protein Protocols Handbook* (pp. 17–24). Humana Press.
https://doi.org/https://doi.org/10.1007/978-1-59745-198-7_4
- Kumar, G. N., & Surapaneni, S. (2001). Role of drug metabolism in drug discovery and development. *Medicinal Research Reviews*, 21(5), 397–411.
<https://doi.org/10.1002/med.1016>

- Kumar, N., Vishwas, K. G., Kumar, M., Reddy, J., Parab, M., Manikanth, C. L., Pavithra, B. S., & Shandil, R. K. (2014). Pharmacokinetics and dose response of anti-TB drugs in rat infection model of tuberculosis. *Tuberculosis*, 94(3), 282–286. <https://doi.org/10.1016/j.tube.2014.02.004>
- Laura Vargas-Parada. (2014). *Proteins and Gene Expression*. Scitable by Nature Education. <https://www.nature.com/scitable/topic/proteins-and-gene-expression-14122688/>
- Liang, Y., Li, S., & Chen, L. (2015). The physiological role of drug transporters. In *Protein and Cell* (Vol. 6, Issue 5, pp. 334–350). Higher Education Press. <https://doi.org/10.1007/s13238-015-0148-2>
- Li, H., Sheng, Y., Li, W., & Yuan, L. (2022). Recent Advances in Molecular Fluorescent Probes for CYP450 Sensing and Imaging. In *Chemosensors* (Vol. 10, Issue 8). MDPI. <https://doi.org/10.3390/chemosensors10080304>
- Li, J., Lin, W., Chen, H., Xu, Z., Ye, Y., & Chen, M. (2020). Dual-target IL-12-containing nanoparticles enhance T cell functions for cancer immunotherapy. *Cellular Immunology*, 349(January). <https://doi.org/10.1016/j.cellimm.2020.104042>
- Lin, J. H. (2006). Tissue Distribution and Pharmacodynamics: A Complicated Relationship. *Current Drug Metabolism*, 7(1), 39–65.
- Liu, X., Feng, Z., Wang, C., Su, Q., Song, H., Zhang, C., Huang, P., Liang, X. J., Dong, A., Kong, D., & Wang, W. (2020a). Co-localized delivery of nanomedicine and nanovaccine augments the postoperative cancer immunotherapy by amplifying T-cell responses. *Biomaterials*, 230, 1–13. <https://doi.org/10.1016/j.biomaterials.2019.119649>
- Liu, X., Feng, Z., Wang, C., Su, Q., Song, H., Zhang, C., Huang, P., Liang, X. J., Dong, A., Kong, D., & Wang, W. (2020b). Co-localized delivery of nanomedicine and nanovaccine augments the postoperative cancer immunotherapy by amplifying T-cell responses. *Biomaterials*, 230(July 2019). <https://doi.org/10.1016/j.biomaterials.2019.119649>
- Liu, X., Wang, Y., Yang, W., Guan, Z., Yu, W., & Liao, D. J. (2016). Protein multiplicity can lead to misconduct in western blotting and misinterpretation of immunohistochemical staining results, creating much conflicting data. In *Progress in Histochemistry and Cytochemistry* (Vol. 51, Issues 3–4, pp. 51–58). Elsevier GmbH. <https://doi.org/10.1016/j.proghi.2016.11.001>
- Li, Y., Meng, Q., Yang, M., Liu, D., Hou, X., Tang, L., Wang, X., Lyu, Y., Chen, X., Liu, K., Yu, A. M., Zuo, Z., & Bi, H. (2019). Current trends in drug metabolism and pharmacokinetics. *Acta Pharmaceutica Sinica B*, 9(6), 1113–1144. <https://doi.org/10.1016/j.apsb.2019.10.001>
- Lohani, S., Cooper, H., Jin, X., Nissley, B. P., Manser, K., Rakes, L. H., Cummings, J. J., Fauty, S. E., & Bak, A. (2014). Physicochemical properties, form, and formulation selection strategy for a biopharmaceutical classification system class II preclinical drug candidate. *Journal of Pharmaceutical Sciences*, 103(10), 3007–3021. <https://doi.org/10.1002/jps.24088>

- Lunney, J. K., Van Goor, A., Walker, K. E., Hailstock, T., Franklin, J., & Dai, C. (2021). Importance of the pig as a human biomedical model. In *Sci. Transl. Med* (Vol. 13). <https://www.science.org>
- Luo, J., Wang, Z., Tang, C., Yin, Z., Huang, J., Ruan, D., Fei, Y., Wang, C., Mo, X., Li, J., Zhang, J., Fang, C., Li, J., Chen, X., & Shen, W. (2023). Animal model for tendinopathy. In *Journal of Orthopaedic Translation* (Vol. 42, pp. 43–56). Elsevier (Singapore) Pte Ltd. <https://doi.org/10.1016/j.jot.2023.06.005>
- Mackenzie, P. I., Somogyi, A. A., & Miners, J. O. (2017). Advances in drug metabolism and pharmacogenetics research in Australia. *Pharmacological Research*, 116, 7–19. <https://doi.org/10.1016/j.phrs.2016.12.008>
- Magaki, S., Hojat, S. A., Wei, B., So, A., & Yong, W. H. (2019). An introduction to the performance of immunohistochemistry. In *Methods in Molecular Biology* (Vol. 1897, pp. 289–298). Humana Press Inc. https://doi.org/10.1007/978-1-4939-8935-5_25
- Mahmood, T., & Yang, P. C. (2012). Western blot: Technique, theory, and trouble shooting. *North American Journal of Medical Sciences*, 4(9), 429–434. <https://doi.org/10.4103/1947-2714.100998>
- Mantione, K. J., Kream, R. M., Kuzelova, H., Ptacek, R., Raboch, J., Samuel, J. M., & Stefano, G. B. (2014). Comparing bioinformatic gene expression profiling methods: microarray and RNA-Seq. *Medical Science Monitor Basic Research*, 20, 138–142. <https://doi.org/10.12659/MSMBR.892101>
- Marth, J. D. (2008). A unified vision of the building blocks of life. In *Nature Cell Biology* (Vol. 10, Issue 9, p. 1015). <https://doi.org/10.1038/ncb0908-1015>
- Martin, K. A., Jesudoss Chelladurai, J. R. J., Sato, Y., & Brewer, M. T. (2020). Syngamus asphyxiation in a captive ring-necked pheasant. *Veterinary Parasitology: Regional Studies and Reports*, 22. <https://doi.org/10.1016/j.vprsr.2020.100493>
- Masereeuw, R., & Russel, F. G. M. (2001). Mechanisms and clinical implications of renal drug excretion. In *Drug Metabolism Reviews* (Vol. 33, Issues 3–4, pp. 299–351). <https://doi.org/10.1081/DMR-120000654>
- Ma, Y., Zhang, Y., Li, X., Zhao, Y., Li, M., Jiang, W., Tang, X., Dou, J., Lu, L., Wang, F., & Wang, Y. (2019). Near-infrared II phototherapy induces deep tissue immunogenic cell death and potentiates cancer immunotherapy. *ACS Nano*, 13(10), 11967–11980. <https://doi.org/10.1021/acsnano.9b06040>
- McCourt, C. M., McArt, D. G., Mills, K., Catherwood, M. A., Maxwell, P., Waugh, D. J., Hamilton, P., O’Sullivan, J. M., & Salto-Tellez, M. (2013). Validation of Next Generation Sequencing Technologies in Comparison to Current Diagnostic Gold Standards for BRAF, EGFR and KRAS Mutational Analysis. *PLoS ONE*, 8(7). <https://doi.org/10.1371/journal.pone.0069604>

- McNally, S., & O'Brien, C. J. (2013). Drug discovery in glaucoma and the role of animal models. In *Drug Discovery Today: Disease Models* (Vol. 10, Issue 4, pp. e207–e214). Elsevier Ltd. <https://doi.org/10.1016/j.ddmod.2013.12.002>
- Mendoza, R., Banerjee, I., Reghupaty, S. C., Yetirajam, R., Manna, D., & Sarkar, D. (2022). Isolation and Culture of Mouse Hepatocytes and Kupffer Cells (KCs). In *Methods in Molecular Biology* (Vol. 2455, pp. 73–84). Humana Press Inc. https://doi.org/10.1007/978-1-0716-2128-8_7
- Mitchell, K., & Iadarola, M. J. (2010). RT-PCR analysis of pain genes: use of gel-based RT-PCR for studying induced and tissue-enriched gene expression. *Methods in Molecular Biology (Clifton, N.J.)*, 617, 279–295. https://doi.org/10.1007/978-1-60327-323-7_21
- Miyauchi, Y., Takechi, S., & Ishii, Y. (2021). Functional Interaction between Cytochrome P450 and UDP-Glucuronosyltransferase on the Endoplasmic Reticulum Membrane: One of Post-translational Factors Which Possibly Contributes to Their Inter-Individual Differences. *Biol. Pharm. Bull.*, 44(11), 1635–1644.
- Mohs, R. C., & Greig, N. H. (2017). Drug discovery and development: Role of basic biological research. *Alzheimer's and Dementia: Translational Research and Clinical Interventions*, 3(4), 651–657. <https://doi.org/10.1016/j.trci.2017.10.005>
- Morgan, E. T. (2001). Minireview REGULATION OF CYTOCHROME P450 BY INFLAMMATORY MEDIATORS: WHY AND HOW? <http://dmd.aspetjournals.org>
- Murty V. Chengalvala, Vargheese M. Chennathukuzhi, Daniel S. Johnston, Panayiotis E. Stevis, & Gregory S. Kopf. (2007a). Gene Expression Profiling and its Practice in Drug Development. *Current Genomics*, 8(4), 262–270. <https://doi.org/10.2174/138920207781386942>
- Murty V. Chengalvala, Vargheese M. Chennathukuzhi, Daniel S. Johnston, Panayiotis E. Stevis, & Gregory S. Kopf. (2007b). Gene Expression Profiling and its Practice in Drug Development. *Current Genomics*, 8(4), 262–270. <https://doi.org/10.2174/138920207781386942>
- Nagar, S., Argikar, U. A., & Tweedie, D. J. (2014). *Enzyme Kinetics in Drug Metabolism Fundamentals and Applications Methods in Molecular Biology 1113* (S. Nagar, U. A. Argikar, & D. J. Tweedie, Eds.; Vol. 1113). Humana Press. <https://doi.org/10.1007/978-1-62703-758-7>
- Nakasa, H., Komiya, M., Ohmori, S., Rikihisa, T., Kiuchi, M., & Kitada, M. (1993). Characterization of human liver microsomal cytochrome P450 involved in the reductive metabolism of zonisamide. *Molecular Pharmacology*, 44(1), 216–221.
- Naritomi, Y., Sanoh, S., & Ohta, S. (2018). Chimeric mice with humanized liver: Application in drug metabolism and pharmacokinetics studies for drug discovery. In *Drug Metabolism and Pharmacokinetics* (Vol. 33, Issue 1, pp. 31–39). Japanese Society for the Study of Xenobiotics. <https://doi.org/10.1016/j.dmpk.2017.11.001>

- Nath, A., & Atkins, W. M. (2006). A theoretical validation of the substrate depletion approach to determining kinetic parameters. *Drug Metabolism and Disposition*, 34(9), 1433–1435. <https://doi.org/10.1124/dmd.106.010777>
- Neirinckx, E., Vervaet, C., De Boever, S., Remon, J. P., Gommeren, K., Daminet, S., De Backer, P., & Croubels, S. (2010). Species comparison of oral bioavailability, first-pass metabolism and pharmacokinetics of acetaminophen. *Research in Veterinary Science*, 89(1), 113–119. <https://doi.org/10.1016/j.rvsc.2010.02.002>
- Noble, B. T., Brennan, F. H., & Popovich, P. G. (2018). The spleen as a neuroimmune interface after spinal cord injury. *Journal of Neuroimmunology*, 321, 1–11. <https://doi.org/10.1016/j.jneuroim.2018.05.007>
- Palandi, J., Mack, J. M., de Araújo, I. L., Farina, M., & Bobinski, F. (2023). Animal models of complex regional pain syndrome: A scoping review. In *Neuroscience and Biobehavioral Reviews* (Vol. 152). Elsevier Ltd. <https://doi.org/10.1016/j.neubiorev.2023.105324>
- Pang, K. S., Maeng, H. J., Fan, J., & Dan, L. L. (2009). Interplay of transporters and enzymes in drug and metabolite processing. In *Molecular Pharmaceutics* (Vol. 6, Issue 6, pp. 1734–1755). <https://doi.org/10.1021/mp900258z>
- Pant, V. (2022). Importance of observing the progress curve during enzyme assay in an automated clinical chemistry analyzer: a case study. *The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine*, 33(1), 56–62.
- Pasanen, M. (2009). 2. *Species differences in CYP enzymes*. <http://drnelson.utmem.edu/CytochromeP450.html>;
- Paul F. Cook, & W. W. Cleland. (2007). *Enzyme Kinetics and Mechanism* (R. Rogers, S. Scholl, & Simon. Hill, Eds.). Garland Science. https://books.google.co.uk/books?id=vywWBAAQBAJ&dq=enzyme+kinetics&lr=&source=gs_navlinks_s
- Paul, S., & Sahoo, G. (2014). SAWSA-LPR: Astochastic search strategy for estimation of maximum likelihood DNA phylogenetic trees. *Applied Soft Computing Journal*, 18, 104–114. <https://doi.org/10.1016/j.asoc.2014.01.023>
- Peng, Y., Chen, L., Ye, S., Kang, Y., Liu, J., Zeng, S., & Yu, L. (2020). Research and development of drug delivery systems based on drug transporter and nano-formulation. *Asian Journal of Pharmaceutical Sciences*, 15(2), 220–236. <https://doi.org/10.1016/j.ajps.2020.02.004>
- Polyak K, & Meyerson M. (2003). Gene Expression: Protein Analysis. In Kufe DW, Pollock RE, & Weichselbaum RR (Eds.), *Holland-Frei Cancer Medicine* (6th ed.). BC Decker. <https://www.ncbi.nlm.nih.gov/books/NBK13114/>
- Pond, S. M., & Tozer, T. N. (1984). First-Pass Elimination. *Clinical Pharmacokinetics*, 9(1), 1–25. <https://doi.org/10.2165/00003088-198409010-00001>

- Poulin, P., Hop, C. E. C. A., Ho, Q., Halladay, J. S., Haddad, S., & Kenny, J. R. (2012). Comparative assessment of In Vitro-In Vivo extrapolation methods used for predicting hepatic metabolic clearance of drugs. *Journal of Pharmaceutical Sciences*, 101(11), 4308–4326. <https://doi.org/10.1002/jps.23288>
- Prabantu, V. M., Naveenkumar, N., & Srinivasan, N. (2021). Influence of Disease-Causing Mutations on Protein Structural Networks. *Frontiers in Molecular Biosciences*, 7. <https://doi.org/10.3389/fmolb.2020.620554>
- Proteintech®. (2024). *Western Blot Troubleshooting: Why Does The Observed Protein Molecular Weight (MW) Differ From The Calculated One?* <https://www.ptglab.com/support/western-blot-protocol/western-blot-troubleshooting-why-does-the-observed-protein-molecular-weight-mw-differ-from-the-calculated-one/>
- Puccinelli, E., Gervasi, Pier. G., & Longo, V. (2011). Xenobiotic Metabolising Cytochrome P450 in Pig, a Promising Animal Model. *Current Drug Metabolism*, 12, 507–525. www.sanger.ac.uk/Projects/S_scrofa/
- Rajman, I., Knapp, L., Morgan, T., & Masimirembwa, C. (2017). African Genetic Diversity: Implications for Cytochrome P450-mediated Drug Metabolism and Drug Development. *EBioMedicine*, 17, 67–74. <https://doi.org/10.1016/j.ebiom.2017.02.017>
- Ren, G., Qin, Z., Yang, N., Chen, H., Fu, K., Lu, C., Lu, Y., Li, N., Zhang, Y., Chen, X., & Zhao, D. (2020). Interactions between Oroxylin A with the solute carrier transporters and ATP-binding cassette transporters: Drug transporters profile for this flavonoid. *Chemico-Biological Interactions*, 324(April). <https://doi.org/10.1016/j.cbi.2020.109097>
- Roberts, S. A. (2005). Drug Metabolism: Overview. In *Encyclopedia of Analytical Science* (Second, pp. 293–305).
- Robinson, P. K. (2015). Enzymes: principles and biotechnological applications. *Essays in Biochemistry*, 59, 1–41. <https://doi.org/10.1042/BSE0590001>
- Rodríguez-Antona, C., Jover, R., Gómez-Lechón, M. J., & Castell, J. V. (2000). Quantitative RT-PCR measurement of human cytochrome P-450s: Application to drug induction studies. *Archives of Biochemistry and Biophysics*, 376(1), 109–116. <https://doi.org/10.1006/abbi.2000.1697>
- Rowland, A., Miners, J. O., & Mackenzie, P. I. (2013). The UDP-glucuronosyltransferases: Their role in drug metabolism and detoxification. In *International Journal of Biochemistry and Cell Biology* (Vol. 45, Issue 6, pp. 1121–1132). <https://doi.org/10.1016/j.biocel.2013.02.019>
- Ruddle, N. H., & Akirav, E. M. (2009). Secondary Lymphoid Organs: Responding to Genetic and Environmental Cues in Ontogeny and the Immune Response. *The Journal of Immunology*, 183(4), 2205–2212. <https://doi.org/10.4049/jimmunol.0804324>
- Ryu, S., Tess, D., Chang, G., Keefer, C., Burchett, W., Steeno, G. S., Novak, J. J., Patel, R., Atkinson, K., Riccardi, K., & Di, L. (2020). Evaluation of Fraction Unbound Across 7 Tissues of 5 Species.

- Journal of Pharmaceutical Sciences*, 109(2), 1178–1190.
<https://doi.org/10.1016/j.xphs.2019.10.060>
- Sadeghirad, H., Bahrami, T., Layeghi, S. M., Yousefi, H., Rezaei, M., Hosseini-Fard, S. R., Radfar, P., Warkiani, M. E., O'Byrne, K., & Kulasinghe, A. (2023). Immunotherapeutic targets in non-small cell lung cancer. In *Immunology* (Vol. 168, Issue 2, pp. 256–272). John Wiley and Sons Inc. <https://doi.org/10.1111/imm.13562>
- Sadler, N. C., Nandhikonda, P., Webb-Robertson, B. J., Ansong, C., Anderson, L. N., Smith, J. N., Corley, R. A., & Wright, A. T. (2016). Hepatic cytochrome P450 activity, abundance, and expression throughout human development. *Drug Metabolism and Disposition*, 44(7), 984–991. <https://doi.org/10.1124/dmd.115.068593>
- Savoji, H., Mohammadi, M. H., Rafatian, N., Toroghi, M. K., Wang, E. Y., Zhao, Y., Korolj, A., Ahadian, S., & Radisic, M. (2019). Cardiovascular disease models: A game changing paradigm in drug discovery and screening. *Biomaterials*, 198, 3–26.
<https://doi.org/10.1016/j.biomaterials.2018.09.036>
- Schelstraete, W., Clerck, L. De, Govaert, E., Millegam, J., Devreese, M., Deforce, D., Bocxlaer, J. Van, & Croubels, S. (2019). Characterization of Porcine Hepatic and Intestinal Drug Metabolizing CYP450: Comparison with Human Orthologues from A Quantitative, Activity and Selectivity Perspective. *Scientific Reports*, 9(1). <https://doi.org/10.1038/s41598-019-45212-0>
- Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M., & Ragg, T. (2006). The RIN: An RNA integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology*, 7.
<https://doi.org/10.1186/1471-2199-7-3>
- Schyman, P., Printz, R. L., Pannala, V. R., AbdulHameed, M. D. M., Estes, S. K., Shiota, C., Boyd, K. L., Shiota, M., & Wallqvist, A. (2021). Genomics and metabolomics of early-stage thioacetamide-induced liver injury: An interspecies study between guinea pig and rat. *Toxicology and Applied Pharmacology*, 430. <https://doi.org/10.1016/j.taap.2021.115713>
- Sepe, P., Mennitto, A., Corti, F., & Procopio, G. (2020). Immunotherapeutic targets and therapy for renal cell carcinoma. *ImmunoTargets and Therapy*, 9, 273–288.
<https://doi.org/10.2147/ITT.S240889>
- Sheppard, J. R., Foker, J. E., Johnson, G. S., Pastan, I. H., Whitfield, J. F., Rixon, R. H., MacManus, J. P., MacManus, G. P., Boynton, A. L., Yondale, T., Swierenga, S., Vitro, I., Braceland, B. M., Whitfield, J. R., Cell Physiol, J., Thrower, S., Ord, M., Walker, P. R., Armato, U., ... Wedner, H. J. (1973). Nucleotides and the Regulation of Cell Growth. In *J. Stambrook, Proc. Natl. Acad. Sci. U.S.A* (Vol. 9). <https://www.science.org>
- Shetty, V., Subramaniam, K., & Rao, R. (2017). Utility of immunofluorescence in dermatology. *Indian Dermatology Online Journal*, 8(1), 1. <https://doi.org/10.4103/2229-5178.198774>

- Singh, K. P., Miaskowski, C., Dhruva, A. A., Flowers, E., & Kober, K. M. (2018). Mechanisms and Measurement of Changes in Gene Expression. *Biological Research for Nursing*, 20(4), 369–382. <https://doi.org/10.1177/1099800418772161>
- Sinz, M., Wallace, G., & Sahi, J. (2008). Current industrial practices in assessing CYP450 enzyme induction: Preclinical and clinical. In *AAPS Journal* (Vol. 10, Issue 2, pp. 391–400). <https://doi.org/10.1208/s12248-008-9037-4>
- Smith, D. A., Beaumont, K., Maurer, T. S., & Di, L. (2019). Clearance in Drug Design. *Journal of Medicinal Chemistry*, 62(5), 2245–2255. <https://doi.org/10.1021/acs.jmedchem.8b01263>
- Smith, S., Stone, A., Oswalt, H., Vaughan, L., Ferdous, F., Scott, T., & Dunn, H. W. (2022). Evaluation of early post-natal pig mammary gland development and human breast cancer gene expression. *Developmental Biology*, 481, 95–103. <https://doi.org/10.1016/j.ydbio.2021.10.004>
- Soars, M. G., Burchell, B., & Riley, R. J. (2002). In Vitro Analysis of Human Drug Glucuronidation and Prediction of in Vivo Metabolic Clearance. *Pharmacology and Experimental Therapeutics*, 301(1), 382–390. <http://jpet.aspetjournals.org>
- Son, S. Y., Shin, D. J., Park, Y. S., Oo, A. M., Jung, D. H., Lee, C. M., Ahn, S. H., Park, D. J., & Kim, H. H. (2017). Spleen-preserving lymphadenectomy versus splenectomy in laparoscopic total gastrectomy for advanced gastric cancer. *Surgical Oncology*, 26(2), 207–211. <https://doi.org/10.1016/j.suronc.2017.04.002>
- Stanley, L. A. (2017). *Pharmacognosy -Part VI: Drug Metabolism: Vol. Chapter 27* (S. Badal & R. Delgoda, Eds.). Elsevier. <https://doi.org/10.1016/C2014-0-01794-7>
- Steiniger, B., Bette, M., & Schwarzbach, H. (2011). The open microcirculation in human spleens: A three-dimensional approach. *Journal of Histochemistry and Cytochemistry*, 59(6), 639–648. <https://doi.org/10.1369/0022155411408315>
- Steiniger, B. S. (2015). Human spleen microanatomy: Why mice do not suffice. *Immunology*, 145(3), 334–346. <https://doi.org/10.1111/imm.12469>
- Steiniger, B. S., Pfeffer, H., Gaffling, S., & Lobachev, O. (2022). The human splenic microcirculation is entirely open as shown by 3D models in virtual reality. *Scientific Reports*, 12(1). <https://doi.org/10.1038/s41598-022-19885-z>
- Sudhir Kumar, Glen Stecher, & Koichiro Tamura. (2015). *MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. Molecular Biology and Evolution (submitted)* (7).
- Sun, Y. L., Choi, I. L., Lee, Y. B., Choi, K. Y., Hong, S. K., & Kang, H. M. (2014). Molecular diversity and phylogentic analysis of Capsicum annuum varieties using the nrDNA ITS region. *Scientia Horticulturae*, 165, 336–343. <https://doi.org/10.1016/j.scienta.2013.11.009>
- Surti, F. (2023, July 18). *Enzyme Kinetics*. Teach Me Physiology. <https://teachmephysiology.com/biochemistry/molecules-and-signalling/enzyme-kinetics/>

- Sze, L. P., Li, H. Y., Lai, K. L. A., Chow, S. F., Li, Q., KennethTo, K. W., Lam, T. N. T., & Lee, W. Y. T. (2019). Oral delivery of paclitaxel by polymeric micelles: A comparison of different block length on uptake, permeability and oral bioavailability. *Colloids and Surfaces B: Biointerfaces*, 184, 1–11. <https://doi.org/10.1016/j.colsurfb.2019.110554>
- Talebi, A., Thiery, J. P., & Kerachian, M. A. (2021). Fusion transcript discovery using RNA sequencing in formalin-fixed paraffin-embedded specimen. In *Critical Reviews in Oncology/Hematology* (Vol. 160). Elsevier Ireland Ltd. <https://doi.org/10.1016/j.critrevonc.2021.103303>
- Tarantino, G., Savastano, S., Capone, D., & Colao, A. (2011). Spleen: A new role for an old player? *World Journal of Gastroenterology*, 17(33), 3776–3784. <https://doi.org/10.3748/wjg.v17.i33.3776>
- Taxak, N., & Bharatam, P. V. (2014, March). Drug Metabolism A Fascinating Link Between Chemistry and Biology. *RESONANCE*, 259–282.
- Thervet, E., Anglicheau, D., King, B., Schlageter, M. H., Cassinat, B., Beaune, P., Legendre, C., & Daly, A. K. (2003). Impact of cytochrome P450 3A5 genetic polymorphism on tacrolimus doses and concentration-to-dose ratio in renal transplant recipients. *Transplantation*, 76(8), 1233–1235. <https://doi.org/10.1097/01.TP.0000090753.99170.89>
- Ung, Y. T., Ong, C. E., & Pan, Y. (2018). Current high-throughput approaches of screening modulatory effects of xenobiotics on cytochrome P450 (CYP) enzymes. In *High-Throughput* (Vol. 7, Issue 4). MDPI AG. <https://doi.org/10.3390/HT7040029>
- van de Waterbeemd, H., & Gifford, E. (2003). ADMET in silico modelling: towards prediction paradise? *Nature Reviews Drug Discovery*, 2(3), 192–204. <https://doi.org/10.1038/nrd1032>
- Vrbanac, J., & Slauter, R. (2016). ADME in Drug Discovery. *A Comprehensive Guide to Toxicology in Nonclinical Drug Development*, 39–67. <https://doi.org/10.1016/B978-0-12-803620-4.00003-7>
- Walker, D. G., Whetzel, A. M., Serrano, G., Sue, L. I., Lue, L. F., & Beach, T. G. (2016). Characterization of RNA isolated from eighteen different human tissues: results from a rapid human autopsy program. *Cell and Tissue Banking*, 17(3), 361–375. <https://doi.org/10.1007/s10561-016-9555-8>
- Wang, D. R., Wu, X. L., & Sun, Y. L. (2022). Therapeutic targets and biomarkers of tumor immunotherapy: response versus non-response. In *Signal Transduction and Targeted Therapy* (Vol. 7, Issue 1). Springer Nature. <https://doi.org/10.1038/s41392-022-01136-2>
- Wang, L., Piao, Y., Guo, F., Wei, J., Chen, Y., Dai, X., & Zhang, X. (2023). Current progress of pig models for liver cancer research. In *Biomedicine and Pharmacotherapy* (Vol. 165). Elsevier Masson s.r.l. <https://doi.org/10.1016/j.biopha.2023.115256>
- Wang, W., He, W., Ruan, Y., & Geng, Q. (2022). First pig-to-human heart transplantation. In *Innovation* (Vol. 3, Issue 2). Cell Press. <https://doi.org/10.1016/j.xinn.2022.100223>

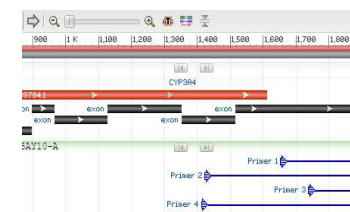
- Wang, X., Wang, X., Zhang, X., He, S., Chen, Y., Liu, X., & Guo, C. (2022). *Genetic Characterization and Variation of African Swine Fever Virus China/GD/2019 Strain in Domestic Pigs*.
<https://doi.org/10.3390/pathogens>
- Wang, Z., Shi, X., Tao, Y., Wu, Q., Bai, Y., Guo, H., & Tang, D. (2019). The complete mitochondrial genome of *Parasesarma pictum* (Brachyura: Grapsoidea: Sesarmidae) and comparison with other Brachyuran crabs. *Genomics*, 111(4), 799–807.
<https://doi.org/10.1016/j.ygeno.2018.05.002>
- Waring, M. J., Arrowsmith, J., Leach, A. R., Leeson, P. D., Mandrell, S., Owen, R. M., Pairaudeau, G., Pennie, W. D., Pickett, S. D., Wang, J., Wallace, O., & Weir, A. (2015). An analysis of the attrition of drug candidates from four major pharmaceutical companies. In *Nature Reviews Drug Discovery* (Vol. 14, Issue 7, pp. 475–486). Nature Publishing Group.
<https://doi.org/10.1038/nrd4609>
- William W. Wilfinger, Karol Mackey, & Piotr Chomczynski. (1997). Short Technical Reports: Effect of pH and Ionic Strength on the Spectro-photometric Assessment of Nucleic Acid Purity. In *BioTechniques* (Vol. 22).
- Won, M. M., Mladenov, G. D., Raymond, S. L., Khan, F. A., & Radulescu, A. (2023). What animal model should I use to study necrotizing enterocolitis? *Seminars in Pediatric Surgery*, 32(3).
<https://doi.org/10.1016/j.sempedsurg.2023.151313>
- Wunderlich, F., Dkhil, M. A., Mehnert, L. I., Braun, J. V., El-Khadragy, M., Borsch, E., Hermesen, D., Benten, W. P. M., Pfeffer, K., Mossmann, H., & Krücken, J. (2005). Testosterone responsiveness of spleen and liver in female lymphotoxin β receptor-deficient mice resistant to blood-stage malaria. *Microbes and Infection*, 7(3), 399–409.
<https://doi.org/10.1016/j.micinf.2004.11.016>
- Xiang, Z., He, X. L., Zhu, C. W., Yang, J. J., Huang, L., Jiang, C., Wu, J., Xiang, Z., He, X. L., Zhu, C. W., Yang, J. J., Huang, L., Jiang, C., & Wu, J. (2023). Animal models of hepatitis E infection: Advances and challenges. In *Hepatobiliary and Pancreatic Diseases International*. Elsevier (Singapore) Pte Ltd. <https://doi.org/10.1016/j.hbpd.2023.10.001>
- Xu, Y., Shrestha, N., Pr  at, V., & Beloqui, A. (2020). Overcoming the intestinal barrier: A look into targeting approaches for improved oral drug delivery systems. In *Journal of Controlled Release* (Vol. 322, pp. 486–508). Elsevier B.V. <https://doi.org/10.1016/j.jconrel.2020.04.006>
- Yaqub, N., Wayne, G., Birchall, M., & Song, W. (2022). Recent advances in human respiratory epithelium models for drug discovery. In *Biotechnology Advances* (Vol. 54). Elsevier Inc. <https://doi.org/10.1016/j.biotechadv.2021.107832>
- You, X., Li, G., Lei, Y., Xu, Z., Zhang, P., & Yang, Y. (2023). Role of genetic factors in different swine breeds exhibiting varying levels of resistance/susceptibility to PRRSV. In *Virus Research* (Vol. 326). Elsevier B.V. <https://doi.org/10.1016/j.virusres.2023.199057>

- Zanger, U. M., & Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology and Therapeutics*, 138(1), 103–141. <https://doi.org/10.1016/j.pharmthera.2012.12.007>
- Zhang, D., Luo, G., Ding, X., & Lu, C. (2012). Preclinical experimental models of drug metabolism and disposition in drug discovery and development. *Acta Pharmaceutica Sinica B*, 2(6), 549–561. <https://doi.org/10.1016/j.apsb.2012.10.004>
- Zhang, F., Lu, G., Wen, X., Li, F., Ji, X., Li, Q., Wu, M., Cheng, Q., Yu, Y., Tang, J., & Mei, L. (2020). Magnetic nanoparticles coated with polyphenols for spatio-temporally controlled cancer photothermal/immunotherapy. *Journal of Controlled Release*, 326(January), 131–139. <https://doi.org/10.1016/j.jconrel.2020.06.015>
- Zhang, L., Xu, P., Cheng, Y., Wang, P., Ma, X., Liu, M., Wang, X., & Xu, F. (2019). Diet-induced obese alters the expression and function of hepatic drug-metabolizing enzymes and transporters in rats. *Biochemical Pharmacology*, 164(May), 368–376. <https://doi.org/10.1016/j.bcp.2019.05.002>
- Zhang, Y., Chen, H., Wang, H., Wang, T., Pan, H., Ji, W., & Chang, J. (2020). A synergistic cancer immunotherapy nano-system for preventing tumor growth. *Chemical Engineering Journal*, 380(July 2019). <https://doi.org/10.1016/j.cej.2019.122472>
- Zhang, Y., & Hagenbuch, B. (2019). Protein-protein interactions of drug uptake transporters that are important for liver and kidney. In *Biochemical Pharmacology* (Vol. 168, pp. 384–391). Elsevier Inc. <https://doi.org/10.1016/j.bcp.2019.07.026>
- Zhang, Z., & Tang, W. (2018). Drug metabolism in drug discovery and development. *Acta Pharmaceutica Sinica B*, 8(5), 721–732. <https://doi.org/10.1016/j.apsb.2018.04.003>
- Zhao, M., Ma, J., Li, M., Zhang, Y., Jiang, B., Zhao, X., Huai, C., Shen, L., Zhang, N., He, L., & Qin, S. (2021). Cytochrome p450 enzymes and drug metabolism in humans. In *International Journal of Molecular Sciences* (Vol. 22, Issue 23). MDPI. <https://doi.org/10.3390/ijms222312808>
- Zhong, Q., Xiao, X., Qiu, Y., Xu, Z., Chen, C., Chong, B., Zhao, X., Hai, S., Li, S., An, Z., & Dai, L. (2023). Protein posttranslational modifications in health and diseases: Functions, regulatory mechanisms, and therapeutic implications. In *MedComm* (Vol. 4, Issue 3). John Wiley and Sons Inc. <https://doi.org/10.1002/mco2.261>
- Zhou, H. (2003). Pharmacokinetic strategies in deciphering atypical drug absorption profiles. *Journal of Clinical Pharmacology*, 43(3), 211–227. <https://doi.org/10.1177/0091270002250613>
- Zhou, S., Chen, W., Cole, J., & Zhu, G. (2020). Delivery of nucleic acid therapeutics for cancer immunotherapy. *Medicine in Drug Discovery*, 6, 100023. <https://doi.org/10.1016/j.medidd.2020.100023>

APPENDIX

A.

B.



Primer pair 1									
	Sequence (5'→3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCTCTGAGAAACCCAGAGACC	Plus	20	1654	1673	60.04	60.00	3.00	0.00
Reverse primer	TCTCTCTAATGTTCGAGGAAGC	Minus	22	2109	2079	58.65	45.45	4.00	2.00
Product length	447								
Product Tm	82.5266								

C.

Descriptions

Graphic Summary

Alignments

Taxonomy

Sequences producing significant alignments

Download

Select columns

Show

100

☒ select all 100 sequences selected

[GenBank](#)
[Graphics](#)
[Distance tree of results](#)
[MSA Viewer](#)

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	Homo sapiens cytochrome P450 family 3 subfamily A member 4 (CYP3A4), transcript variant 2, mRNA	Homo sapiens	40.1	40.1	100%	0.027	100.00%	2778	NM_001202855.3
☒	Homo sapiens cytochrome P450 family 3 subfamily A member 4 (CYP3A4), transcript variant 1, mRNA	Homo sapiens	40.1	40.1	100%	0.027	100.00%	2781	NM_017460.6

Figure. A1 Primer design procedure: (A-C) Primers designed from rat and pig mRNA sequences using BLAST algorithm to confirm specificity for selected CYP450s. **A.** choosing parameters for primer design, **B.** Output of primers within selected parameters, **C.** confirmation of designed primer specificity via BLAST search for 100 % percentage identity and low E value.

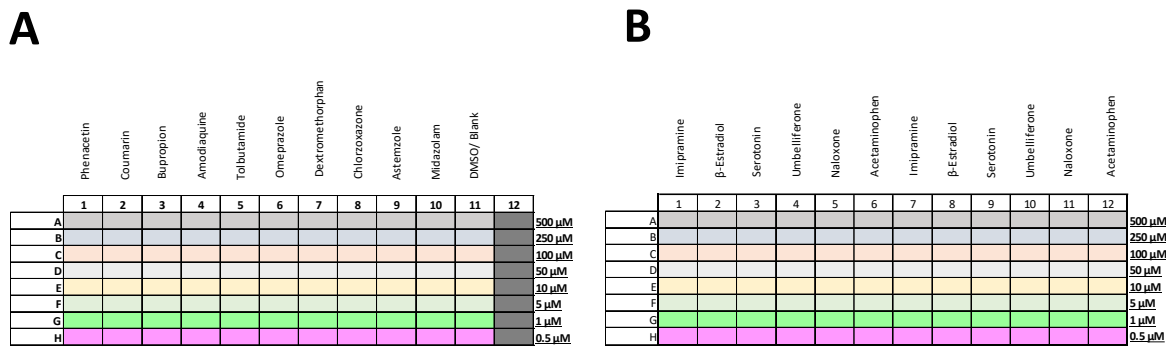


Figure A2 Test Compound plate layout for the prepared working concentrations of the drug substrates in their corresponding wells (1-11/12) for **A.** CYP450 (includes phenacetin, coumarin, bupropion, amodiaquine, tolbutamide, omeprazole, dextromethorphan, chlorzoxazone, astemizole, midazolam, blank dmso) and **B.** UGT (includes imipramine, β -estradiol, serotonin, umbelliferone, acetaminophen; repeated again in exact same order)

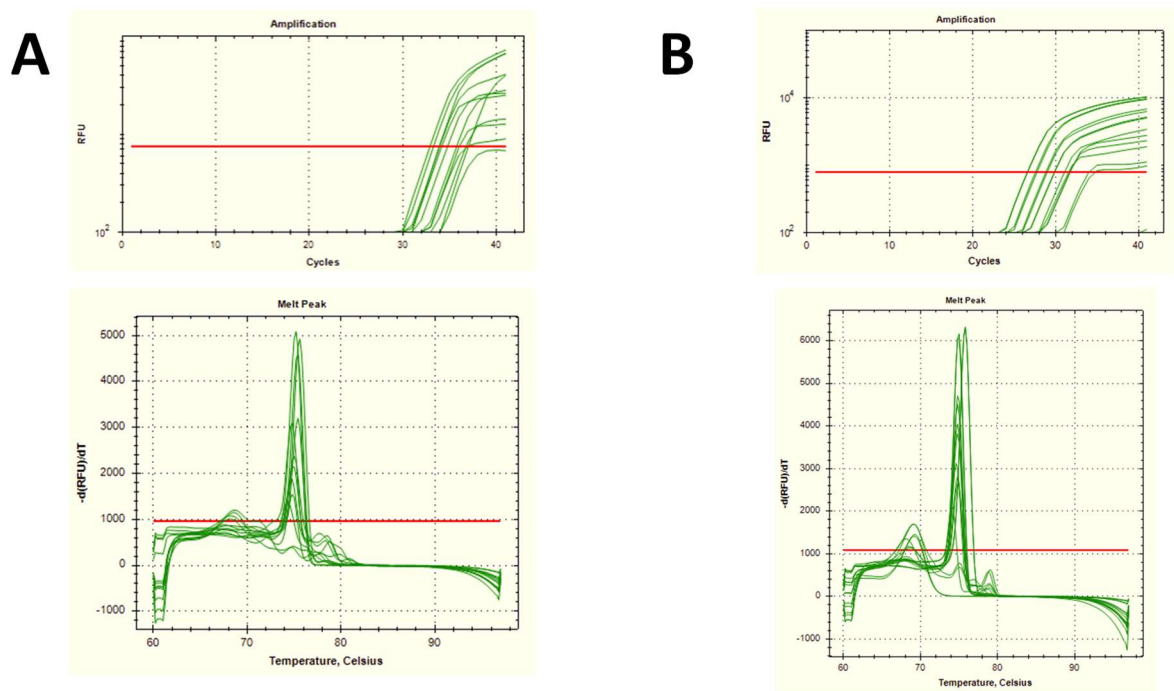


Figure A3 The amplification curve and melt peak of *Abcb1* gene in **A.** pig hepatocytes **B.** pig splenocytes.

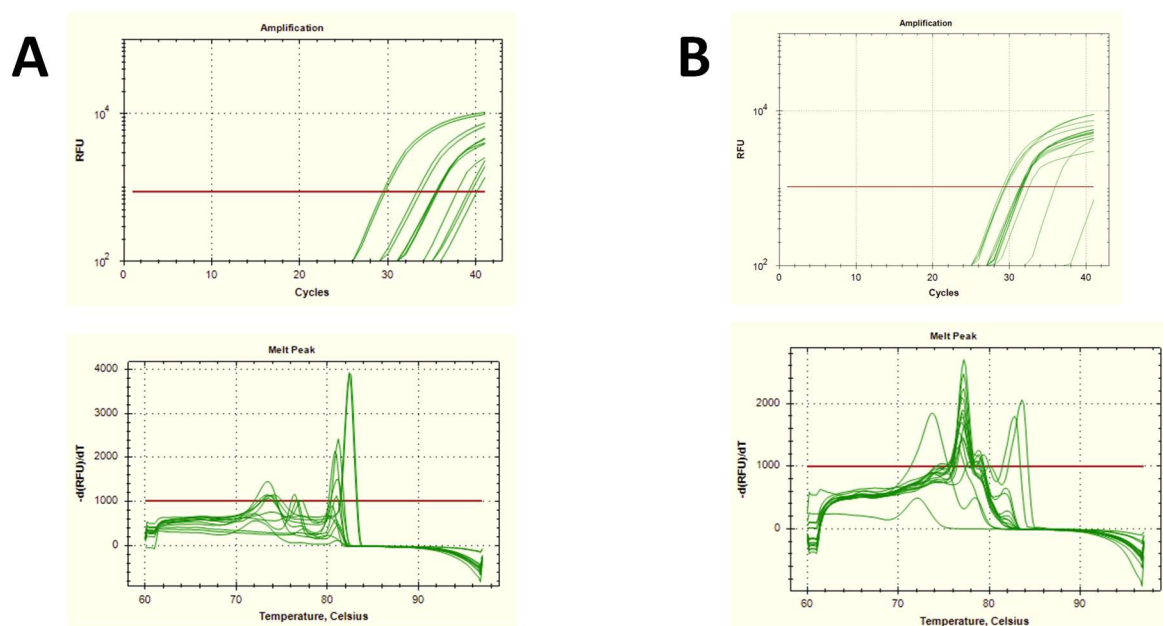


Figure A4 The amplification curve and melt peak of *Cyp2a19* gene in **A.** pig hepatocytes **B.** pig splenocytes.

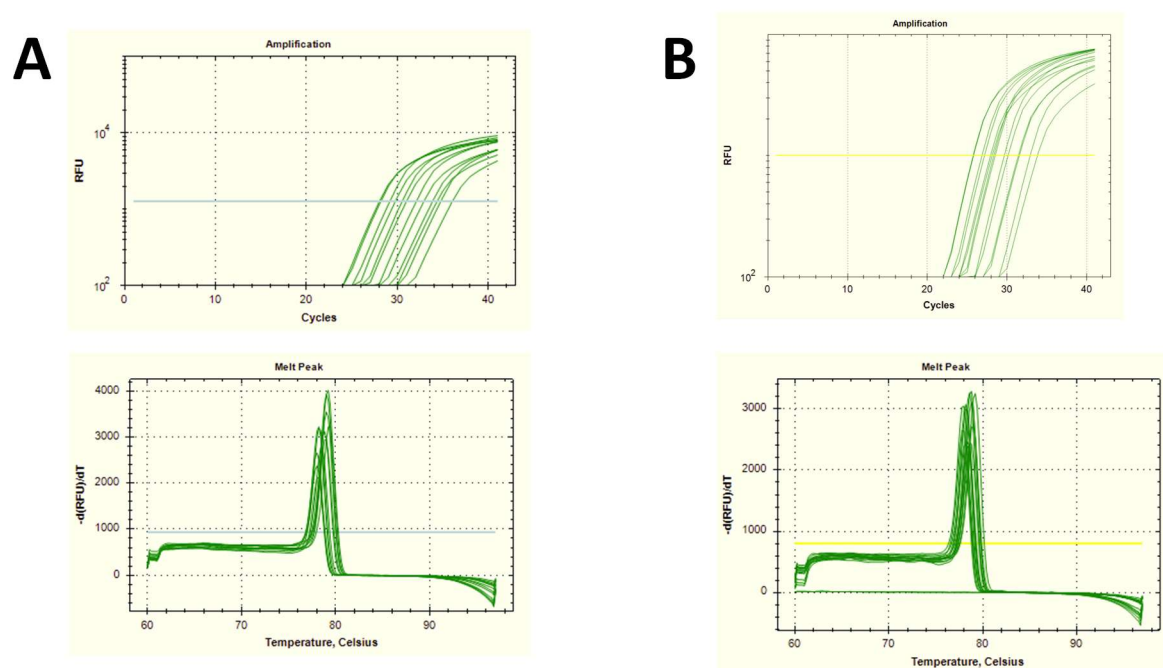


Figure A5 The amplification curve and melt peak of *Cyp2e1* gene in **A.** pig hepatocytes **B.** pig splenocytes.

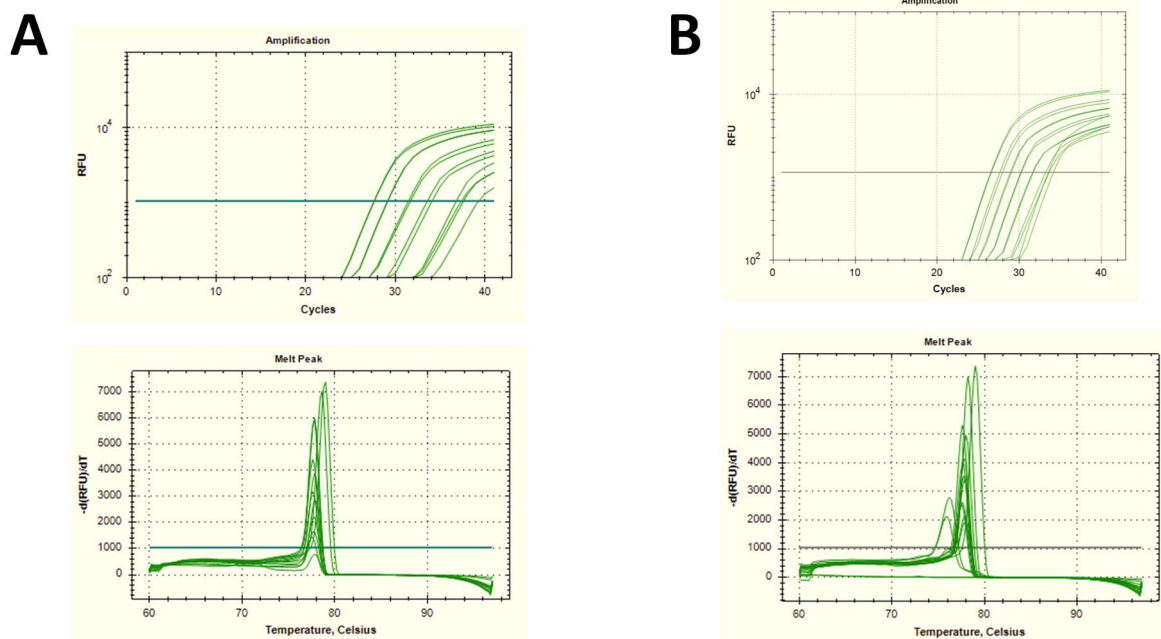


Figure A6 The amplification curve and melt peak of *Cyp3a29* gene in **A.** pig hepatocytes **B.** pig splenocytes

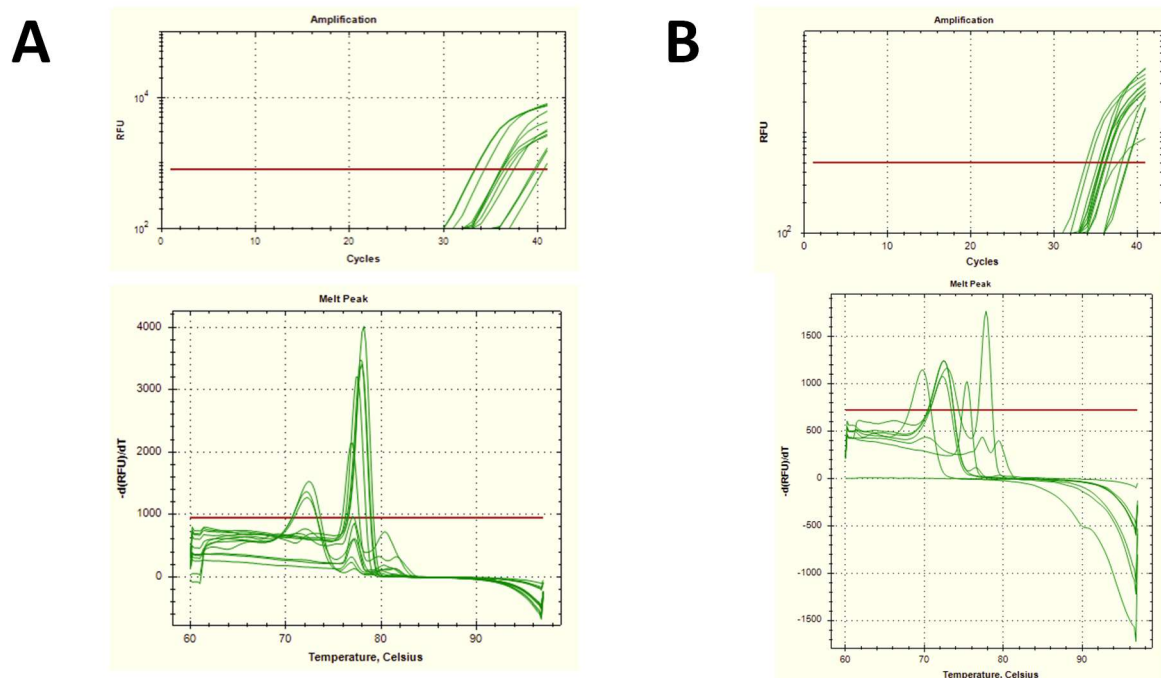


Figure A7 The amplification curve and melt peak of *Ugt8* gene in **A.** pig hepatocytes **B.** pig splenocytes.

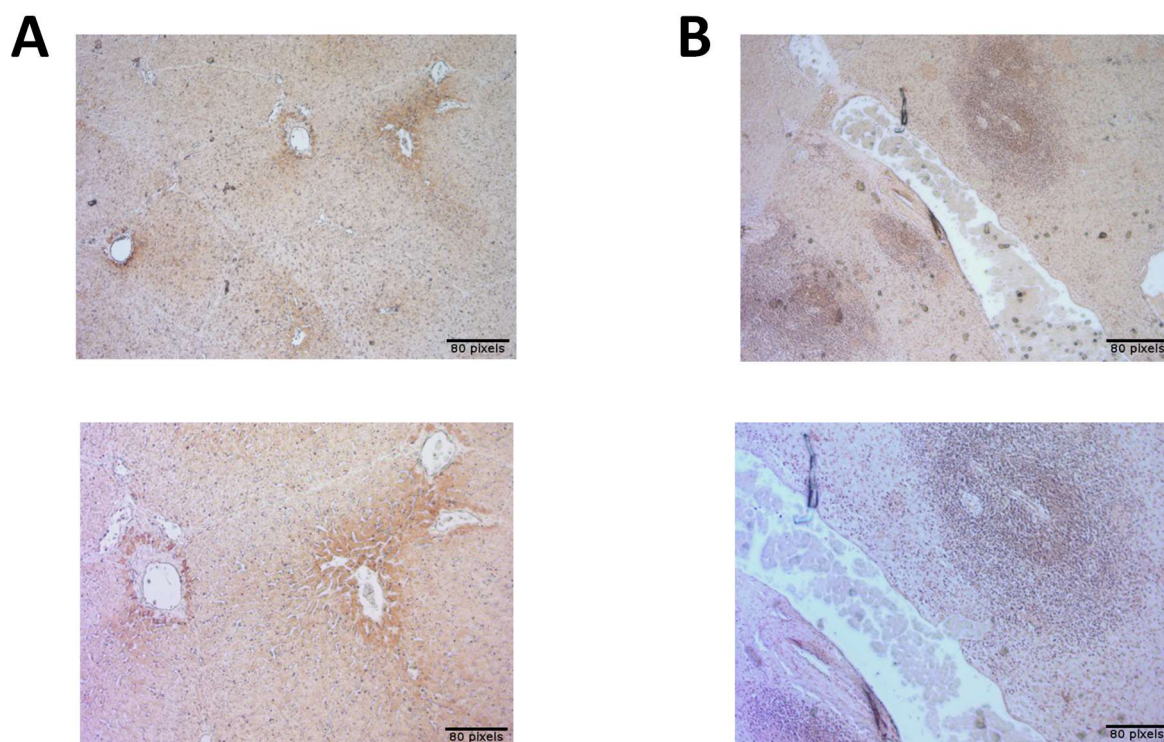


Figure A8 IHC staining probing for target *Cyp2e1* protein expression in **A.** pig liver tissue section and **B.** pig spleen tissue section. Scale bar is 80 pixels.

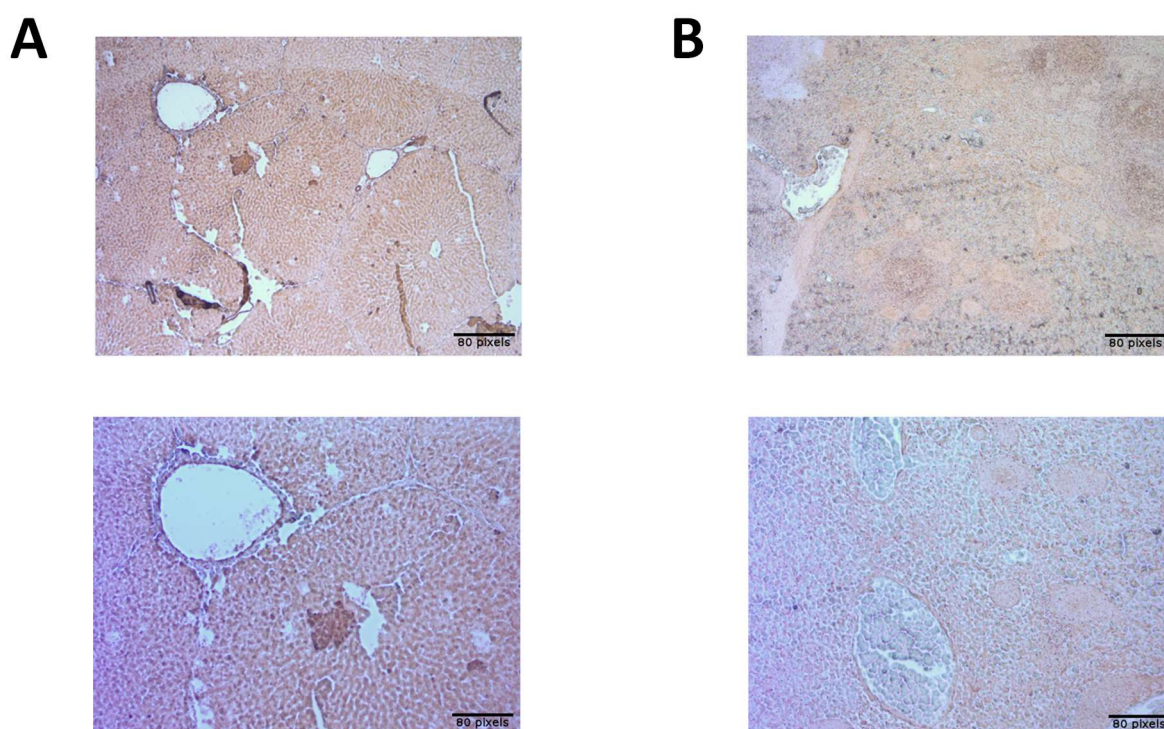


Figure A9 IHC staining probing for target *Ugt8* protein expression in **A.** pig liver tissue section and **B.** pig spleen tissue section. Scale bar is 80 pixels.

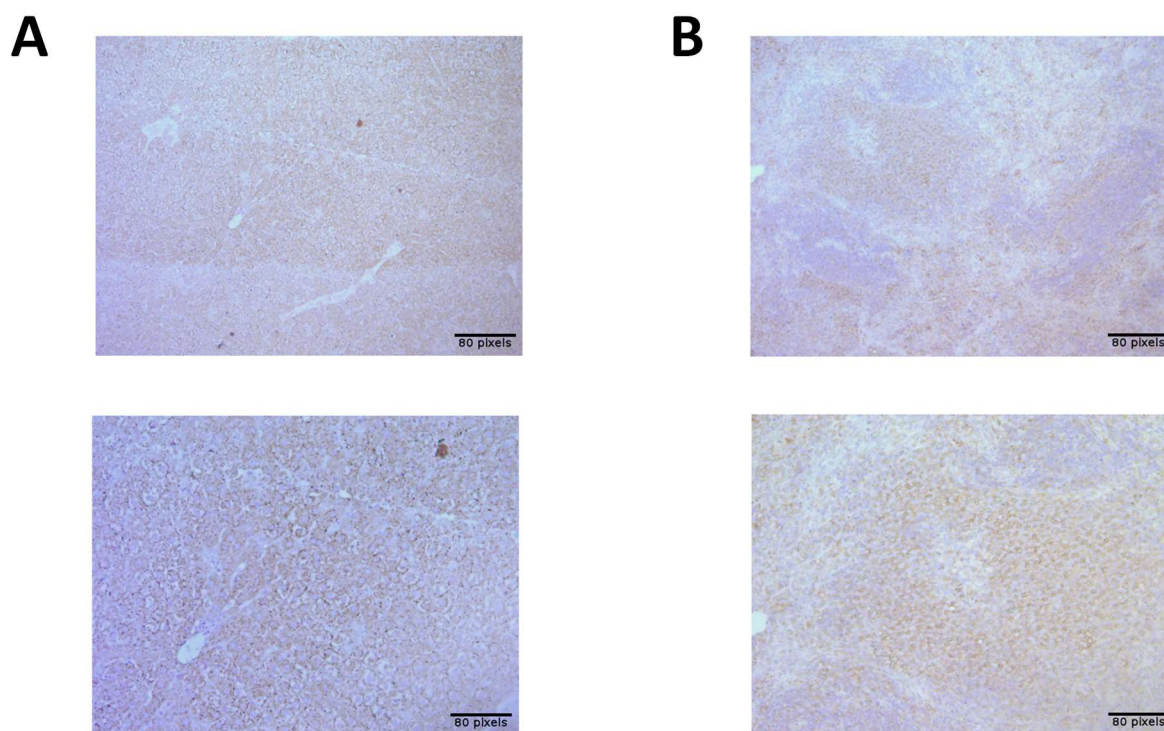


Figure A10 IHC staining probing for target *Ugt1a6* protein expression in **A.** rat liver tissue section and **B.** rat spleen tissue section. Scale bar is 80 pixels.

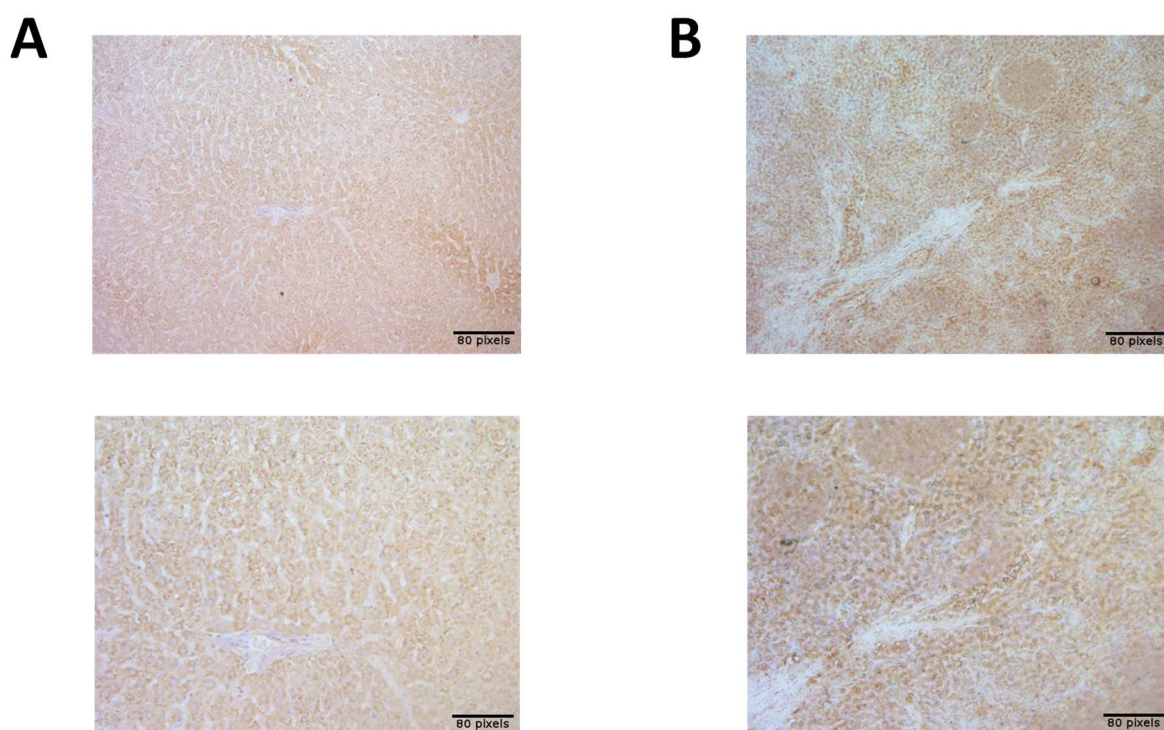


Figure A11 IHC staining probing for target *Cyp2e1* protein expression in **A.** rat liver tissue section and **B.** rat spleen tissue section. Scale bar is 80 pixels.

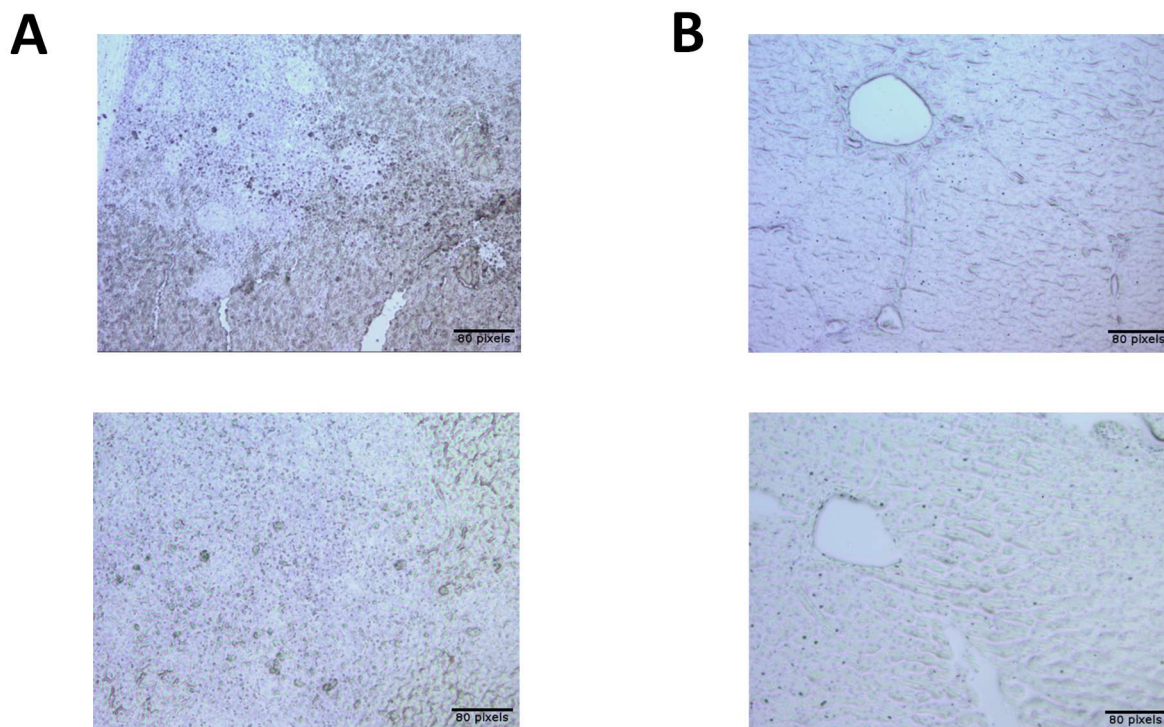


Figure A12 IHC staining of a (no primary antibody) negative control in **A.** pig liver tissue section and **B.** pig spleen tissue section. Scale bar is 80 pixels.

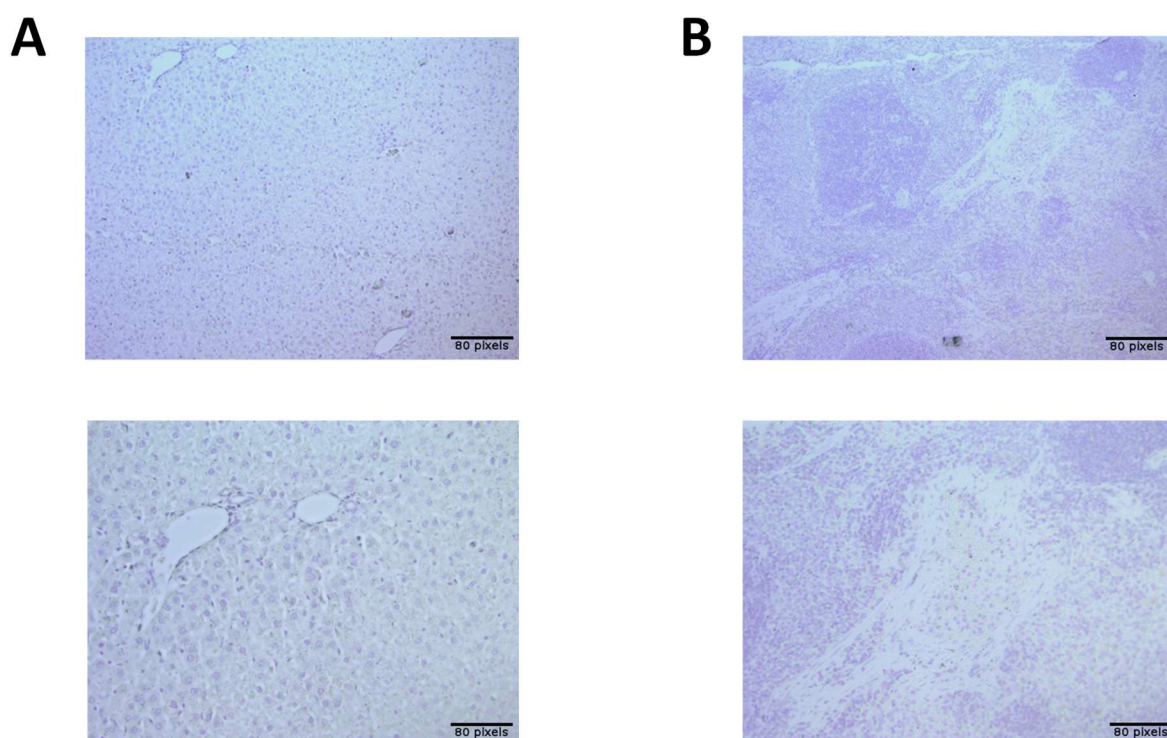


Figure A13 IHC staining of a (no primary antibody) negative control in **A.** rat liver tissue section and **B.** rat spleen tissue section. Scale bar is 80 pixels.

Table A1.0-1.11 Bioinformatics data from RNA-seq transcriptomic analysis of listed (top 12) phase I and II metabolic enzymes, including transporter proteins identified by NGS in rat and pig (liver and spleen tissues)

Table A1.0

PIG LIVER - Bioinformatics data from untreated animal (BASAL)							
Phase I Metabolic Enzymes: Calculated %NumReads							
Gene Symbol							
CYP450	FMO	MAO	AO	ADH	ALDH	AKR	NQO
CYP3A29 (H)	FMO1 (H)	MAOb (H)	AOX1 (H)	ADH1C (H)	ALDH1A1 (H)	AKR1D1 (H)	NQO1 (M-L)
CYP2E1 (H)	FMO5 (H)			ADH4 (H)	ALDH1L1 (H)	AKR1C2 (H)	NQO2 (M)
CYP2A19 (H)	FMO3 (M)			ADH5 (M)	ALDH7A1 (H)	AKR1A1 (M)	
CYP2D25 (H)	FMO4 (M)				ALDH8A1 (H)	AKR7A2 (M)	
CYP27A1 (H)	FMO2 (M-L)				ALDH2 (H)	AKR1E2 (M-L)	
CYP1A2 (H)					ALDH4A1 (H)	AKR1B1 (M-L)	
CYP2C42 (H)					ALDH3A2 (H)		
CYP4A24 (M)					ALDH6A1 (M)		
CYP51A1 (M)					ALDH5A1 (M)		
CYP4V2 (M)					ALDH1B1 (M)		
CYP7A1 (M)					ALDH16A1 (M)		
CYP7B1 (M)					ALDH1L2 (M-L)		

Table A1.1

RAT LIVER - Bioinformatics data from untreated animal (BASAL)							
Phase I Metabolic Enzymes: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts							
CYP450	FMO	MAO	AO	ADH	ALDH	AKR	NQO
CYP3a23/3a1 (H)	FMO5 (H)	MAOa (M)	AOX1 (H)	ADH6 (M)	ALDH2 (H)	AKR1c14 (H)	NQO1 (M-L)
CYP2c11 (H)	FMO3 (H)	MAOb (M)	AOX3 (M-L)	ADH5 (M)	ALDH6a1 (H)	AKR1d1 (H)	NQO2 (M)
CYP2c13 (H)	FMO1 (M)		AOX2 (L)	ADH7 (L)	ALDH7a1 (H)	AKR1c12 (H)	
CYP2c7 (H)	FMO2 (M)				ALDH9a1 (H)	AKR1a1 (H)	
CYP4a2 (H)	FMO4 (L)				ALDH8a1 (H)	AKR1d1 (M)	
CYP2d2 (H)					ALDH1l1 (H)	AKR1c1 (M)	
CYP2e1 (H)					ALDH1l2 (M)	AKR7a2 (M)	
CYP2c23 (H)					ALDH3a2 (M)	AKR7a3 (M)	
CYP2c6v1 (H)					ALDH1a1 (M)	AKR1e2 (M)	
CYP2c22 (H)					ALDH5a1 (M)	AKR1c2 (M)	
CYP4f1 (H)					ALDH1b1 (M-L)	AKR1c13 (M)	
CYP3a18 (H)							

Table A1.2

PIG SPLEEN - Bioinformatics data from untreated animal (BASAL)							
Phase I Metabolic Enzymes: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts							
CYP450	FMO	MAO	AO	ADH	ALDH	AKR	NQO
CYP1B1 (M)	FMO4 (M)	MAOb (M-L)	AOX1 (M-L)	ADH5 (M)	ALDH7A1 (M)	AKR1A1 (M)	NQO1 (M-L)
CYP27A1 (M)	FMO5 (M-L)			ADH1C (L)	ALDH3A2 (M)	AKR1B1 (M)	NQO2 (M-L)
CYP2S1 (M)	FMO2 (M-L)				ALDH16A1 (M)	AKR7A2 (M-L)	
CYP20A1 (M)	FMO1 (L)				ALDH18A1 (M)	AKR1C2 (M-L)	
CYP51A1 (M-L)					ALDH5A1 (M)	AKR1D1 (L)	
CYP2R1 (M-L)					ALDH2 (M)		
CYP2E1 (M-L)					ALDH6A1 (M)		
CYP4V2 (M-L)					ALDH1L2 (M)		
CYP7B1 (M-L)					ALDH4A1 (M)		
CYP2U1 (M-L)					ALDH1A3 (M)		
CYP2A19 (M-L)					ALDH1B1 (M-L)		
CYP3A29 (M-L)					ALDH1A1 (M-L)		

Table A1.3

RAT SPLEEN - Bioinformatics data from untreated animal (BASAL)							
Phase I Metabolic Enzymes: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts							
CYP450	FMO	MAO	AO	ADH	ALDH	AKR	NQO
CYP1b1 (M)	FMO2 (M)	MAOa (M)	AOX1 (M-L)	ADH5 (M)	ALDH2 (H)	AKR1a1 (H)	NQO1 (M-L)
CYP51 (M)	FMO5 (M-L)	MAOb (M-L)	AOX3 (L)	ADH6 (M-L)	ALDH9a1 (H)	AKR1b1 (H)	NQO2 (M-L)
CYP4v3 (M)	FMO3 (M-L)			ADHfe1 (L)	ALDH16a1 (M)	AKR7a2 (M)	
CYP2s1 (M)	FMO4 (M-L)			ADH6a (L)	ALDH3a2 (M)	AKR1e2 (M-L)	
CYP20a1 (M)	FMO1 (L)				ALDH6a1 (M)	AKR1c19 (M-L)	
CYP2ab1 (M)					ALDH7a1 (M)	AKR1b8 (M-L)	
CYP27a1 (M)					ALDH1a3 (M)	AKR7a3 (M-L)	
CYP4f17 (M)					ALDH5a1 (M-L)	AKR1c13 (M-L)	
CYP2d3 (M)					ALDH3a1 (M-L)	AKR1c12 (L)	
CYP4f5 (M)					ALDH16a1 (M-L)	AKR1b7 (L)	
CYP2u1 (M-L)					ALDH18a1 (M-L)		
CYP4f6 (M-L)					ALDH8a1 (L)		

Table A1.4

PIG LIVER - Bioinformatics data from untreated animal (BASAL)			
Phase II Metabolic Enzymes: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts			
UGT	GST	SULT	NAT
UGT2B31 (H)	GSTA1 (H)	SULT2A1 (H)	NAT9 (M)
UGT8 (L)	GSTK1 (H)	SULT1A3 (M)	NAT10 (M-L)
	GSTZ1 (H)	SULT1C2 (M-L)	NAT14 (M-L)
	GSTO1 (M)	SULT1B1 (M-L)	NAT8L (L)
	GSTT4 (M)	SULT1C4 (L)	
	GSTA4 (M-L)		
	GSTM3 (M-L)		
	GSTO2 (L)		

Table A1.5

RAT LIVER - Bioinformatics data from untreated animal (BASAL)			
Phase II Metabolic Enzymes: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts			
UGT	GST	SULT	NAT
UGT2b15 (H)	GSTm1 (H)	SULT1a1 (H)	NAT10 (M)
UGT2b35 (H)	GSTa5 (H)	SULT1e1 (H)	NAT9 (M)
UGT1a6 (H)	GSTm7 (H)	SULT1c3 (H)	NAT1 (M)
UGT2b10 (M)	GSTa1 (H)	SULT1b1 (H)	NATd1 (M-L)
UGT2a3 (M)	GSTz1 (H)	SULT1c2a (M)	NAT2 (M-L)
	GSTt3 (M)	SULT2a1 (M)	NAT14 (L)
	GSTa2 (M)	SULT2b1 (L)	NAT8l (L)
	GSTk1 (M)	SULT1c2 (L)	NAT8f1 (L)
	GSTo1 (M)		
	GSTt1 (M)		
	GSTM4 (M)		
	GSTa1 (M)		

Table A1.6

PIG SPLEEN - Bioinformatics data from untreated animal (BASAL)			
Phase II Metabolic Enzymes: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts			
UGT	GST	SULT	NAT
UGT8 (L)	GSTA4 (M)	SULT1A3 (M)	NAT9 (M)
	GSTO1 (M-L)	SULT2B1 (M-L)	NAT10 (M)
	GSTK1 (M-L)	SULT1C2 (L)	NAT14 (M-L)
	GSTZ1 (M-L)	SULT4A1 (L)	NAT8L (M-L)
	GSTM3 (M-L)		
	GSTA1 (L)		

Table A1.7

RAT SPLEEN - Bioinformatics data from untreated animal (BASAL)			
Phase II Metabolic Enzymes: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts			
UGT	GST	SULT	NAT
UGT1a6 (M)	GSTp1 (H)	SULT1a1 (M-L)	NAT10 (M)
UGT8 (L)	GSTt3 (M)	SULT1c2a (L)	NAT1 (M-L)
	GSTm1 (M)	SULT2b1 (L)	NAT8b (M-L)
	GSTa1 (M)		NAT8f3 (L)
	GSTo1 (M)		NAT8f5 (L)
	GSTa6 (M)		
	GSTt1 (M)		
	GSTm4 (M)		
	GSTm7 (M-L)		
	GSTk1 (M-L)		

Table A1.8

PIG LIVER - Bioinformatics data from untreated animal (BASAL)		
Transporter Proteins: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts		
ABC	SLC	PgP
*ABCB11 (H)	SLC27A5 (H)	PgP (M)
ABCA6 (H)	SLC38A3 (H)	
ABCA8 (M)	SLC7A2 (H)	
*ABCC2 (M)	SLC10A1 (H)	
ABCF1 (M)	SLC37A4 (H)	
ABCD4 (M)	SLC38A4 (H)	
ABCF3 (M)	SLC5A6 (H)	
ABCD3 (M)	SLC26A1 (H)	
ABCB8 (M)	SLC6A12 (H)	
ABCA1 (M)	SLC17A2 (H)	
ABCG8 (M)	SLC22A1 (H)	
ABCB7 (M)	SLC25A39 (H)	

ABCB11 - BSEP

ABCC2 - MRP2

Table A1.9

RAT LIVER - Bioinformatics data from untreated animal (BASAL)		
Transporter Proteins: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts		
ABC	SLC	PgP
*ABCB11 (H)	SLC39a14 (H)	*PgP (M-L)
ABCa1 (H)	SLC9a3r1 (H)	
*ABCC2 (H)	SLC38a3 (H)	
*ABCB4 (H)	SLC41a2 (H)	
ABCe1 (H)	SLC27a2 (H)	
ABCC6 (H)	SLCo1b2 (H)	
ABCa8a (M)	SLC25a15 (H)	
ABCF1 (M)	SLC38a4 (H)	
ABCa6 (M)	SLC25a5 (H)	
ABCD3 (M)	SLC10a1 (H)	
ABCF3 (M)	SLC16a1 (H)	
ABCa3 (M)	SLC27a5 (H)	

ABCB11 - BSEP

ABCB4 - MDR3

ABCC2 - MRP2

PgP - ABCB1

Table A1.10

PIG SPLEEN - Bioinformatics data from untreated animal (BASAL)		
Transporter Proteins: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts		
ABC	SLC	PgP
ABCC10 (M)	SLC25A6 (H)	PgP (M-L)
ABCF1 (M)	SLC40A1 (H)	
ABCF3 (M)	SLC25A3 (H)	
ABCA1 (M)	SLC11A1 (M)	
ABCC5 (M)	SLC25A39 (M)	
ABCD1 (M)	SLC9A3R2 (M)	
ABCG1 (M)	SLC38A10 (M)	
ABCG4 (M)	SLC5A10 (M)	
ABCC1 (M)	SLC43A2 (M)	
ABCB7 (M)	SLC44A2 (M)	
ABCB8 (M)	SLC9A3R1 (M)	
ABCD4 (M)	SLC48A1 (M)	

Table A1.11

RAT SPLEEN - Bioinformatics data from untreated animal (BASAL)		
Transporter Proteins: Calculated %NumReads (estimates based on relative abundance of expression) from the identified sequenced transcripts		
ABC	SLC	PgP
ABCG1 (H)	SLC4a1 (H)	PgP (M)
ABCF1 (H)	SLC44a2 (H)	
ABCE1 (H)	SLC25a5 (H)	
ABCA2 (H)	SLC25a3 (H)	
ABCA3 (M)	SLC25a39 (H)	
ABCA1 (M)	SLC39a1 (H)	
ABCF3 (M)	SLC12a6 (H)	
ABCD1 (M)	SLC38a10 (H)	
ABCF2 (M)	SLC38a2 (H)	
ABCC1 (M)	SLC23a2 (H)	
ABCB10 (M)	SLC2a1 (H)	
*ABCB4 (M)	SLC1a5 (H)	

ABCB4 - MDR3

Table A2.0-2.11 The peak area responses of selected drug substrates from UPLC/MS/MS analysis demonstrating CYP450 and UGT-mediated drug disappearance in pig liver and spleen microsomes

Table A2.0

Amodiaquine - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	700	475	3015	292	1548	231	815	282
0.01	1313	926	1816	2760	1554	1154	1649	387
0.05	6183	4258	5920	3213	5706	1864	6509	1533
0.1	13151	9388	11276	5038	11591	3022	12945	2568
0.5	71413	44034	56469	23134	57659	12678	62574	11020
1	139311	100847	122070	43886	120778	24656	123940	22410
2.5	326774	237797	301454	118473	295132	68879	313253	55997
5	643435	493883	601165	271698	580004	167780	641929	118807

Table A2.1

Amodiaquine - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	1048	1044	1035	1235	2968	10921	1332	1542
0.01	2043	1835	2693	3130	3340	4122	2139	2300
0.05	8487	8007	9096	9448	11287	11902	8862	9366
0.1	16453	16412	17565	18263	23099	23982	17679	18901
0.5	80670	76227	84806	83224	101727	105199	97625	102979
1	160024	158706	171652	174731	210048	219829	198688	210942
2.5	355352	329083	369815	386580	456850	478434	456756	471939
5	721192	698362	725957	739904	848745	876514	844316	885358

Table A2.2

Omeprazole - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	1099	1199	1164	1626	1153	1222	1352	1341
0.01	2185	2236	2110	2113	1955	1956	2479	2199
0.05	9991	10733	9891	10018	9356	9263	12078	10986
0.1	21919	21891	19877	20513	19014	19006	24064	21186
0.5	115163	119114	108119	113855	95077	91705	119326	104988
1	232265	259054	221401	222348	186706	182780	232722	213086
2.5	511371	560524	507182	903148	202488	487580	515514	477581
5	922400	945549	903148	927820	872402	812228	952476	943678

Table A2.3

Omeprazole - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	1592	1567	1902	2139	2350	2736	1480	1517
0.01	2979	2928	3329	3057	4079	3669	2794	2996
0.05	13771	13189	13291	13728	17355	16607	13870	14618
0.1	26308	26087	26416	26484	35275	35268	27382	27792
0.5	120629	125743	128908	135427	169944	161381	142122	157281
1	244337	248403	272077	258668	350710	343871	289376	301154
2.5	306958	448903	577847	662830	173164	Error	603472	630990
5	938807	908099	1093720	1041579	1259870	1266147	1095048	1129266

Table A2.4

Astemizole - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	216	176	475	68	133	74	455	174
0.01	498	362	322	657	461	710	466	623
0.05	2584	1854	896	1380	2077	1939	1652	1954
0.1	5674	4581	1841	1715	4229	3744	3611	4185
0.5	31814	20769	8278	9274	27644	23995	19182	16124
1	54661	47619	19383	16891	53658	35605	41214	37600
2.5	134404	112780	53772	45555	120829	96321	103446	89420
5	309574	327534	111111	147289	256750	215040	249165	382788

Table A2.5

Astemizole - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	1092	679	1611	714	2022	728	1074	1319
0.01	1668	1897	2201	2516	2740	3632	1647	2402
0.05	7620	8033	7486	8521	10466	12144	8134	10424
0.1	15239	16718	15223	17419	21388	31009	16365	23187
0.5	76540	81957	78371	94481	101738	140391	88630	131889
1	153323	171009	154505	194649	229023	294991	185828	254914
2.5	348206	383241	328545	440203	469743	637598	374531	537564
5	692166	874142	717747	970131	947217	1252626	793034	1144204

Table A2.6

Dextromethorphan - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	620	359	361	138	504	703	475	384
0.01	1043	516	790	390	681	325	680	238
0.05	4074	1504	2676	541	1877	339	2554	305
0.1	8225	2913	5371	722	3733	407	5103	368
0.5	43188	14230	28090	2504	19247	803	24897	839
1	92678	31288	61013	4546	43098	1297	51728	1333
2.5	220279	91566	164673	14008	126553	3101	128605	2704
5	478272	257148	373696	65127	333607	13004	303332	7882

Table A2.7

Dextromethorphan - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	738	734	846	1009	1062	837	640	724
0.01	1217	1096	1349	1386	1589	1635	1040	1099
0.05	4855	4649	5220	4997	6138	6512	4687	4995
0.1	9766	9030	10796	10597	12718	13400	9345	9978
0.5	46830	43441	50151	49710	57136	61933	50230	54301
1	98167	88717	100306	96714	128383	133697	105086	109514
2.5	221050	205663	234940	234996	286213	295765	238007	255165
5	424201	419268	458017	464384	558179	576108	485420	494526

Table A2.8

Midazolam - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	305	272	505	362	351	265	394	291
0.01	559	533	524	479	601	385	679	392
0.05	2499	2455	2242	1920	2615	1690	3317	1571
0.1	5360	5147	4815	3996	5268	3326	6520	3265
0.5	29964	24189	25756	22325	26868	17994	32293	15274
1	61706	58471	54731	42466	57571	36826	67287	32023
2.5	136502	141850	129678	126266	143143	102133	158822	90465
5	263383	276446	266375	285748	292299	235105	321121	214200

Table A2.9

Midazolam - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	744	696	740	877	924	1167	701	702
0.01	1348	1124	1189	1294	1564	1480	1076	1235
0.05	4976	4337	4832	4939	6124	6144	4832	5337
0.1	9640	8646	9546	10307	12518	13979	9383	10341
0.5	44097	41372	46094	45838	60111	64705	49910	58412
1	97244	82463	93177	96256	122516	131180	103276	117806
2.5	200602	186379	203665	208095	261226	276803	225514	252692
5	368827	356162	389161	402804	479341	505740	417557	459992

Table A2.10

Serotonin - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	137	133	78	74	112	74	64	NA
0.01	230	190	204	191	193	151	99	58
0.05	793	804	705	922	909	767	445	291
0.1	1658	1336	1352	1156	1397	1107	926	498
0.5	7896	7379	6572	5917	6688	5463	4877	2999
1	16074	14769	12829	11748	12860	10575	10061	5892
2.5	39908	37771	31340	29354	28714	24111	27663	17019
5	75834	71025	61270	56962	58516	47407	53971	34140

Table A2.11

Serotonin - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	124	146	130	174	135	123	306	247
0.01	238	264	209	263	244	183	525	542
0.05	926	915	895	1121	793	661	859	825
0.1	1914	1788	1865	1604	1547	1742	1295	1291
0.5	9773	8976	9012	7815	7625	6390	4924	4878
1	19121	18278	18413	16266	15576	13087	9419	8893
2.5	45604	45658	44310	40097	29894	26323	24286	24485
5	92927	90404	87958	77250	71797	62125	51762	51602

Table A3.0-3.11 The peak area responses of selected drug substrates from UPLC/MS/MS analysis demonstrating CYP450 and UGT-mediated metabolite appearance in pig liver and spleen microsomes.

Table A3.0

Dextrorphan

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	326	1115	524	1039	549	840	456	858
0.01	643	2042	870	1895	951	1495	804	1621
0.05	3105	9475	3950	8959	4663	7800	4230	7838
0.1	6254	19787	8256	18181	9116	15760	8440	16068
0.5	26326	98517	39663	93241	44809	78916	40975	73171
1	52064	189096	77292	189901	87354	166283	79653	145209
2.5	89203	390438	152362	431752	183077	389400	180790	345996
5	122844	596651	224913	752811	286676	740638	328616	617268

Table A3.1

Dextrorphan - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.5	< LOD	< LOD	< LOD	< LOD	10	53	1	29
1	< LOD	< LOD	12	70	18	84	6	72
2.5	43	151	15	210	17	257	31	226
5	26	118	47	197	38	186	35	175

Table A3.2

Desmethylastemizole - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	< LOD	< LOD	< LOD	< LOD	28	71	< LOD	< LOD
0.1	12	86	5	46	24	117	15	58
0.5	71	377	39	196	51	286	57	251
1	55	319	77	404	89	462	101	463
2.5	114	573	154	954	179	1017	203	1208
5	185	892	259	1732	366	1774	401	7231

Table A3.3

Desmethylastemizole - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.5	< LOD	< LOD	76	61	< LOD	< LOD	78	94
1	< LOD	< LOD	120	54	53	191	90	107
2.5	< LOD	< LOD	59	83	35	97	72	120
5	45	38	103	58	34	100	90	132

Table A3.4

Desethylamodiaquine - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	29	49	30	30	< LOD	< LOD	< LOD	< LOD
0.1	54	87	42	55	15	46	21	43
0.5	162	441	216	274	205	101	167	107
1	341	852	435	599	444	238	381	241
2.5	727	2284	966	1706	1067	759	1089	642
5	1264	3690	1702	3379	1841	1702	2116	1246

Table A3.5

Desethylamodiaquine - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.5	40	75	45	61	30	105	8	71
1	98	86	66	101	72	166	35	113
2.5	156	133	168	211	196	372	100	231
5	331	311	315	373	356	560	175	412

Table A3.6

5-Hydroxyomeprazole - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	94	81	20	70	28	102	54	206
0.1	87	140	39	166	50	219	93	338
0.5	369	678	212	899	421	1099	428	1164
1	636	1331	570	1817	814	2287	899	2699
2.5	1375	2944	1396	4096	2014	5152	1966	6279
5	2315	4462	2107	6584	3708	8853	3798	9316

Table A3.7

5-Hydroxyomeprazole - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	42	51	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.1	73	58	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.5	366	429	193	238	100	134	57	65
1	289	296	183	206	145	162	103	93
2.5	676	729	409	457	337	373	205	238
5	1238	1373	875	934	648	779	425	438

Table A3.8

1Hydroxymidazolam

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	< LOD	< LOD	11	35	12	55	31	54
0.1	3	45	25	79	21	90	44	144
0.5	50	207	101	405	157	483	279	668
1	93	410	218	742	305	876	521	1266
2.5	182	695	405	1456	694	1744	1119	2871
5	278	1049	753	2285	1081	2788	2071	5225

Table A3.9

1Hydroxymidazolam - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.05	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.5	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2.5	84	2	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5	126	85	< LOD	< LOD	11	48	4	33

Table A3.10

Serotonin- β -D-glucuronide - Liver

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
0.01	< LOD	< LOD	< LOD	< LOD	20	15	20	15
0.05	< LOD	< LOD	< LOD	< LOD	18	35	21	34
0.1	< LOD	< LOD	< LOD	< LOD	33	71	32	59
0.5	7.0	32	< LOD	< LOD	46	191	68	289
1	0.6	60	30	148	79	377	140	679
2.5	18	156	89	401	174	1125	320	1882
5	16	175	92	585	411	2320	787	4070

Table A3.11

Serotonin- β -D-glucuronide - Spleen

	0.1 mg/mL		0.25 mg/mL		0.5 mg/mL		1 mg/mL	
	5 min	45 min	5 min	45 min	5 min	45 min	5 min	45 min
0.005	< LOD	< LOD	151	189	342	483	48	91
0.01	< LOD	< LOD	209	169	231	345	39	55
0.05	< LOD	< LOD	163	130	212	308	29	43
0.1	49	71	195	167	242	280	35	80
0.5	30	70	191	148	225	206	41	70
1	43	53	128	107	194	140	28	59
2.5	55	73	124	79	214	167	31	71
5	57	56	137	125	269	206	19	77

1. Pierce co-immunoprecipitation kit protocol (ThermoFisher)

A. Antibody Immobilization

1. Equilibrate the AminoLink Plus Coupling Resin and reagents to room temperature.
2. Prepare 2mL of 1X Coupling Buffer for each co-IP reaction by diluting the 20X Coupling Buffer with ultrapure water.
3. Gently swirl the bottle of AminoLink Plus Coupling Resin to obtain an even suspension. Using a wide-bore or cut pipette tip, add 50µL of the resin slurry into a Pierce Spin Column. Place column into a microcentrifuge tube and centrifuge at 1000 × g for 1 minute. Discard the flow-through.
4. Wash resin twice by adding 200µL of 1X Coupling Buffer, centrifuge and discard the flow-through.
5. Gently tap the bottom of the spin column on a paper towel to remove any excess liquid and insert the bottom plug.
6. Prepare 10-75µg of affinity-purified antibody for coupling by adjusting the volume to 200µL, using sufficient ultrapure water and 20X Coupling Buffer to produce 1X Coupling Buffer. For example add 10µL of 20X Coupling Buffer, 180µL of ultrapure water and 10µL of antibody at 1µg/1µL. Add the ultrapure water, 20X Coupling Buffer and affinity-purified antibody directly to the resin in the spin column.
7. In a fume hood, add 3µL of the Sodium Cyanoborohydride Solution for every 200µL of reaction volume. Note: Sodium cyanoborohydride is highly toxic. Wear gloves and use caution when handling.
8. Attach the screw cap to the column and incubate on a rotator or mixer at room temperature for 90-120 minutes, ensuring that the slurry remains suspended during incubation.
9. Remove and retain the bottom plug and loosen the screw cap. Place the spin column into a collection tube and centrifuge. Save the flow-through to verify antibody coupling.
10. Remove the screw cap, add 200µL of 1X Coupling Buffer, centrifuge and discard the flow-through. Repeat this step once.
11. Add 200µL of Quenching Buffer to the column, centrifuge and discard the flow-through.
12. Tap the bottom of the column on a paper towel to remove excess liquid and insert the bottom plug. Add 200µL of Quenching Buffer to the resin.
13. In a fume hood, add 3µL of Sodium Cyanoborohydride Solution and attach the screw cap. Incubate for 15 minutes with gentle shaking or end-over-end mixing.
14. Remove plug and loosen the screw cap. Place spin column in a collection tube, centrifuge and discard the flow-through.
15. Remove screw cap, wash the resin twice with 200µL of 1X Coupling Buffer, centrifuging after each wash.
16. Wash the resin six times with 150µL of Wash Solution, centrifuging after each wash.

17. Either proceed to cell lysis and co-IP

B. Mammalian Cell Lysis

1. Carefully remove culture medium from cells.
2. Wash the cells once with 1X Modified Dulbecco's PBS.
3. Add ice-cold IP Lysis/Wash Buffer (Table 2) to the cells. Incubate on ice for 5 minutes with periodic mixing.
4. Transfer the lysate to a microcentrifuge tube and centrifuge at $\sim 13,000 \times g$ for 10 minutes to pellet the cell debris.
5. Transfer supernatant to a new tube for protein concentration determination and further analysis.

C. Pre-clear lysate using the Control Agarose Resin

1. For 1mg of lysate, add 80 μ L of the Control Agarose Resin slurry (40 μ L of settled resin) into a spin column.
2. Centrifuge column to remove storage buffer.
3. Add 100 μ L of 1X Coupling Buffer to the column, centrifuge and discard the flow-through.
4. Add 1mg of lysate to the column containing the resin and incubate at 4°C for 30 minutes to 1 hour with gentle end-overend mixing.
5. Centrifuge column at $1000 \times g$ for 1 minute. Discard the column containing the resin and save the flow-through, which will be added to the immobilized antibody for the co-IP.

D. Co-IP

1. Mix the bait and prey proteins (if separate) and prepare appropriate experimental controls.
2. Dilute the bait:prey protein mixture and controls in IP Lysis/Wash Buffer, or other suitable buffer. Recommended total sample volume in the spin column is 100-500 μ L.
3. Wash the resin twice by adding 200 μ L of IP Lysis/Wash Buffer to the spin column containing the antibody-coupled resin, centrifuge and discard the flow-through.
4. Gently tap the bottom of the spin column on a paper towel to remove excess liquid and insert the bottom plug.
5. Add the bait:prey protein mixture and controls to the appropriate resin. Attach cap and incubate with gentle mixing or rocking for 1-2 hours or overnight at 4°C.
6. Remove the bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge the spin columns. Save the flow-through for future analysis.

7. Remove the screw cap, place the column into a new tube, add 200µL of IP Lysis/Wash Buffer and centrifuge.

8. Wash the sample two more times with 200µL IP Lysis/Wash Buffer and centrifuge after each wash.

E. Elution of Co-IP

1. Place the spin column into a new collection tube. Add 10µL of Elution Buffer and centrifuge.

2. Keep the column in the tube and add 50µL of Elution Buffer. Incubate for 5 minutes at room temperature. The column does not need to be closed or mixed.

3. Centrifuge the tube and collect the flow-through. Analyze the flow-through for protein. Perform additional elutions.

2. NucleoSpin® RNA Isolation kit protocol (MACHEREY-NAGEL)

1. Disrupt up to 30 mg of tissue or Up to 5 x 10⁶ eukaryotic cultured cells lysed by addition of Buffer RA1 directly.

2. Add 350 µL Buffer RA1 and 3.5 µL β-mercaptoethanol (β-ME) to the cell pellet or to ground tissue and vortex vigorously.

3. Reduce viscosity and clear the lysate by filtration through NucleoSpin® Filter (violet ring): Place NucleoSpin® Filter in a Collection Tube (2 mL), apply the mixture, and centrifuge for 1 min at 11,000 x g.

4. Discard the NucleoSpin® Filter and add 350 µL ethanol (70 %) to the homogenized lysate and mix by pipetting up and down (5 times). Alternatively, transfer flow through into a new 1.5 mL microcentrifuge tube (not provided), add 350 µL ethanol (70 %), and mix by vortexing (2 x 5 s).

5. For each preparation take one NucleoSpin® RNA Column (light blue ring) placed in a Collection Tube. Pipette lysate up and down 2–3 times and load the lysate to the column. Centrifuge for 30 s at 11,000 x g. Place the column in a new Collection Tube (2 mL).

6. Add 350 µL MDB (Membrane Desalting Buffer) and centrifuge at 11,000 x g for 1 min to dry the membrane.

7. Prepare DNase reaction mixture in a sterile 1.5 mL microcentrifuge tube (not provided): For each isolation, add 10 µL reconstituted rDNase (also see section 3) to 90 µL Reaction Buffer for rDNase. Mix by flicking the tube.

8. Add 200 µL Buffer RAW2 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 x g. Place the column into a new Collection Tube (2 mL).

9. Add 600 µL Buffer RA3 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 x g. Discard flowthrough and place the column back into the Collection Tube.
10. Add 250 µL Buffer RA3 to the NucleoSpin® RNA Column. Centrifuge for 2 min at 11,000 x g to dry the membrane completely. Place the column into a nuclease free Collection Tube.
11. Elute the RNA in 60 µL RNase-free H₂O, (supplied) and centrifuge at 11,000 x g for 1 min.

3. NucleoSpin® Gel and PCR Clean-up kit protocol (MACHEREY-NAGEL)

1. Mix 1 volume of sample with 2 volumes of Buffer NT1 (e.g., mix 100 µL PCR reaction and 200 µL Buffer NT1).
2. Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 µL sample. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.
3. Add 700 µL Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.
4. Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.
5. Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube (not provided). Add 15–30 µL Buffer NE and incubate at room temperature (15–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x g.

4. Invitrogen SuperScript™ III Reverse Transcriptase kit protocol (ThermoFisher)

1. Add the following components to a nuclease-free microcentrifuge tube:
 - 1 µl of oligo(dT)20 (50 µM); or 200–500 ng of oligo(dT)12-18; or
 - 50–250 ng of random primers; or 2 pmol of gene-specific primer
 - 10 pg–5 µg total RNA or 10 pg–500 ng mRNA
 - 1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) Sterile, distilled water to 13 µl
2. Heat mixture to 65°C for 5 minutes and incubate on ice for at least 1 minute
3. Collect the contents of the tube by brief centrifugation and add:

4 µl 5X First-Strand Buffer

1 µl 0.1 M DTT

1 µl RNaseOUT™ Recombinant RNase Inhibitor

1 µl of SuperScript™ III RT (200 units/µl)

4. Mix by pipetting gently up and down. If using random primers, incubate tube at 25°C for 5 minutes.

5. Incubate at 50°C for 30–60 minutes. Increase the reaction temperature to 55°C for gene-specific primer.

6. Inactivate the reaction by heating at 70°C for 15 minutes.