Optimisation of *In Vitro* Methodology for Drug Metabolism Studies to Improve Prediction of Hepatic Drug Clearance

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List of Abbreviations

6GDH	6-gluconate phosphate dehydrogenase
ADP	Adenosine 5'-diphosphate
AFE	Average fold error
ANOVA	Analysis of variance
APS	Adenosine 5’-phosphosulphate
ATP	Adenosine 5'-triphosphate
BDDCS	Biopharmaceutical Drug Disposition Classification System
CL_{app}	$CL_{int,met}$ restricted by both CL_{mem} and CL_{UWL}
$CL_{app,mem}$	$CL_{int,met}$ restricted by CL_{mem}
CL_b	Blood clearance
CL_h	Hepatic clearance
CL_{int}	Intrinsic clearance
$CL_{int,met}$	Intrinsic metabolic clearance
$CL_{int,u}$	Unbound intrinsic clearance
CL_{mem}	Clearance through the plasma membrane
CL_p	Plasma clearance
CL_{UWL}	Clearance through the unstirred water layer
CV	Coefficient of variation
CYP	Cytochrome P450
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s phosphate buffered saline
EBSS	Earl’s balanced salt solution
EGTA	Ethylene glycol tetraacetic acid
ESF	Empirical scaling factor
fm_{UGT}	Fraction metabolised by UGT
fu_b	Fraction of unbound drug in the blood
fu_{heps}	Fraction of unbound drug in hepatocyte incubations
fu_{mic}	Fraction of unbound drug in microsomal incubations
fu_p	Fraction of unbound drug in the plasma
G6PDH	Glucose-6-phosphate dehydrogenase
Gly	Glycine
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HLM	Human liver microsomes
HPLC	High-performance liquid chromatography

ICPc	Cytosolic NADP+-dependent isocitrate dehydrogenase
IVIVE	*In vitro-in vivo* extrapolation
k	Elimination rate constant
K_a	Microsomal binding affinity equilibrium constant
KHB	Krebs-Heinseleit Buffer
K_m	Michaelis constant
$K_{m,u}$	K_m corrected for protein binding
K_p	Hepatocyte/medium concentration ratio
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
L-Cys	L-Cysteine
L-Glu	L-Glutamate
LW	Liver weight
MDCK	Madin-Darby canine kidney
MEPc	Cytosolic NADP+-dependent malic enzymes
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
NA	Nicotinic acid
NAAD	Nicotinic acid adenine dinucleotide
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADK	NAD+ kinases
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Nam	Nicotinamide
NAMN	Nicotinic acid mononucleotide
NAR	Nicotinic acid riboside
NMN	Nicotinamide mononucleotide
NR	Nicotinamide riboside
OCT-2	Human Organic Cation Transporter 2
P	Microsomal protein concentration
PAMPA	Parallel artificial membrane permeability assay
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBSF	Physiologically based scaling factor
Pi	Inorganic phosphate
PPi	Pyrophosphate
PT	Parallel tube
QA	Quinolinic acid
Q_H	Hepatic blood flow
r	Pearson product-moment correlation coefficients
r^2	Coefficient of determination
R^2	Goodness of fit of power function

R_b	Blood/plasma concentration ratio
RLM	Rat liver microsomes
RMSE	Root mean squared error
[S]	Substrate concentration
SD	Standard deviation
SULT	Sulphotransferase
t½	Half-life
Trp	Tryptophan
UDP	Uridine 5’-diphosphate
UDPG	Uridine 5'-diphosphoglucose
UDPGA	Uridine 5'-diphospho-glucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UMP	Uridine 5’-monophosphate
UTP	Uridine 5’-triphosphate
UWL	Unstirred water layer
v	Reaction velocity
V	Incubation volume
vol.	Volume containing either 1 mg microsomal protein or 10^6 cells
V_{max}	Maximum initial velocity of a reaction
V_R	Volume ratio of hepatocytes to medium
V_s	Sample volume
V_t	Total volume
WME	Williams’ medium E
WS	Well-stirred

Abstract

As a critical parameter in pharmacokinetics, prediction of clearance is an integral aspect of drug discovery programmes. Since the liver is the major site of xenobiotic biotransformation, accurate prediction of hepatic clearance (CL_h) is vital. The use of cellular and subcellular *in vitro* systems for this purpose is common practice; however, prediction accuracy tends to be poor. The aim of this thesis was to explore potential contributing factors to the underprediction of *in vivo* clearance, specifically with relation to the *in vitro* methodology of hepatocyte clearance assays, which is largely unstandardised.

Literature data analyses highlighted an overall clearance-dependent trend of underprediction in both human and rat hepatocytes, indicating a fundamental *in vitro* system bias which is independent of species. During initial investigation of incubation conditions, the format of hepatocyte assays (suspension in microcentrifuge tubes, 96-well plates, 24-well plates and short-term monolayer culture) was demonstrated to influence determined intrinsic clearance (CL_{int}). Differences in midazolam CL_{int} were observed not only between suspended and short-term cultured hepatocytes, but also between suspended hepatocytes in different vessels/plate formats. The applicability of 1 µM as a generic substrate incubation concentration for determination of CL_{int} by substrate depletion was evaluated in rat hepatocytes using nine well-characterised drugs. For seven of the nine drugs, a statistically significantly ($p < 0.05$) higher CL_{int} was observed in determinations of 0.1 µM substrate as opposed to 1 µM, highlighting the potential for false determinations using current practices.

Cofactor depletion in isolated hepatocytes was investigated based on previous speculation as the cause of clearance-dependent underprediction of *in vivo* clearance. Although moderate increases in CL_{int} were observed with the addition of NADPH to hepatocyte incubations, this was subsequently attributed to the replenishment of NADPH in membrane-damaged hepatocytes. Retained functionality of metabolic enzymes in cells which would generally be considered non-viable by trypan blue exclusion was indicated in comparisons of unpurified and Percoll-purified cryopreserved hepatocytes. This phenomenon was conclusively demonstrated in incubations of permeabilised hepatocytes supplemented with NADPH, revealing a need for re-evaluation of the use of plasma membrane integrity (trypan blue exclusion) as a measure of viability in metabolic studies. ATP content was considered as a potential alternative measure; however no significant correlations were found between ATP content, trypan blue exclusion and the CL_{int} of nine drugs in associated preparations.

The effect of shaking on CL_{int} in rat hepatocytes was also examined. For 10 out of 12 drugs, CL_{int} determined at 900 rpm was significantly ($p < 0.05$) higher than in static incubations. Three potential mechanisms were hypothesised: plasma membrane damage, increased substrate distribution throughout the bulk medium and reduction in the depth of the unstirred water layer (UWL) surrounding cells. Shaking of saponin-permeabilised hepatocytes (supplemented with NADPH to maintain metabolism) also increased the determined CL_{int} of saquinavir, indicating a rate-limitation other than membrane permeation. However, shaking of ultra-sonicated hepatocytes in which the plasma membrane was entirely destroyed (also supplemented with NADPH) did not change the determined CL_{int} of saquinavir, revealing the rate-limitation of UWL permeation in both intact and permeabilised cells. The depth of such an UWL *in vitro* is likely to be artificially greater than *in vivo*; therefore reduction of UWL depth through incubation shaking is proposed as a physiologically sound approach by which to increase *in vitro* CL_{int}. In addition, a framework of experiments and related equations is presented by which intact and permeabilised hepatocytes in static and shaken conditions may be utilised to identify the rate-determining process and contribution of individual processes to the *in vitro* CL_{int} of a drug.

The effects of substrate concentration and shaking were also evaluated in human hepatocytes. Significant increases in determined CL_{int} of drugs with use of 0.1 µM substrate (as opposed to 1 µM) and shaking at 900 rpm were demonstrated, confirming equivalent potential *in vitro* sources of underprediction between rat and human.

These highly significant findings reveal the existing limitations of *in vitro* assays and potential flaws in current practice in *in vitro* determinations of CL_{int}. Appropriate consideration of the properties of *in vitro* systems, including the presence of the UWL, should lead to improved predictions of *in vivo* clearance.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

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The Author

The author graduated from The University of Leeds with a First Class Honours B.Sc. in Biological Sciences with Industrial Placement. As part of this degree, the author spent one year working as an industrial placement student in the Drug Metabolism and Pharmacokinetics group at (OSI) Prosidion (Oxford, UK). During this time, the author undertook a project focussed on the design, development and automation of cytochrome P450 inhibition assays. Upon completion of her degree in 2011, the author was awarded the Donald Nicholson Prize for best overall mark in the B.Sc. Biological Sciences programme.

In September 2012, the author joined the Centre for Applied Pharmacokinetic Research at The University of Manchester to begin a Ph.D. under the supervision of Prof. J. Brian Houston and Dr David Hallifax.

Chapter 1. Introduction

Creation of an innovative drug, effective at targeting an unmet clinical need whilst retaining a near impeccable safety profile, is proving increasingly difficult in today's pharmaceutical industry. The challenge of developing treatments of more complex diseases is exaggerated by rising costs and tightening new chemical entity approval regulations. Prediction of human pharmacokinetics is a vital aspect of all drug discovery and development programmes. Inaccuracies in such predictions, specifically with relation to small molecules, can result in false representations of the pharmacokinetic profile, routes and rates of elimination and drug-drug interaction potentials. Consequently, incorrect decisions concerning a compound's progression or attrition, or even dosing strategies should a drug reach first in human trials, could be made (Fagerholm, 2007).

Pharmacokinetic parameters to be assessed during the development of a drug include volume of distribution, bioavailability, plasma half-life, route of elimination and clearance (hepatic and/or renal), as well as potential drug-drug interactions associated with both enzymes and transporters. Amongst such parameters, hepatic clearance (CL_h), defined as the volume of blood or plasma cleared of drug by the liver over a specified time (Pang and Rowland, 1977), is an extremely important consideration. CL_h can substantially influence the concentration of a drug in the blood and consequently its bioavailability and pharmacodynamics (Iwatsubo et al., 1997a; Gomez-Lechon et al., 2007). Adequate metabolic stability is a key requirement of a successful drug candidate and therefore hepatic metabolism screening programmes are an integral part of the drug discovery process. Additionally, hepatic metabolism may play a significant role in the toxicity of a drug, for example via metabolism to a toxic metabolite, drug-stimulated inhibition or induction of metabolic enzymes affecting both self- and other xenobiotic metabolism, or through metabolic enzyme polymorphisms which result in significant interindividual variability and therefore require dosage adjustments (Hewitt et al., 2007).

Currently, prediction of human CL_h is largely informed by human-derived *in vitro* data. However, before the widespread availability of human tissue, predictions of clearance in humans were based on direct allometric scaling of *in vivo* clearance in animals. Allometric scaling utilises the relationship between the size and functional capacities of organs relative to species body weight (Mordenti, 1986; Lave et al., 1999) and is based upon the shared fundamental anatomy, physiology and biochemical processes between humans and the animal models used (Mordenti, 1986; Lin, 1998). Early successes in the prediction of human renal clearance using the allometric approach (Swabb and Bonner, 1983; Mordenti, 1985; Mordenti, 1986; Lin, 1995) however, were not translated to metabolic clearance (Lave et al., 1997; Zuegge et al., 2001; Naritomi et al., 2003; Ito and Houston, 2005). Metabolic clearance is fundamentally influenced by enzyme activities and biochemical parameters; as these properties and pathways vary substantially between

species, allometric scaling is usually unsuccessful (Lave et al., 1997; Obach et al., 1997; Lin, 1998; Lave et al., 1999; Ito and Houston, 2005). This is particularly apparent for compounds with a low or intermediate hepatic extraction ratio, where clearance is not limited by hepatic blood flow (Lin, 1995; Lave et al., 1997). The poor performance of pure allometric approaches (Lave et al., 1997; Obach et al., 1997; Lin, 1998; Lave et al., 1999; Ito and Houston, 2005) and improved predictions of human clearance with incorporation of human *in vitro* data (Lave et al., 1999) shifted the focus towards *in vitro-in vivo* extrapolation (IVIVE) strategies. Indeed, the large reduction in late-stage compound attrition previously attributed to poor pharmacokinetics (Kola and Landis, 2004; Arrowsmith, 2011; Cook et al., 2014) is at least in part accredited to the increased availability of human tissue for *in vitro* studies (Chiba et al., 2009).

1.1 *In vitro* characterisation of clearance

The metabolic activity of animal hepatocytes has been investigated for almost half a century (Schenkma et al., 1967; Moldeus et al., 1974; Grudin et al., 1975), but whilst methods to predict *in vivo* CL_h from *in vitro* systems were first discussed in the 1970s (Rane et al., 1977), it was not until 1994 that a significant and comprehensive report of such a strategy was published (Houston, 1994). IVIVE is based upon the premise that the activity of a drug-metabolising enzyme (or transporter) will be the same *in vitro* as *in vivo*. In the case of metabolism, the two circumstances are linked by the parameter intrinsic clearance (CL_{int}), which solely defines the enzymatic metabolism of a drug (Houston, 1994). Theoretically, CL_{int} can be measured *in vitro* using cellular or sub-cellular systems and subsequently scaled to a value representing *in vivo* CL_{int} by accounting for the difference between the amount of enzyme present in the *in vitro* assay and that in a human or animal liver (Houston, 1994) (described in Section 1.2).

In vitro CL_{int} can be measured by one of three approaches: determination of Michaelis-Menten kinetic parameters V_{max} (maximum initial velocity of a reaction) and K_m (Michaelis constant, defined as the substrate concentration at which the reaction rate is half of V_{max}) via measurement of either metabolite formation or substrate depletion over a range of substrate concentrations, or alternatively by monitoring substrate depletion time profiles (Rane et al., 1977; Houston, 1994). Whilst metabolite formation is assumed to provide the most accurate determinations, this method is not always feasible due to the need for radiolabelled compounds or known metabolite standards. The substrate depletion over time approach lacks these constraints and requires the least experimental input, making it especially popular in the drug discovery setting (Obach et al., 1997; Obach and Reed-Hagen, 2002; Jones and Houston, 2004; Jacobson et al., 2007). This method, commonly referred to as the '*in vitro* t½ method', is derived from an integration of the Michaelis-Menten equation (Equation 1.1), which at substrate concentrations ([S]) considerably below the K_m reduces to Equation 1.2, as [S] becomes negligible.

Equation 1.1

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where v is reaction velocity.

Equation 1.2

$$v = \frac{V_{max}}{K_m}$$

Under these conditions (very low substrate concentrations), the *in vitro* half-life (t½) can be determined from the first order rate constant (k) for the removal of parent drug (assumed to be via metabolism) from the system (Equation 1.3) (Obach et al., 1997).

Equation 1.3

$$t½ = \frac{0.693}{k}$$

Equation 1.3 relates to the Michaelis-Menten equation through Equation 1.4.

Equation 1.4

$v = \text{vol.} \cdot k$

where vol. is the volume containing either 1 mg microsomal protein or 10^6 cells.

Rearrangement and substitution of the above equations yields Equation 1.5 (Obach et al., 1997; Edwards et al., 2011).

Equation 1.5

$$\frac{V_{max}}{K_m} = \frac{\text{vol} \cdot 0.693}{t½} = CL_{int}$$

The accuracy of this approach is reliant on use of a substrate concentration of around 10% or less of the K_m, as in this scenario the rate of reaction is proportional to substrate concentration (a first order reaction). With increases in substrate concentration towards and thereafter above the K_m, the metabolic enzyme becomes increasingly saturated with drug and the rate of reaction approaches maximal velocity. Once this point has been reached, any further increases in substrate concentration will not affect the rate of reaction; it has progressed from first order to zero order (Jia and Liu, 2007) (Figure 1-1).

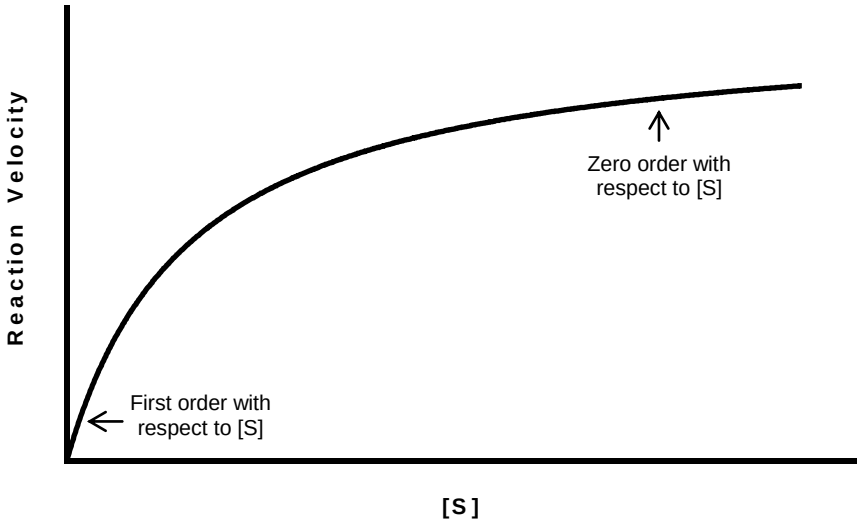


Figure 1-1. Example of Michaelis-Menten enzyme kinetics illustrating first order and zero order reaction phases.

Often however, a certain degree of analytical expediency may occur; when K_m values are unavailable a generic protocol for substrate depletion assays is employed, resulting in potential violation of the stipulation regarding substrate concentration. The consequences of such an oversight, in terms of accurate prediction of CL_{int}, can be substantial. Figure 1-2 provides a theoretical illustration of the change in measured CL_{int} as a percentage of true CL_{int} with changes in substrate concentration relative to the K_m (Klopf and Worboys, 2010).

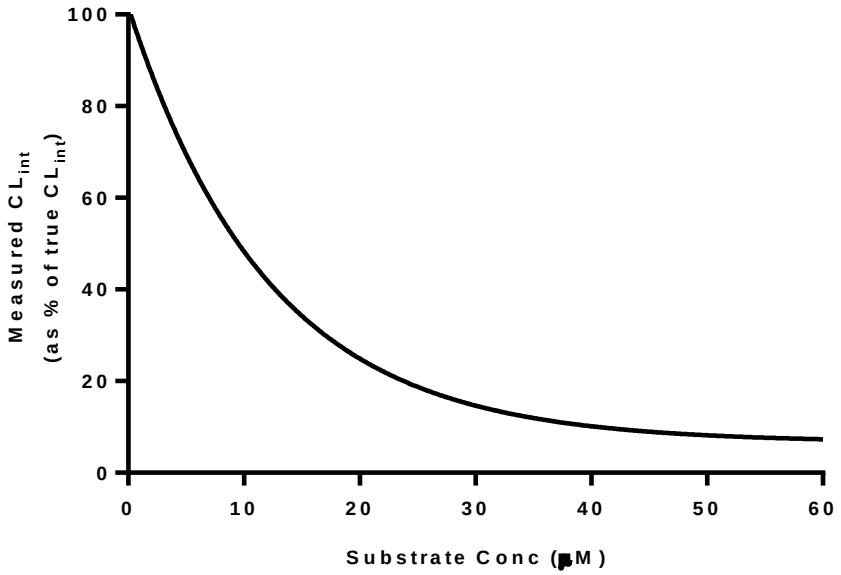


Figure 1-2. Theoretical illustration of the effect of substrate concentration on measured CL_{int} for a substrate with a K_m of 10 µM.

1.2 *In vitro* systems for studying metabolism

The most common *in vitro* systems used for prediction of CL_{int} are liver microsomes and isolated hepatocytes.

1.2.1 Liver microsomes

Microsomes are a subcellular fraction consisting of the endoplasmic reticulum and associated metabolic enzymes including cytochrome P450s (CYPs) and uridine 5'-diphospho-glucuronosyltransferases (UGTs). Simple preparation and preservation procedures (relative to isolated hepatocytes) increased the accessibility of liver microsomes and led to their widespread use for *in vitro* metabolic clearance determinations, especially in high throughput systems. Additionally, the availability of large donor pools of human liver microsomes (HLM) reduces the influence of any single individual's metabolising profile. By their very nature however, microsomes contain only endoplasmic reticulum-bound metabolic enzymes and are devoid of associated cofactors. The addition of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (the cofactor for CYP-mediated reactions) or an NADPH-regenerating system to microsomal incubations is essential to enable these phase I metabolic reactions to proceed (Carlile et al., 1998; Obach, 1999; Jones and Houston, 2004; Lu et al., 2006; Brown et al., 2007b; Sohlenius-Sternbeck et al., 2010). UGT-mediated metabolism can also be evaluated in microsomes (Fisher et al., 2000; Soars et al., 2001; Soars et al., 2002; Cubitt et al., 2009; Kilford et al., 2009; Gertz et al., 2010; Gill et al., 2012), however this requires addition of not only the associated cofactor uridine 5'-diphospho-glucuronic acid (UDPGA), but also a membrane permeabilising agent such as alamethicin, as UGT enzymes are localised on the luminal surfaces of the endoplasmic reticulum and microsomes (Fisher et al., 2000; Soars et al., 2003). In many circumstances, evaluation of phase II metabolism is not performed however (Obach, 1999; Naritomi et al., 2001; Jones and Houston, 2004; Lu et al., 2006; Sohlenius-Sternbeck et al., 2010). The absence of such cofactors not only prohibits assessment of any direct substrate conjugation, but a lack of subsequent metabolism of phase I metabolites can lead to their accumulation, competition with, and interference of metabolism of the parent compound - a phenomenon known as end product inhibition (Jones et al., 2005).

Thus, the application of microsomes is restricted to characterisation of specific metabolic pathways only; metabolism by cytosolic enzymes such as sulphotransferases (SULTs), aldehyde oxidase and carboxylesterases for example cannot be determined, and the contribution of uptake processes (passive or active) to a compound's disposition is also unaccounted for. Considering these limitations, it is largely unlikely that the clearance in microsomal assays will be reflective of the *in vivo* situation. Indeed, underprediction of *in vivo* clearance from both animal and human microsomal studies has been widely reported (Houston, 1994; Houston and Carlile, 1997; Obach, 1999; Boase and Miners, 2002; Soars et al., 2002; Ito and Houston, 2004; Engtrakul et al., 2005; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007b; Kilford et al., 2009; Hallifax et al., 2010).

1.2.2 Hepatocytes

Hepatocytes are viewed as an accessible system closely representative of the liver and are considered the gold standard for determination of hepatic metabolic clearance (Li et al., 1999a; Hewitt et al., 2007). Unlike microsomes, hepatocytes are expected to contain the full complement of hepatic metabolic enzymes and uptake transporter proteins that a drug is exposed to *in vivo* at physiologically relevant quantities and cellular localisations (Li et al., 1999a; Hewitt et al., 2007; Giacomini et al., 2010). With recent advances enabling successful routine cryopreservation of hepatocytes and the preparation of large human donor pools (pools of up to 100 donors at the time of writing, (Tebu-Bio Ltd., Cambridgeshire, UK)), the once unequivocal advantages of microsomes have become much less pertinent.

Early success of *in vivo* CL_{int} prediction was achieved with rat hepatocytes (Houston, 1994; Houston and Carlile, 1997) and consequently hepatocytes appeared to be a promising alternative to liver microsomes (Houston, 1994; Ashforth et al., 1995; Houston and Carlile, 1997; Ito and Houston, 2004). However, whilst human hepatocytes have also consistently been found to outperform microsomes in the prediction of human *in vivo* clearance (Soars et al., 2002; Engtrakul et al., 2005; Riley et al., 2005; Miners et al., 2006; Brown et al., 2007b; Hallifax et al., 2010), such predictions are typically less successful than those previously described in rat. Although some studies have reported good predictions of CL_h from scaled human hepatocyte CL_{int} (Bayliss et al., 1999; Soars et al., 2002; McGinnity et al., 2004), in many instances an underprediction of *in vivo* clearance is observed (Shibata et al., 2002; Hallifax et al., 2005; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007b; Stringer et al., 2008; Hallifax et al., 2010). A large scale literature analysis of hepatocyte data revealed this underprediction to exhibit a trend of clearance-dependence (Hallifax et al., 2010). Possible explanations for this difference between prediction accuracy of rat and human hepatocytes include the more prevalent use of cryopreserved human hepatocytes (discussed in the following sub-section), and the impact of greater interindividual variability in human (discussed in Section 1.4).

1.2.2.1 Metabolic activity of fresh and cryopreserved hepatocytes

Whilst cryopreserved hepatocytes of many species are utilised, preservation of metabolic activity in cryopreserved cells is especially relevant to prediction of human clearance. As the availability of human tissue is sporadic and unpredictable, routine experiments using freshly isolated human hepatocytes is not possible and in the majority of circumstances, cryopreserved preparations are used. There are differing standpoints on whether cryopreserved hepatocytes are able to be used with the same confidence as fresh hepatocytes, each supported by experimental evidence highlighting the resulting differences (or lack of) in the prediction of *in vivo* clearance. Similar *in vitro* CL_{int} values between fresh and cryopreserved hepatocytes have been reported for rat, dog, monkey and human (Diener et al., 1995; Li et al., 1999b; Hewitt et al., 2000; Lau et al., 2002;

Naritomi et al., 2003; McGinnity and Riley, 2004; Blanchard et al., 2005; Floby et al., 2009). However, a number of other studies appear to indicate that whilst cryopreserved hepatocytes (both human and animal) show similar phase I metabolic activity to freshly isolated hepatocytes (Swales and Utesch, 1998; Li et al., 1999b; Steinberg et al., 1999; Jouin et al., 2006; Lu et al., 2006), phase II metabolic activity is not well retained (Powis et al., 1987; Diener et al., 1993; Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Sohlenius-Sternbeck and Schmidt, 2005; Wang et al., 2005). In addition, the activity of the conjugating enzyme glutathione S-transferase (GST), has been shown to be significantly reduced in cryopreserved human hepatocytes compared to freshly isolated (Coundouris et al., 1993; Steinberg et al., 1999; Sohlenius-Sternbeck and Schmidt, 2005).

1.2.2.2 Metabolic activity of suspended and cultured hepatocyte preparations

Typically, hepatocyte suspensions as opposed to cultures are used for metabolic clearance studies as CYP enzyme activity and expression are known to decrease in long-term cultured hepatocytes (Lake and Paine, 1982; Steward et al., 1985; Binda et al., 2003). Consistent with this, previous studies have reported significantly lower CL_{int} of tested compounds in cultured rat hepatocytes compared to suspended hepatocytes (Blanchard et al., 2004; Griffin and Houston, 2005) and different metabolic enzyme activity profiles between the two formats (Utesch et al., 1991; Hewitt and Utesch, 2004). In contrast, two studies in human (Blanchard et al., 2005; Jouin et al., 2006) and another in rat (Lundquist et al., 2014) reported comparable CL_{int} in short-term cultured and suspended hepatocyte preparations.

1.2.3 Alternative *in vitro* systems

Whilst suspended hepatocytes and liver microsomes are the major *in vitro* systems used for determination of hepatic metabolic clearance, alternative systems are occasionally employed. Liver slices were originally thought to be a potentially good model owing to their ease of preparation and preservation of tissue architecture (Houston and Carlile, 1997). However, it was realised that the fundamental tissue structure impedes access of compound to the inner hepatocytes, thereby rate-limiting the metabolic clearance and negatively influencing predictions of *in vivo* CL_{int} (Worboys et al., 1996; Houston and Carlile, 1997). Since then, the use of liver slices for such purposes has been extremely limited, although not altogether abolished (Lake and Price, 2013).

Recombinant enzymes are an alternative *in vitro* system which, due to expression of activity per unit of enzyme, allow increased incorporation of interindividual variability in such enzymatic expression into predictions of human clearance (Iwatsubo et al., 1997b; Galetin et al., 2004; Rostami-Hodjegan and Tucker, 2007; Stringer et al., 2009).

Since the accurate prediction of *in vivo* clearance from traditional *in vitro* systems remains elusive, novel technologies and approaches are continually being explored. An example of such is the immortalised hepatoma cell line, HepaRG. Research has indicated that these

cells demonstrate comparable CYP activity (Lubberstedt et al., 2011) and perform similarly in prediction of *in vivo* clearance to primary human hepatocytes, but likewise display a clearance-dependent trend of underprediction (Zanelli et al., 2012). Use of bioreactors (multi-compartment three-dimensional dynamic culture systems) can be useful in determination of CL_{int} of slowly metabolised compounds, as this system has been shown to facilitate retention of both enzymatic and transporter activities of primary human hepatocytes for up to seven days (Darnell et al., 2012; Ulvestad et al., 2012). In addition, perfused three-dimensional culture systems such as LiverChip™ have been developed which deliver a continuous flow of medium across the scaffold and attached cells(Vivares et al., 2015). In the LiverChip™ system maintenance of metabolic enzyme and transporter gene expression for up to seven days and drug metabolism activity for up to four days has been reported (Vivares et al., 2015). Formation of *in vivo* tissue-like structures, as well as maintenance of viability and liver-specific biochemical properties has even been demonstrated for up to seven weeks in another such perfused culture system (Choi et al., 2014a). Alternative long-term culture systems such as HepatoPac® (hepatocytes co-cultured with fibroblasts) are also under investigation and have shown greater accuracy compared to suspended hepatocytes in characterisation of low clearance compounds (Chan et al., 2013; Ramsden et al., 2014).

1.3 Scaling of *in vitro* clearance

In vitro CL_{int} is scaled to the whole liver equivalent (*in vivo* CL_{int}) by correcting for the difference between the quantity of enzyme present in the *in vitro* assay and that in the respective species' liver. This is achieved through use of physiologically based scaling factors (PBSFs) describing either hepatocellularity (number of hepatocytes per gram of liver) for hepatocyte incubations or microsomal protein content (per gram of liver) for microsomal incubations, together with a measure of liver weight relative to body weight (Houston, 1994). CL_h is derived from *in vivo* CL_{int} using a liver model which incorporates liver blood flow and in most instances blood or plasma protein binding (Houston, 1994). Although mechanistically logical, this method has resulted in consistent underprediction of *in vivo* CL_{int} (Naritomi et al., 2001; Shibata et al., 2002; Hallifax et al., 2005; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007b; Stringer et al., 2008), which may, in part, be a reflection of the imprecision of scaling factor values used (Bayliss et al., 1999; Wilson et al., 2003).

1.3.1 Models of hepatic drug clearance

There are three liver models generally utilised to derive CL_h from *in vivo* CL_{int} (or the reverse); the well-stirred (WS), parallel tube (PT) and dispersion models (Houston and Carlile, 1997). Whilst all represent liver blood flow as the limiting factor of CL_h, the fundamental difference between them lies in the way each models the distribution of a drug within the liver. The WS model suggests that this process is instantaneous and represents

the liver as one well-mixed compartment; the PT model describes a reduction in drug concentration as the blood passes through the liver via the sinusoids with no mixing in the lateral direction; and the dispersion model is an intermediate between the two (Pang and Rowland, 1977; Houston and Carlile, 1997; Liu and Pang, 2006; Soars et al., 2009). Being mathematical models, none are able to describe the hepatic clearance of drugs entirely accurately.

1.3.2 Incorporation of protein binding parameters

Within each liver model, a value for the fraction of unbound drug in the blood (fu_b) is incorporated, the rationale being that according to the 'free drug hypothesis' only unbound drug is believed to be able to cross the plasma membrane and therefore available for metabolism by hepatic enzymes (Lin, 1995). Consequently, to ascertain the true clearance, calculations must be based upon the concentration of free drug in the blood (Pang and Rowland, 1977; Obach, 1999). Correspondingly, *in vitro* CL_{int} should also be corrected for non-specific binding in the incubation, such that the *in vitro* and *in vivo* circumstances are directly comparable through a mutual parameter, unbound CL_{int} ($CL_{int,u}$) (Obach, 1999). A previous proposal to disregard both *in vitro* and *in vivo* binding parameters based on the assumption of equivalence (Obach et al., 1997) was rejected due to demonstrations of improved accuracy of *in vivo* clearance predictions with incorporation of both fu_b and a measure of *in vitro* binding (Obach, 1996; Obach, 1999; Riley et al., 2005; Stringer et al., 2008). Correction for protein binding of both *in vivo* and *in vitro* CL_{int} and other concentration related pharmacokinetic parameters is now considered standard practice.

1.3.3 Empirical scaling

Despite invested efforts in the use of PBSFs for IVIVE, underpredictions of *in vivo* clearance prevail (Shibata et al., 2002; Hallifax et al., 2005; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007b; Stringer et al., 2008; Hallifax et al., 2010). Empirical scaling, which modifies *in vivo* CL_{int} estimates by a factor determined from previous experimental and/or preclinical data has been suggested as a means by which to improve predictions of *in vivo* clearance (Ito and Houston, 2005; Hallifax and Houston, 2009; Hallifax et al., 2010). Such correction is designed to provide adjustment for any systematic difference between the *in vitro* and *in vivo* situations which is unaccounted for. This may include membrane permeability, interindividual variation and the potential effects of cryopreservation on the metabolic capacity of cells (Iwatsubo et al., 1996; Zuegge et al., 2001; Shibata et al., 2002). Empirical scaling has been shown to improve overall prediction of *in vivo* $CL_{int,u}$ (Shibata et al., 2002; Hallifax and Houston, 2009; Foster et al., 2011; Poulin and Haddad, 2013) and outperform other methods of scaling (Zuegge et al., 2001; Ito and Houston, 2005; Sohlenius-Sternbeck et al., 2010), but whilst providing an immediate (partial) resolution, such scaling approaches offer no understanding of the mechanisms underlying the underprediction of *in vivo* clearance (Ito and Houston, 2005). Further investigation is

necessary to identify the limitations of *in vitro* assays and to resolve or mechanistically adjust for such factors.

1.4 Consideration of drug uptake

The topic of hepatic uptake transporters has been of particular interest when examining the underprediction of *in vivo* clearance from hepatocyte assays (Iwatsubo et al., 1997a; Riley et al., 2005; Soars et al., 2007b; Chiba et al., 2009; Giacomini et al., 2010; Chu et al., 2013). In one study, analysis of compounds showing the greatest underprediction by hepatocyte metabolic clearance assays revealed these to be either substrates for hepatic transporters or to have considerable renal excretion (Soars et al., 2007b). Since prediction of transporter-mediated clearance is not the focus of this project, this subject will not be discussed in detail. In general, it is agreed that for a certain subset of drugs (those which are actively transported into hepatocytes, but have a low metabolic and/or biliary clearance), hepatic uptake can be a determining factor of overall CL_h and therefore it should be specifically considered (Shitara et al., 2006; Webborn et al., 2007; Parker and Houston, 2008; Gardiner and Paine, 2011; Menochet et al., 2012a; Menochet et al., 2012b; Zamek-Gliszczynski et al., 2013). However, the majority of compounds included in large dataset analyses of prediction of *in vivo* $CL_{int,u}$ are not thought to be limited by hepatic uptake transport (McGinnity and Riley, 2004; Stringer et al., 2008; Hallifax et al., 2010) and therefore this cannot explain the general trend of underprediction of *in vivo* $CL_{int,u}$ observed.

1.5 Interindividual variability

It is likely that interindividual differences in humans are a considerable source of variation of *in vitro* CL_{int} and this has previously been proposed to (at least partially) account for the underprediction of *in vivo* $CL_{int,u}$ (Iwatsubo et al., 1996; Iwatsubo et al., 1997a; Wilson et al., 2003; Ito and Houston, 2005; Riley et al., 2005; Hallifax and Houston, 2009). Many studies have reported significant variation between the metabolic activities of different donors determined from *in vitro* assays (Mertes et al., 1985; Diener et al., 1994; Shibata et al., 2002; Soars et al., 2002; Naritomi et al., 2003; Rawden et al., 2005; Jouin et al., 2006) and one study even reported a range of hepatocellularities of liver donors (Wilson et al., 2003). Interindividual variation is not only limited to metabolic enzymes, but has also been clearly documented with respect to transporters (Badolo et al., 2011; De Bruyn et al., 2011; Menochet et al., 2012b). Such disparity is thought to arise from the inherent differences in the human population; a result of both genetic and environmental factors. Yet intrinsic variability of enzymatic activity would be expected to manifest as both over- and underpredictions of *in vivo* clearance, but a negative bias of *in vitro* predictions is observed (Chiba et al., 2009). Hallifax and Houston (2009) suggested that a possible reason for the discrepancy between predicted and observed *in vivo* clearance values is donor mismatch, whereby the biochemical characteristics of the tissue(s) used for *in vitro* assays are significantly different to those of the clinical study participants. One possible reason for this

is the source of liver tissue: either resection from patients undergoing an operation to remove part of this organ due to disease, or whole livers which are unsuitable for transplantation. Disease state and medication history are further sources of variability which may impact metabolic activity. Studies which have retrospectively analysed liver tissue donor descriptors including age, gender, ethnicity, disease, prior medication and alcohol or tobacco consumption however, found no significant correlations with hepatocyte yield, viability, attachment rate, CYP-dependent functions (Alexandre et al., 2002a), microsomal and cytosolic epoxide hydrolase activities (Mertes et al., 1985) or aldehyde oxidase activity (Hutzler et al., 2015).

1.5.1 Extrinsic variability

Differential tissue isolation, cryopreservation and thawing procedures have been suggested as sources of extrinsic variability in metabolic and transporter activity of hepatocytes (Coundouris et al., 1993; Iwatsubo et al., 1996; Iwatsubo et al., 1997a; Guillouzo et al., 1999; Ito and Houston, 2005; Terry et al., 2006; Brown et al., 2007b; Badolo et al., 2011; De Bruyn et al., 2011). An extended period between removal of the liver (section) and preparation of hepatocytes (greater than 24 hours), and a prolonged phase of warm ischemia after resection (greater than 30 minutes) have been identified as detrimental to viability and/or yield of hepatocyte preparations (Guillouzo et al., 1999; Li et al., 1999a; Richert et al., 2004). Additionally, aspects of the digestion protocol including duration, collagenase preparation and perfusion flow rate can also affect cell viability and/or function (Richert et al., 2004; Terry et al., 2006). The choice and concentration of cryoprotectant may be crucial in maintaining membrane integrity throughout the freezing and thawing processes (Guillouzo et al., 1999; Lloyd et al., 2003) and has been shown to influence the glutathione (GSH) content in human hepatocytes, as well as the viability (Coundouris et al., 1993). Other aspects of the protocol such as cooling and thawing rates controlled via temperature changes across time are also thought to be important in maintenance of viability (Diener et al., 1993; Guillouzo et al., 1999; Alexandre et al., 2002b).

1.6 *In vitro* methodology

Despite extensive use of hepatocytes for predictions of *in vivo* clearance for over a decade, much of the *in vitro* methodology remains unstandardised. Variations between protocols for metabolic assays are numerous and extensive; viability cut-off limits and purification techniques, incubation vessel, incubation medium and additions (including serum), pre-incubation and incubation time, incubation atmosphere, and the use of shaking are just some of the parameters which vary widely, not only between groups in academia and the pharmaceutical industry, but researchers within these groups (Shibata et al., 2000; Blanchard et al., 2004; Brown et al., 2007b; Soars et al., 2007a; Parker and Houston, 2008; Foster et al., 2011). Such differences can clearly confound comparison of CL_{int} values from

different studies and may influence the overall representation of *in vivo* clearance prediction in a compilation of multiple datasets (Soars et al., 2007b).

1.6.1 Viability of isolated hepatocytes and Percoll purification

Reported viability of isolated hepatocytes is typically a measurement of plasma membrane integrity as determined by trypan blue exclusion. The diazo dye trypan blue is a vital stain (does not kill living cells) which is ordinarily unable to penetrate the plasma membrane of eukaryotes. Cells with a damaged membrane however, do not exclude this dye and therefore are stained blue in colour (Evans and Schulemann, 1914). In many instances, a pre-defined viability threshold is reported whereby hepatocyte preparations below this viability were not used (for example Jones and Houston (2004)). Such cut-offs appear to be implemented with the conception that preparations of lower than this viability will be less representative of the *in vivo* situation, whether in terms of metabolic, uptake or other capacity. However, there is no conclusive evidence to support the implication of reduced activity of remaining viable cells. Furthermore, it has been suggested that plasma membrane integrity is a poor representative of metabolic activity (Cook and Mitchell, 1989; Page et al., 1992; Sandker et al., 1993) and alternative measures such as adenosine 5'-triphosphate (ATP) content (Page et al., 1992) and tetrazolium dye reduction (Schiller et al., 1992) have been recommended. In the instance that viability is below the set threshold, Percoll purification is a technique frequently employed to remove non-viable cells and thereby increase the percentage viability of the suspension. Enrichment of a specific population of hepatocytes however, could lead to altered properties of the resulting preparation. As well as increased percentage viability, increased DNA repair capability (Kreamer et al., 1986) and metabolic activity (Diener et al., 1993; Diener et al., 1994; Hengstler et al., 2000) of Percoll-purified preparations have been reported. Viability cut-off limits, the use of Percoll purification and ATP content as an alternative measure of viability are discussed further in Chapter 4.

1.6.2 Incubation conditions

Incubation variables including volume, vessel, agitation rate, medium composition and solvent can influence the progression of a metabolic reaction *in vitro* and consequently determinations of CL_{int} (Griffin and Houston, 2004; Miners et al., 2006; Soars et al., 2007b). Yet, as noted previously, the specific incubation conditions of suspended hepatocyte metabolic clearance studies vary considerably. Differences in suspended cell assay format are undoubtedly a consequence of technical progression; early experiments were often performed in flasks (McLean, 1978; Koike et al., 1981; Pang et al., 1985); these were subsequently scaled down to microcentrifuge tubes (Griffin and Houston, 2004; Griffin and Houston, 2005; Parker and Houston, 2008) or vials (Soars et al., 2007a) and the requirement for lower volume, higher-throughput formats has led to the use of 24- (Brown et al., 2007b; Foster et al., 2011), 48- (Shibata et al., 2000) and 96-well plates (Jouin et al., 2006; Jacobson et al., 2007). However, studies of equivalence in measured CL_{int} across

such formats have not been published. With high throughput screening, the use of generic hepatocyte incubation protocols rather than those optimised for individual compounds has also increased. 1 µM is a typically utilised substrate concentration in the drug discovery setting, most likely to ensure sufficient quantification of substrate depletion without requirement of high analytical sensitivity (Klopf and Worboys, 2010). However, the use of this concentration is based upon the assumption that 1 µM will be far enough below the K_m of the measured metabolic reactions to enable accurate determination of CL_{int} using the substrate depletion approach (detailed in Section 1.1). It is possible that for some compounds this will not be the case.

1.6.2.1 Incubation shaking

Berry et al. (1991) noted the need to modify shaking conditions relative to the incubation vessel and volume, in order to maintain both even suspension and viability of hepatocytes, however this recommendation does not appear to be widely followed. Shaking of hepatocyte incubations is often not reported or poorly recorded; descriptions such as 'low speed' (Hallifax et al., 2008) and 'gentle' (Sohlenius-Sternbeck et al., 2012) shaking illustrate a lack of perceived influence of this variable. Indeed there is very little literature which examines the effect of shaking on metabolic clearance determinations. This is most likely due to the fact that prior to the investigation of hepatic drug uptake, which lagged behind metabolic studies, there was an assumption of non rate-limiting diffusion of drug into cells/the liver. In a comparison of fresh and cryopreserved hepatocytes, Griffin and Houston (2004) noted that the observed differences between *in vitro* CL_{int} values may be due to the difference in incubation conditions including shaking of fresh, but not of cryopreserved hepatocytes. The authors suggested that agitation may result in even distribution of substrate throughout the medium and therefore enhanced entry into hepatocytes.

In addition, it has been widely reported that the depth of the unstirred water layer (UWL) surrounding cells is reduced by shaking or agitation (Barry and Diamond, 1984; Williams et al., 1990; Avdeef et al., 2004; Ghosh et al., 2014; Shibayama et al., 2015). The existence of an UWL, described as an undefined region adjacent to a biological cellular membrane in which the solvent travels markedly slower than in the bulk solution (Korjamo et al., 2009), is a much-discussed phenomenon (Winne, 1973; Barry and Diamond, 1984; Williams et al., 1990; Naruhashi et al., 2003; Avdeef et al., 2004; Shibayama et al., 2015). The UWL reportedly acts as a diffusional barrier to passage both into and out of a cell, the effects of which are more pronounced for highly permeable compounds (Barry and Diamond, 1984; Naruhashi et al., 2003; Korjamo et al., 2009). Despite consideration of the effect of the UWL on membrane permeability assays (Naruhashi et al., 2003; Avdeef et al., 2004), this research has not been translated to drug metabolism studies. The principles are however, applicable to both scenarios. In the case of hepatocyte metabolic clearance assays, the UWL may significantly influence the permeation of highly permeable compounds into

isolated hepatocytes and therefore it is conceivable that this may become the (unidentified) rate-determining process for some compounds. Indeed, one proposed explanation for the clearance-dependent underprediction of *in vivo* clearance observed in hepatocytes is permeability rate-limitation (Hallifax et al., 2010).

1.6.2.2 Incubation media

Elaut et al. (2005) investigated the effects of incubation media on the viability, phase I and phase II metabolic capacities, intracellular ATP levels and GSH content of freshly isolated rat hepatocytes in suspension. GSH is an important protectant against oxidative stress and its depletion can lead to cellular death (Anderson, 1998; Elaut et al., 2005). Of major importance in hepatocyte incubations is the need to maintain physiological pH. Bicarbonate based buffers such as Williams' medium E (WME) and Krebs-Heinseleit Buffer (KHB) require carbogen (95:5 (v:v) air/CO_2) gassing in order to retain pH buffering capacity. 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) is a zwitterionic buffering agent which may be added to saline or other incubation media, able to effectively buffer such solutions at atmospheric levels of CO_2. Elaut et al. (2005) found HEPES and WME buffers to be superior to KHB in prevention of spontaneous cell death(Elaut et al., 2005). Additionally, only WME (containing both GSH and GSH precursors) was able to prevent loss of GSH from the isolated cells (Elaut et al., 2005). Greater phase II metabolic capacity was observed for hepatocytes suspended in KHB compared to HEPES; this was attributed to the presence of extracellular sulphate and glucose in this medium (Elaut et al., 2005). Hallifax et al. (2008) also noted increased CL_{int} in human hepatocytes with use of KHB as opposed to WME; this was apparent for all pathways of midazolam metabolism, but specific to 10'-hydroxylation of triazolam. The increased metabolic capacity of selected enzymes achieved by incubation in different media may not only alter the rate of metabolism, but consequently the metabolic profile (Elaut et al., 2006). These findings highlight the importance of incubation media selection and indicate a potential deficit of cofactors in isolated hepatocytes. Cofactor depletion has been suggested as a potentially major source of underprediction of *in vivo* clearance from hepatocyte incubations (Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Wang et al., 2005; Hallifax et al., 2010; Foster et al., 2011).

1.7 Cofactor depletion

The rate of many xenobiotic metabolism reactions is determined not only by the activity of the metabolic enzyme, but also by the availability of the required cofactor (Dalhoff, 1996); a deficit of this cofactor could therefore reduce the observed rate of metabolism. Should cofactor depletion occur, it may not be to such an extent as to affect the measured *in vitro* CL_{int} of a low clearance drug, however, supply of cofactor could become rate-limiting for a high clearance drug and therefore significantly impact upon the measured *in vitro* CL_{int}.

This provides a potential explanation for the clearance-dependent trend of underprediction of *in vivo* $CL_{int,u}$ in hepatocytes.

Notably, much of the research on cofactor depletion has focussed on comparison of freshly isolated and cryopreserved hepatocytes, the rationale being that depletion is largely due to metabolic and oxidative stress encountered during cryopreservation and thawing. Indeed, there have been reports of a reduction in both phase I (Powis et al., 1987; Hewitt et al., 2000; Hewitt and Utesch, 2004) and phase II (Powis et al., 1987; Steinberg et al., 1999; Hengstler et al., 2000; Hewitt et al., 2000; Rialland et al., 2000) metabolic activities of cryopreserved hepatocytes relative to those of freshly isolated cells. By comparison of intact hepatocytes and hepatocyte sonicates (or permeabilised hepatocytes) supplemented with cofactors (NADPH, UDPGA, and/or 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the cofactors for CYP, UGT and SULT enzymes respectively), a number of studies have demonstrated that the reduction in metabolic activity in cryopreserved hepatocytes is not due to loss of enzyme capacity and therefore suggest rather, a loss of cofactor (Swales et al., 1996; Swales and Utesch, 1998; Hewitt et al., 2000; Hewitt and Utesch, 2004). In addition, studies which compared the metabolic profile of testosterone in both freshly isolated and cryopreserved hepatocytes found no changes in the relative amounts, but a reduction in the absolute amounts of metabolites produced in cryopreserved cells, implying that the activities of the CYP isozymes responsible for testosterone metabolism are reduced by the same degree (Utesch et al., 1992; Swales and Utesch, 1998; Hewitt et al., 2000). As the CYP content was shown not to be significantly different after cryopreservation (Utesch et al., 1992), a plausible explanation could be a loss of NADPH.

Determining if cofactor depletion occurs, and exploring potential approaches by which such metabolic changes can be reversed, may go some way to improving the prediction of metabolic clearance. Previously described attempts to increase intracellular cofactor levels have included: supplementing hepatocyte incubations with cofactor (Wang et al., 2005; Kuester and Sipes, 2007); incubation of hepatocytes with precursors of cofactors (Schwarz, 1980; Aw and Jones, 1984; Sweeny and Reinke, 1988; Sohlenius-Sternbeck and Schmidt, 2005); and introducing a recovery period in which the cells may regenerate their own cofactors (Swales and Utesch, 1998; Cross and Bayliss, 2000; Ostrowska et al., 2000).

1.7.1 ATP

ATP is known to play a key role within the cell, acting both as an energy currency and a signalling molecule. As depicted in Figure 1-3, synthesis of a number of cofactors involved in xenobiotic metabolism is ATP-dependent. Notably, it was found that when administered to rats, xenobiotics which lower the ATP/adenosine 5'-diphosphate (ADP) ratio in the liver such as ethionine and fructose, caused a decrease in UDPGA, PAPS and GSH concentrations of between 30-50% (Dills and Klaassen, 1986). This was further shown to translate to limited glucuronidation and sulphation of exogenous compounds *in vivo* (Dills and Klaassen, 1986). Should depletion of one or more metabolic cofactors be observed,

the complexity and connectivity between the syntheses of these molecules can make it difficult to distinguish the underlying cause, especially, for example, in terms of ATP/ADP and nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide (NAD+/NADH) ratios which are closely linked (Dills and Klaassen, 1986).

It has been reported that during cold storage of organs, up to 90% of ATP can be lost (Vreugdenhil et al., 1991). However, this does not appear to be translated to isolated hepatocytes; Pang et al. (1996) stated that cryopreserved rat hepatocytes exhibit 'normal' ATP levels, and Ostrowska et al. (2000) reported that cryopreserved human hepatocytes retained 50-72% of the ATP content of freshly isolated hepatocytes. Nevertheless, cells must be able to retain not only ATP subsequent to cryopreservation, but also the ability to replenish this cofactor. Elaut et al. (2005) found that during incubation in buffers lacking glucose, freshly isolated rat hepatocytes were only able to maintain initial ATP levels for approximately 120 minutes and were unable to increase energy stores.

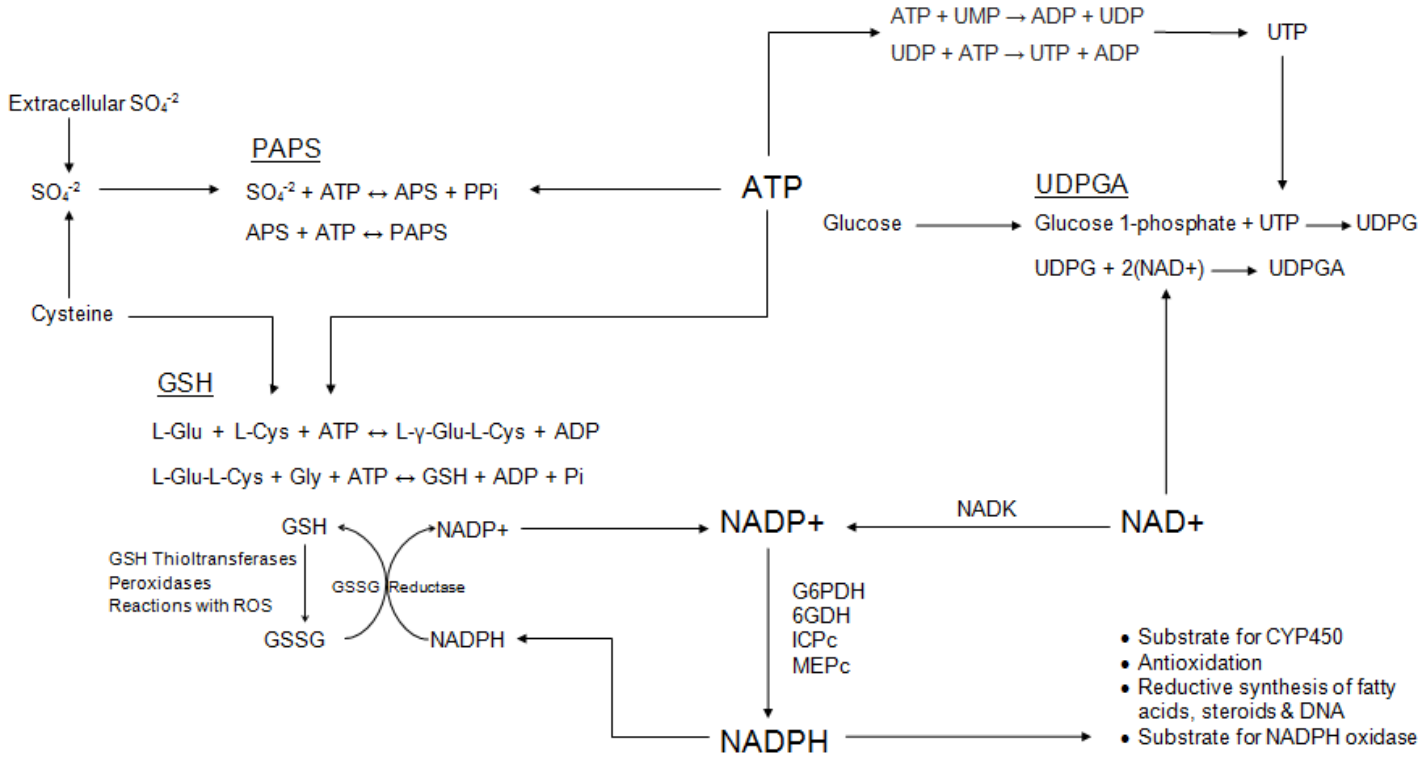


Figure 1-3. Interconnectivity between the syntheses of metabolic cofactors and ATP. Abbreviations: 6GDH, 6-gluconate phosphate dehydrogenase; ADP, adenosine 5'-diphosphate; APS, adenosine 5'-phosphosulphate; ATP, adenosine 5'-triphosphate; G6PDH, glucose 6-phosphate dehydrogenase; Gly, glycine; GSH, glutathione; GSSG, glutathione disulphide; ICPc, cytosolic NADP+-dependent isocitrate dehydrogenase; L-Cys, L-Cysteine; L-Glu, L-Glutamate; MEPc, cytosolic NADP+-dependent malic enzymes; NAD+, nicotinamide adenine dinucleotide; NADK, NAD+ kinases; NADP+, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Pi, inorganic phosphate; PPi, pyrophosphate; UDP, uridine 5'-diphosphate; UDPG, uridine 5'-disphosphoglucose; UDPGA, uridine 5'-disphospho-glucuronic acid; UMP, uridine 5'-monophosphate; UTP, uridine 5'-triphosphate.

1.7.2 NAD+

NAD+ can be synthesised from one of five metabolic precursors, namely nicotinic acid, nicotinamide, nicotinic acid riboside, nicotinamide riboside and tryptophan (Nikiforov et al., 2011) (Figure 1-4). There remains controversy over whether extracellular NAD+ is able to enter cells directly or must first be broken down into one of the afore-mentioned precursors (Pittelli et al., 2011). Nikiforov et al. (2011) stated that extracellular NAD+ must be converted to nicotinamide mononucleotide and subsequently nicotinamide riboside before entering the cell. However, others have described the existence and function of connexin 43 hemichannels which can facilitate NAD+ influx and efflux across the cell membrane of astrocytes, cardiac myocytes and fibroblasts (Bruzzone et al., 2001; Ying et al., 2003; Pillai et al., 2005; Billington et al., 2008; Wang et al., 2013). The presence and/or function of these hemichannels in hepatocytes has not been investigated. Regardless of the mechanism of uptake, incubation of cells with NAD+ appears to be an effective method of increasing intracellular NAD+ concentrations (Ying et al., 2003; Pillai et al., 2005; Billington et al., 2008). Furthermore, Diener et al. (1993) reported that incorporation of an NADPH generating system within the incubation medium of previously cryopreserved rat hepatocytes resulted in increases in benzo[a]pyrene metabolism to levels comparable to fresh cells.

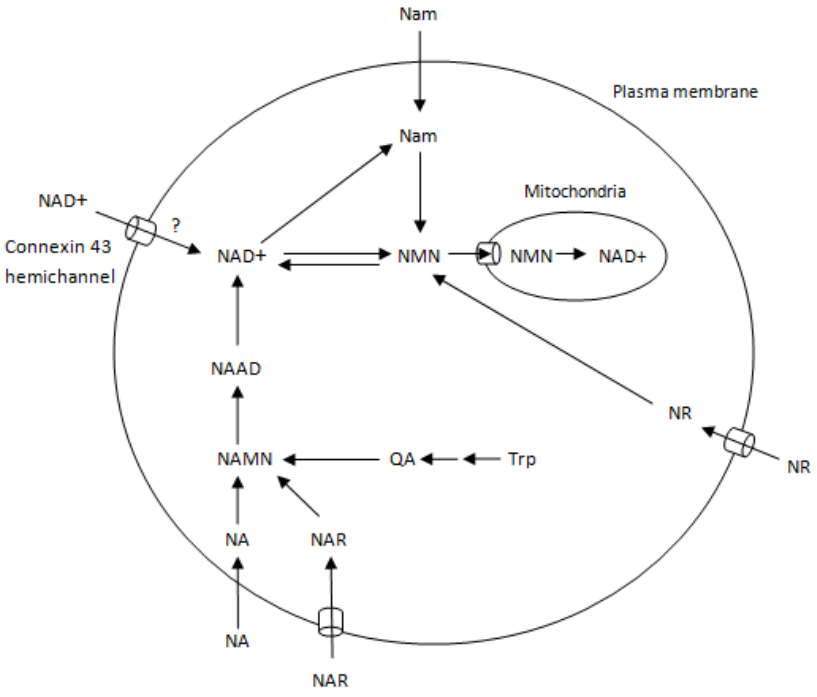


Figure 1-4. Schematic of NAD+ biosynthesis within cells and potential existence of connexin 43 hemichannels facilitating transport of extracellular NAD+ across the plasma membrane. Abbreviations: NA, nicotinic acid; NAAD, nicotinic acid adenine dinucleotide; Nam, nicotinamide; NAMN, nicotinic acid mononucleotide; NAR, nicotinic acid riboside; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; QA, quinolinic acid; Trp, tryptophan. Adapted from Nikiforov et al. (2011).

1.8 Aims

The underprediction of *in vivo* $CL_{int,u}$ from *in vitro* assays using both human liver microsomes and isolated hepatocytes is well-documented (Shibata et al., 2002; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007b; Stringer et al., 2008; Hallifax et al., 2010). Elucidating and subsequently resolving the issues underlying this underprediction is of vital importance to enable accurate assessment of pharmacokinetics in the preclinical setting. The overall aim of this thesis was to explore potential contributing factors to the underprediction of *in vivo* $CL_{int,u}$, specifically with relation to the *in vitro* methodology of hepatocyte assays.

The first aim (described in Chapter 2) was to provide an up-to-date representation of the literature regarding prediction of both human and rat *in vivo* $CL_{int,u}$ from *in vitro* systems, namely suspended hepatocytes and liver microsomes. The accuracy of $CL_{int,u}$ predictions from fresh and cryopreserved human hepatocytes was also compared.

The second aim (described in Chapter 3) was to assess the impact of assay format and initial substrate concentration on determination of *in vitro* CL_{int} in rat hepatocytes. Based on speculations of cofactor depletion as the source of clearance-dependent underprediction of *in vivo* $CL_{int,u}$ (Hallifax et al., 2010; Foster et al., 2011), preliminary investigations into cofactor supplementation of isolated rat hepatocytes were also undertaken.

The third aim (described in Chapter 4) was to evaluate the concept of viability in the consideration of whether use of sub-optimal hepatocyte preparations could be a cause of the underprediction of *in vivo* $CL_{int,u}$. The hypothesis of cofactor depletion was further explored through examination of the effect of Percoll purification and NADPH supplementation on cryopreserved rat hepatocytes. Plasma membrane integrity as a reflection of metabolic capacity was assessed through the use of deliberate gross membrane destruction and plasma membrane permeabilisation by sonication and saponin treatment respectively. ATP content was considered as an alternative measure of viability and the relationship between ATP content, plasma membrane integrity and metabolic activity examined.

The fourth aim (described in Chapter 5) was to investigate the effect of shaking on *in vitro* CL_{int} determinations. The CL_{int} of 12 drugs in rat hepatocytes in static and shaken conditions was compared. Following observations of increased CL_{int} with shaking, experiments designed to elucidate the underlying mechanism including testing notions of plasma membrane damage, increased substrate distribution throughout the bulk medium and reduction in the depth of the UWL were undertaken. In addition, the utility of different shaking conditions and hepatocyte preparations including permeabilised and ultra-sonicated cells in the identification of rate-determining processes of *in vitro* CL_{int} was explored.

The final aim of this project was to determine if the factors found to be limiting *in vitro* CL_{int} in rat hepatocytes similarly affect CL_{int} in human hepatocytes.

Chapter 2. Underprediction of metabolic clearance from *in vitro* systems: a systematic analysis of the literature

2.1 Introduction

The underprediction of *in vivo* clearance (CL_h/CL_{int}) in both human hepatocytes (Shibata et al., 2002; Hallifax et al., 2005; Riley et al., 2005; Brown et al., 2007b; Stringer et al., 2008) and HLM (Ito and Houston, 2005; Riley et al., 2005; Stringer et al., 2008) is widely reported. A literature review by Hallifax et al. (2010) gave the average fold-underprediction of *in vivo* $CL_{int,u}$ in hepatocytes and HLM as 3.9 and 5.2, representing predictions 26% and 19% of observed values respectively. Within the same analysis a trend of increasing underprediction with increasing *in vivo* $CL_{int,u}$ (henceforth referred to as clearance-dependent underprediction) was observed in hepatocytes, but not microsomes.

Much of the previous investigation regarding improvement of *in vivo* clearance predictions has focussed on the IVIVE methodology, including evaluation of different liver models (Ito and Houston, 2004). As many studies have not found a difference in the overall accuracy of the three models in prediction of CL_h from *in vivo* CL_{int} (Houston and Carlile, 1997; Bayliss et al., 1999; Boase and Miners, 2002; Ito and Houston, 2004), the WS model is recommended for simplicity (Houston and Carlile, 1997; Hallifax et al., 2010). Often however, it is CL_{int} rather than CL_h which is compared; as the WS model is more sensitive to CL_h values approaching hepatic blood flow (Q_H) (Pang and Rowland, 1977), use of the PT model has been suggested for high clearance drugs (Ito and Houston, 2004; Houston and Galetin, 2008; Stringer et al., 2008; Kilford et al., 2009). There has also been much discussion concerning the predictive accuracy of cryopreserved as opposed to freshly isolated hepatocytes (Diener et al., 1995; Li et al., 1999b; Hewitt et al., 2000; Lau et al., 2002; Naritomi et al., 2003; McGinnity and Riley, 2004; Blanchard et al., 2005; Floby et al., 2009), in particular the reportedly reduced phase II metabolic activities of cryopreserved hepatocytes (Powis et al., 1987; Diener et al., 1993; Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Sohlenius-Sternbeck and Schmidt, 2005; Wang et al., 2005). Accordingly, the human hepatocyte database compiled in this study will be used for comparison of: the WS and PT liver models, predictive accuracy of freshly isolated and cryopreserved hepatocytes and predictions of CYP and UGT substrates.

Early predictions of *in vivo* clearance in rat hepatocytes were largely successful (Houston, 1994; Houston and Carlile, 1997; Ito and Houston, 2004), however, in later years, underprediction in this system became more widely reported (Naritomi et al., 2003; Blanchard et al., 2004; Huang et al., 2010; Sohlenius-Sternbeck et al., 2012). Comparison of prediction bias of *in vitro* systems across species would be useful in elucidating the underlying mechanism of underprediction of *in vivo* clearance.

2.2 Aims

In order to determine and potentially resolve the underprediction of *in vivo* clearance, it is important to have an accurate appreciation of the performance of *in vitro* systems. The primary aim of this chapter was to provide an up-to-date representation of the literature regarding prediction of human *in vivo* $CL_{int,u}$ from suspended hepatocytes and liver microsomes. As the greatest underpredictions of *in vivo* clearance in hepatocytes are observed for high clearance drugs, reassessment of the WS and PT models was undertaken before selection of a liver model for use throughout this project.

A secondary aim was to investigate the presence of any trends relating to cryopreservation of hepatocytes or glucuronidation of substrates. In contrast to the compilation by Hallifax et al. (2010), in this study CL_{int} data from both freshly isolated and cryopreserved hepatocytes was considered. Following previous evaluations of the applicability of cryopreserved hepatocytes as a substitute for fresh (McGinnity et al., 2004; Hallifax et al., 2008; Floby et al., 2009; Akabane et al., 2012; Hutzler et al., 2014), the accuracy of $CL_{int,u}$ predictions from fresh and cryopreserved hepatocytes was more extensively compared. Additionally, drugs subject to glucuronidation were differentiated from those metabolised via other enzymes (predominantly CYP) to allow comparison of prediction accuracy between metabolic pathways.

A further aim of this chapter was to compile equivalent databases regarding prediction of *in vivo* $CL_{int,u}$ in the same *in vitro* systems in rat. This enabled evaluation of prediction accuracy across species and the *in vivo* data provided comparative information for much of the *in vitro* data generated throughout this project.

2.3 Methods

2.3.1 Data collation

An extensive search of the literature was performed to obtain determinations of *in vitro* CL_{int} and *in vivo* CL_h; both approved pharmaceuticals and investigatory proprietary compounds were considered provided that complementary *in vitro* and *in vivo* data were available. *In vivo* data were only considered if the reported blood clearance (CL_b) or plasma clearance (CL_p) was determined from intravenous dosing and CL_b did not exceed Q_H. *In vitro* CL_{int} data determined from either substrate depletion or metabolite formation over a range of substrate concentrations, or from single concentration substrate depletion time profiles were considered. Hepatocyte data represents that from suspended hepatocytes incubated in the absence of serum (serum has been advocated as an alternative to traditional incubation medium, but the effects on *in vitro* CL_{int} are not fully established (Shibata et al., 2000; Bachmann et al., 2003; Blanchard et al., 2004; Blanchard et al., 2005; Blanchard et al., 2006)). The use of fresh or cryopreserved cells was also recorded. Previously published papers regarding glucuronidation of drugs were used to identify UGT substrates and the

fraction metabolised by UGT (fm_{UGT}). Knowledge of the fm_{UGT} and indeed the fraction(s) metabolised by other enzymes provides a useful indication of the potential for drug-drug interactions. fm_{UGT} estimates were obtained from both *in vitro* and *in vivo* data, the sources for which are given in Appendix Table 8-2. Microsomal data represents that from preparations with added NADPH (phase I metabolism) only.

2.3.2 *In vitro* fraction unbound

In studies where the fraction unbound in either microsomes (fu_{mic}) or hepatocytes (fu_{heps}) was experimentally determined and reported alongside CL_{int}, this value was applied in prediction of *in vivo* $CL_{int,u}$ (Section 2.3.3). Where fu_{mic} or fu_{heps} were unreported, these values were estimated using lipophilicity relationship algorithms proposed by Hallifax and Houston (2006a) (Equation 2.1) and Kilford et al. (2008) (Equation 2.2) respectively.

Equation 2.1

$$fu_{mic} = \frac{1}{1 + P \cdot 10^{0.072 \cdot \log P/D^2 + 0.067 \cdot \log P/D - 1.126}}$$

Equation 2.2

$$fu_{heps} = \frac{1}{1 + 125 \cdot V_R \cdot 10^{0.072 \cdot \log P/D^2 + 0.067 \cdot \log P/D - 1.126}}$$

where P is the microsomal protein concentration, logP/D is the logP value for basic drugs and the logD value for acidic and neutral drugs and V_R is the volume ratio of hepatocytes to medium (0.005 for 1×10^6 cells/ml) (Brown et al., 2007b).

2.3.3 Scaling *in vitro* intrinsic clearance to whole liver

In vitro CL_{int} values were scaled to the *in vivo* whole liver equivalent using Equation 2.3 (Hallifax et al., 2010), where the PBSF is the microsomal average recovery factor for microsomal predictions and hepatocellularity for hepatocyte predictions, and LW is the liver weight/kg bodyweight.

Equation 2.3

$$\text{Predicted } \textit{in vivo}\ CL_{int,u} = \frac{\textit{in vitro}\ CL_{int} \cdot PBSF \cdot LW}{fu_{mic} \text{ or } fu_{heps}}$$

Microsomal recovery factors of 40 mg microsomal protein/g liver (Hakooz et al., 2006; Barter et al., 2007) and 60 mg microsomal protein/g liver (Houston and Carlile, 1997) were used for human and rat respectively; hepatocellularity of 120×10^6 hepatocytes/g liver was used for both human (Hakooz et al., 2006) and rat (Bayliss et al., 1999) and LW was 21.4 g/kg bodyweight for human and 40 g/kg bodyweight for rat (Davies and Morris, 1993).

2.3.4 Determination of *in vivo* intrinsic clearance

In vivo $CL_{int,u}$ was derived from CL_h (blood), fu_b and Q_H with a given value of 20.7 ml/min/kg for human (Davies and Morris, 1993) and 100 ml/min/kg for rat (Ito and Houston, 2004) using the WS (Equation 2.4) and PT (Equation 2.5) liver models. Where applicable and data available, CL_h was determined by subtracting renal clearance from total CL_b. In instances where this correction was unclear, drugs with an *in vivo* clearance greater than Q_H were omitted to remove any ambiguity from extra-hepatic clearance and due to mathematical impossibility. Where required, CL_b and fu_b were calculated from CL_p and fraction unbound in plasma (fu_p) using the blood/plasma concentration ratio (R_b) (CL_p/R_b and fu_p/R_b respectively). In the event an R_b value was unavailable, it was assumed to be equal to 1 for a basic or neutral drug and 0.55 (1 - haematocrit) for an acidic drug (as acidic drugs bind more strongly to plasma proteins and minimally partition into red blood cells (Kulmatycki et al., 2014)). If data from multiple studies were available for the same drug, the arithmetic means of CL_b and fu_b were used in the calculation of $CL_{int,u}$.

Equation 2.4 (**WS liver model)**

$$\textit{In vivo}\ CL_{int,u} = \frac{CL_h}{fu_b \cdot \left(1 - \frac{CL_h}{Q_H}\right)}$$

Equation 2.5 (PT liver model)

$$\textit{In vivo}\ CL_{int,u} = \frac{Q_H}{fu_b} \cdot \mathrm{Ln}\left(1 - \frac{CL_h}{Q_H}\right)$$

2.3.5 Assessment of accuracy and precision of predictions

The overall bias in predictions was assessed by calculation of the average fold error (AFE) (Equation 2.6). Root mean squared error (RMSE) (Equation 2.7) was used as a measure of precision (Hallifax et al., 2010).

Equation 2.6

$$AFE = 10^{\frac{\sum \log \frac{predicted}{observed}}{n}}$$

Equation 2.7

$$RMSE = \sqrt{\frac{1}{n}\sum (predicted\text{-}observed)^2}$$

where n = number of predictions.

The percentage of $CL_{int,u}$ predictions within two-fold of *in vivo* was used as an additional indicator of predictive accuracy consistent with previous publications (Obach, 1999; Naritomi et al., 2001; McGinnity et al., 2004; Stringer et al., 2008; Sohlenius-Sternbeck et al., 2012;

Chan et al., 2013). The percentage of predictions above- and below two-fold of *in vivo* were also calculated as further descriptors of prediction accuracy.

2.3.6 Calculation of empirical scaling factors

The empirical scaling factors (ESFs) required to equate predicted $CL_{int,u}$ with observed $CL_{int,u}$ for individual drugs within each dataset were calculated using Equation 2.8.

Equation 2.8

$$ESF = \frac{\text{observed } CL_{int,u}}{\text{predicted } CL_{int,u}}$$

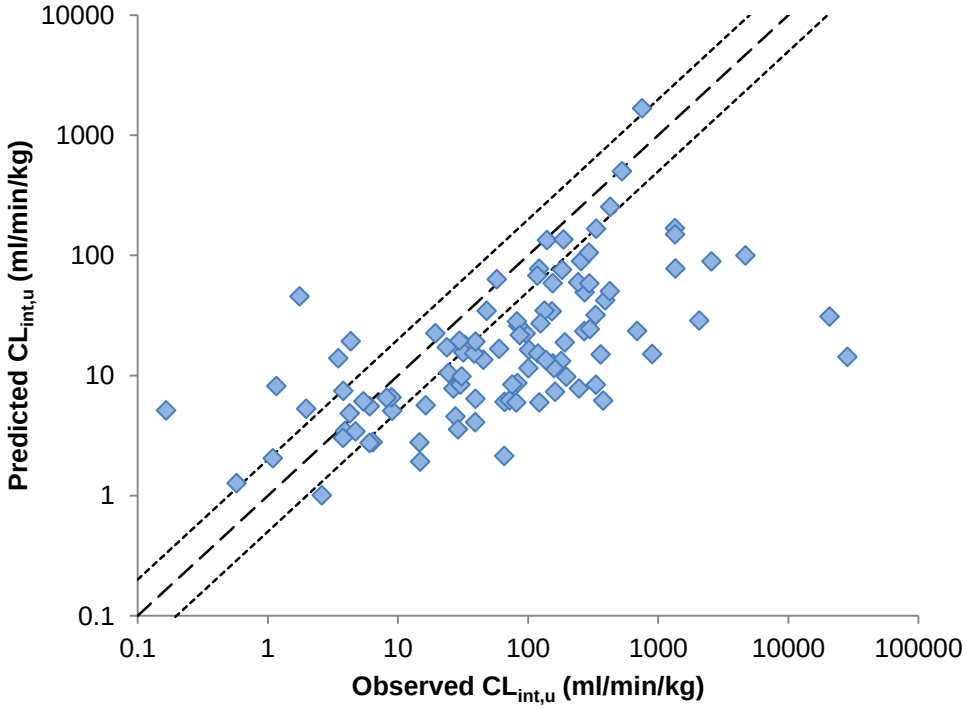
The empirical relationship between ESF and *in vivo* $CL_{int,u}$ was assessed by least squares regression of the power function ESF = a · *in vivo* $CL_{int,u}^{b}$ using Microsoft Excel 2010 (v14.0, Microsoft, Washington, USA). The goodness of fit of the power function is reported as R^2.

2.4 Results

2.4.1 Human data

Using the criteria detailed in Section 2.3, *in vivo* $CL_{int,u}$ predictions were made for 101 drugs from human hepatocyte data (Figure 2-1) and for 73 drugs from HLM data (Figure 2-2); 60 drugs were common to both systems. *In vivo* and *in vitro* clearance data for individual drugs is given in Appendix 8.1. To illustrate the resulting effects on prediction accuracy, comparisons to *in vivo* $CL_{int,u}$ derived using the WS and PT models are represented graphically for both *in vitro* systems (Figures 2-1 and 2-2).

A



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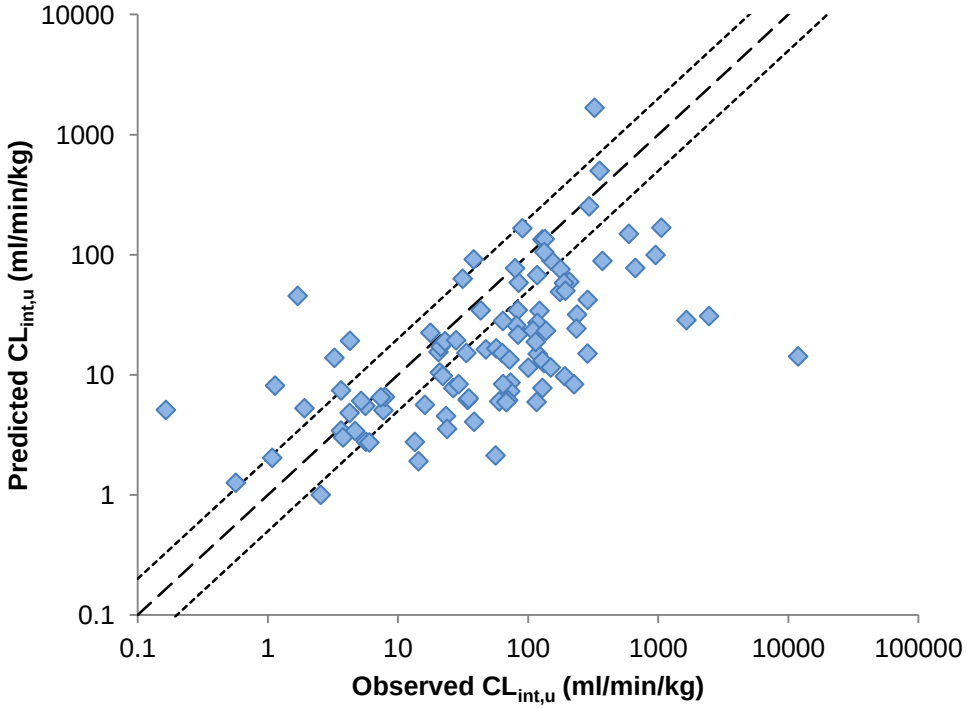
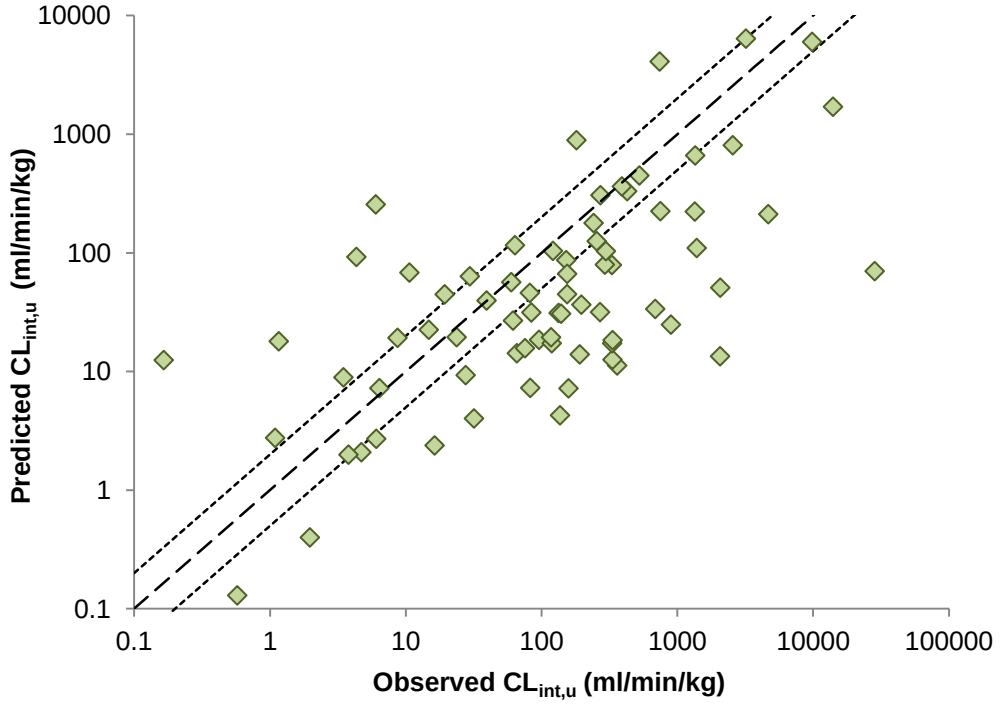


Figure 2-1. Comparison of predicted *in vivo* $CL_{int,u}$ in human hepatocytes with observed *in vivo* $CL_{int,u}$ derived using the (A) WS and (B) PT liver model; n = 101. Dashed lines represent unity and dotted lines a two-fold margin of error.

A



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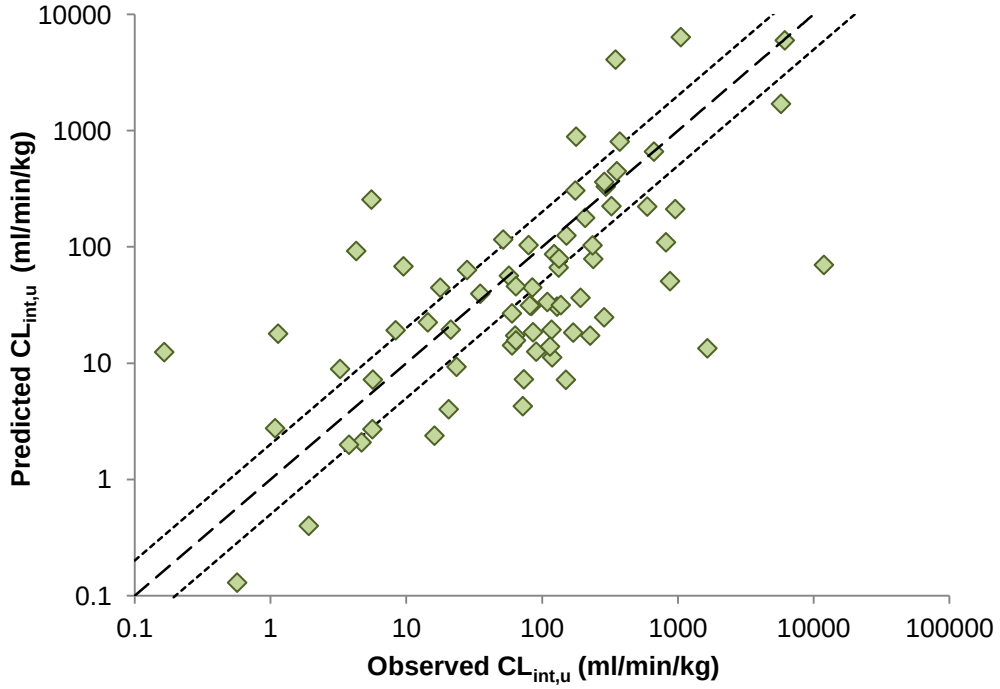


Figure 2-2. Comparison of predicted *in vivo* $CL_{int,u}$ in HLM with observed *in vivo* $CL_{int,u}$ derived using the (A) WS and (B) PT liver model; n = 73. Dashed lines represent unity and dotted lines a two-fold margin of error.

2.4.1.1 Influence of liver model

For both *in vitro* systems, use of the PT model reduced the average fold-underprediction (1/AFE) by approximately one third compared to the WS model (Table 2-1). The RMSE was also lower (indicating greater precision) with use of the PT model by approximately two- and three-fold for microsomes and hepatocytes respectively. Despite small improvements with use of the PT model however, the percentage of predictions within two-fold of observed was 30% or less for both hepatocytes and microsomes. This highlights a considerable need to improve *in vitro* predictions of *in vivo* $CL_{int,u}$ irrespective of liver model. As the differences between bias of the WS and PT models evaluated using two large datasets were not considered to be substantial, consistent with other published studies (Jones and Houston, 2004; Riley et al., 2005; Brown et al., 2007b), the WS model will be used for all subsequent calculations of $CL_{int,u}$ from *in vivo* data.

Table 2-1. Accuracy and precision of *in vivo* $CL_{int,u}$ predictions in human hepatocytes and HLM as represented by AFE, average fold-underprediction, RMSE and percentage of predictions that fall within-, above- and below two-fold of observed *in vivo* $CL_{int,u}$ derived using the WS and PT models, n = number of drugs.

	Hepatocytes n = 101		Microsomes n = 73	
	WS	PT	WS	PT
AFE	0.24	0.35	0.36	0.54
Average fold-underprediction	4.1	2.9	2.8	1.9
RMSE	3549	1234	3752	1670
% predictions within two-fold of observed	24	28	25	30
% predictions above two-fold of observed	8	10	15	19
% predictions below two-fold of observed	68	62	60	51

2.4.1.2 Comparison of *in vitro* systems: hepatocytes and microsomes

Both human hepatocytes and HLM underpredict the *in vivo* $CL_{int,u}$ of the majority of drugs by greater than two-fold (Figures 2-1 and 2-2). Microsomal predictions of *in vivo* $CL_{int,u}$ appear to show less overall bias than those from hepatocytes, with average fold-underpredictions of 2.8 and 4.1 for HLM and human hepatocytes respectively using the WS model (Table 2-1). However, since the percentage of predictions within two-fold of observed and RMSE are very similar between the two systems, the lower average fold-underprediction in HLM is most likely due to the increased percentage of overpredictions (Table 2-1), which to a degree negate the underpredictions in calculation of averages. HLM display a wider range of

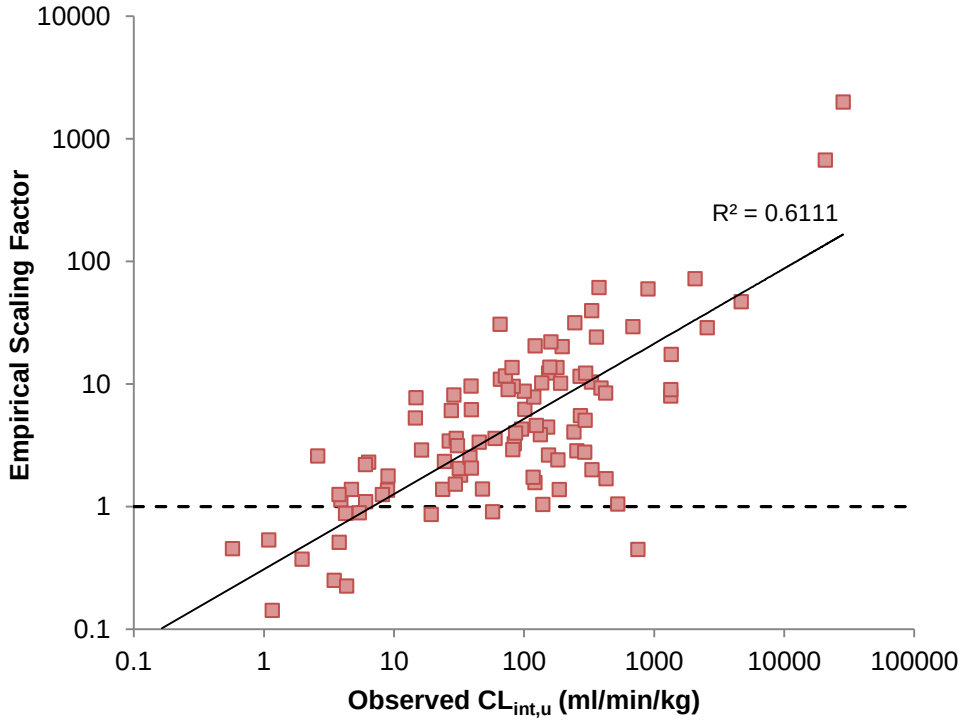
clearance predictions, from approximately 0.1-10,000 ml/min/kg, compared to approximately 1-1000 ml/min/kg for hepatocytes. This threshold of 1000 ml/min/kg appears to indicate the existence of a clearance-limitation in hepatocytes, but not microsomes.

To further evaluate the differences in underprediction of *in vivo* $CL_{int,u}$ between human hepatocytes and microsomes, ESFs for individual drugs were plotted as a function of observed $CL_{int,u}$ (Figure 2-3). A strong trend of increasing ESF with increasing *in vivo* $CL_{int,u}$ was observed for hepatocytes (R^2 = 0.6111) (Figure 2-3A) exemplifying clearance-dependent underprediction. Whilst a similar trend was also observed for microsomes, the correlation was much weaker (R^2 = 0.3245) (Figure 2-3B), indicating a lesser dependence on clearance of underpredictions in this system. Calculation of the average ESF across different levels of *in vivo* $CL_{int,u}$ revealed a similar pattern of underprediction between hepatocytes and microsomes up to 1000 ml/min/kg. Above this value, the ESF for hepatocytes was much larger than that for microsomes (Table 2-2) further supporting a maximal CL_{int} limitation in hepatocytes.

Table 2-2. Average empirical scaling factor (± standard deviation (SD)) for predicted $CL_{int,u}$ across level of observed $CL_{int,u}$ for human hepatocytes and HLM.

Observed $CL_{int,u}$ (ml/min/kg)	Empirical Scaling Factor	
	Hepatocytes	Microsomes
< 10	1.0 ± 0.8	1.5 ± 1.7
10-100	5.6 ± 5.7	3.0 ± 3.0
100-1000	12 ± 15	9.2 ± 11
>1000	355 ± 698	60 ± 123

A



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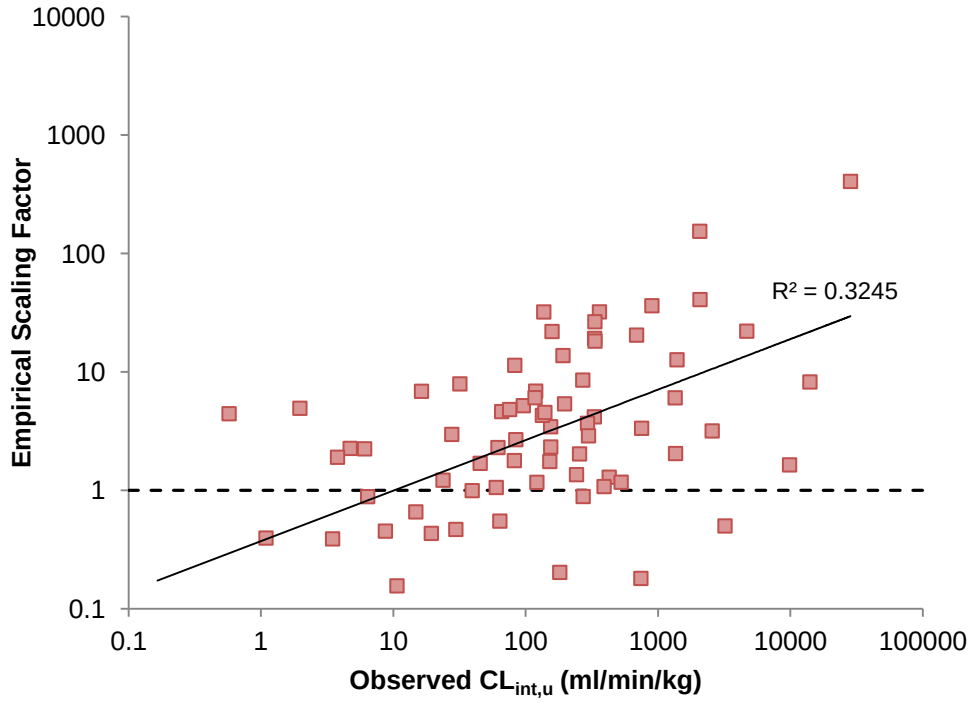


Figure 2-3. Relationship between empirical scaling factor and observed $CL_{int,u}$ for (A) human hepatocytes and (B) HLM. Dashed lines represent exact predictions; solid lines represent fitted power functions.

2.4.1.3 Comparison of predictions from fresh and cryopreserved hepatocytes

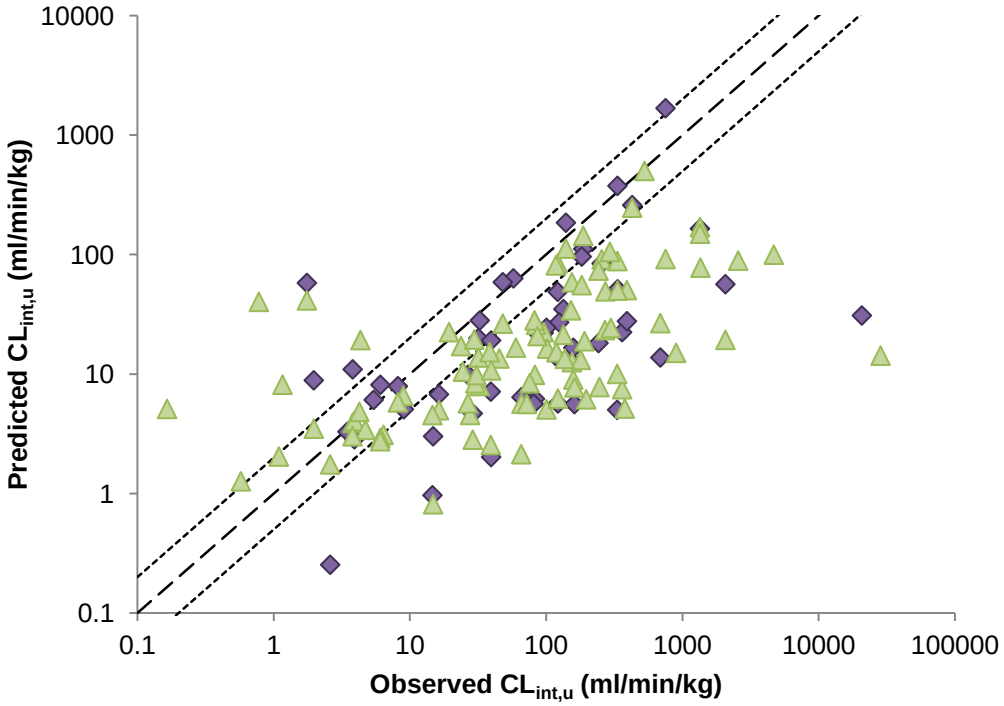
Unlike the most recently published literature compilation of human hepatocyte CL_{int} (Hallifax et al., 2010), this database includes values from both fresh and cryopreserved hepatocytes. Division of the complete dataset into *in vivo* $CL_{int,u}$ predictions from fresh and cryopreserved hepatocytes revealed minimal differences in both prediction bias and precision (Table 2-3, Figure 2-4A). This was further confirmed by analysis of a reduced dataset comprising only of drugs common to both types of preparation (Table 2-3, Figure 2-4B).

Table 2-3. Accuracy and precision of *in vivo* $CL_{int,u}$ predictions in fresh and cryopreserved human hepatocytes as represented by AFE, average fold-underprediction, RMSE and percentage of predictions that fall within-, above- and below two-fold of observed *in vivo* $CL_{int,u}$. Analyses of the complete dataset and of a reduced dataset of common drugs only are presented, n = number of compounds.

	Complete Dataset		Common Drugs[§]	
	Fresh	Cryopreserved	Fresh	Cryopreserved
n	52	92	43	43
AFE	0.26	0.24	0.25	0.22
Average fold-underprediction	3.9	4.2	4.0	4.5
RMSE	2891	3030	390	395
% predictions within two-fold of observed	29	22	26	26
% predictions above two-fold of observed	8	7	7	2
% predictions below two-fold of observed	63	72	67	72

[§] Common drugs refers to drugs which are present in both the fresh and cryopreserved hepatocyte datasets.

A



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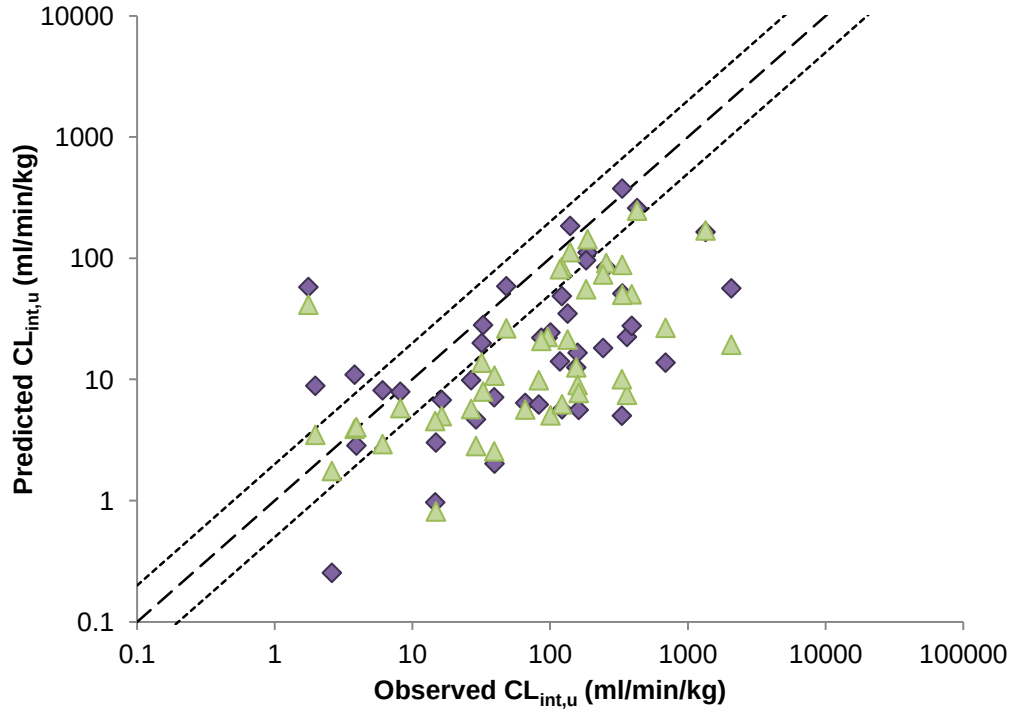


Figure 2-4. Comparison of predicted *in vivo* $CL_{int,u}$ in fresh (◆) and cryopreserved (▲) human hepatocytes with observed *in vivo* $CL_{int,u}$ data for (A) the complete dataset and (B) drugs common to both preparations only. Dashed lines represent unity and dotted lines a two-fold margin of error.

2.4.1.4 Prediction of *in vivo* clearance of UGT substrates

Within the current dataset, drugs subject to glucuronidation were identified and categorised as high (≥ 0.75), medium high (0.50-0.75), medium low (0.25-0.50) or low (<0.25) fm_{UGT} (Figure 2-5). (Values and references for fm_{UGT} for individual drugs are given in Appendix Table 8-2). As a single group, glucuronidated drugs span a similar range of *in vivo* $CL_{int,u}$ and show comparable underprediction to drugs metabolised via other pathways (predominantly CYP). There also appears to be no relationship between fm_{UGT} and underprediction of *in vivo* $CL_{int,u}$ (Figure 2-5).

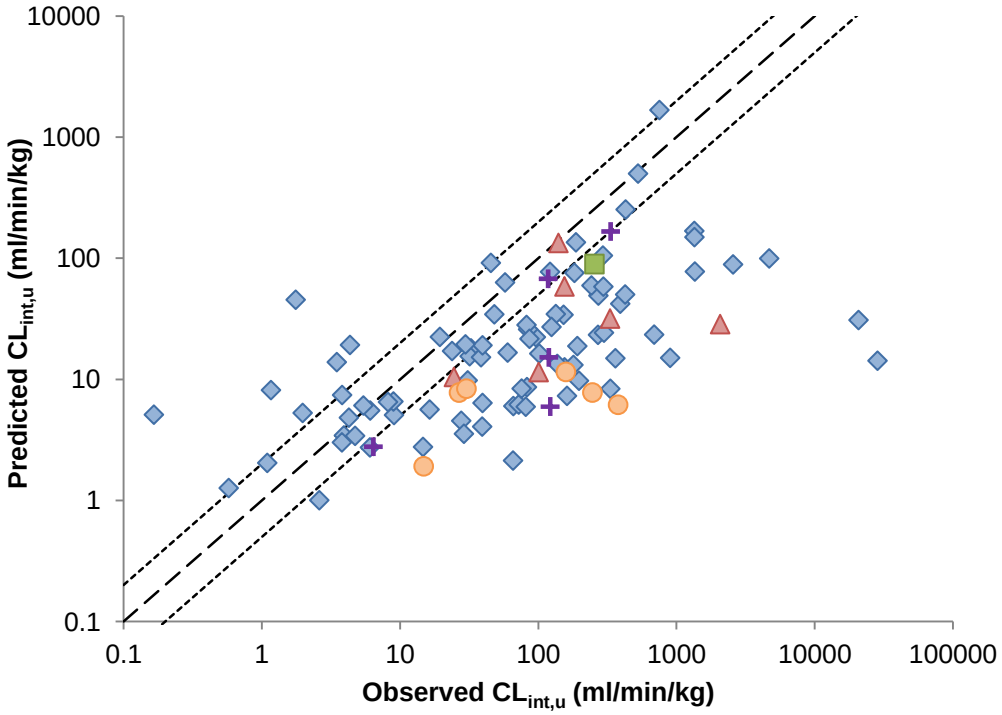


Figure 2-5. Comparison of predicted $CL_{int,u}$ in human hepatocytes with observed $CL_{int,u}$. Drugs subject to glucuronidation are identified as high (≥ 0.75) (●), medium high (0.50-0.75) (+), medium low (0.25-0.50) (▲) and low (<0.25) (■) fm_{UGT}; the remaining drugs are represented as (◆).

2.4.2 Rat data

2.4.2.1 Comparison of *in vitro* systems: hepatocytes and microsomes

In vivo $CL_{int,u}$ predictions for 128 compounds were made from rat hepatocyte data and for 71 compounds from rat microsomal data; 52 compounds were common to both datasets. *In vivo* and *in vitro* clearance data for individual drugs is given in Appendix 8.3. Plots of predicted against observed *in vivo* $CL_{int,u}$ for suspended rat hepatocytes and rat liver microsomes (RLM) are given in Figures 2-6 and 2-7 respectively.

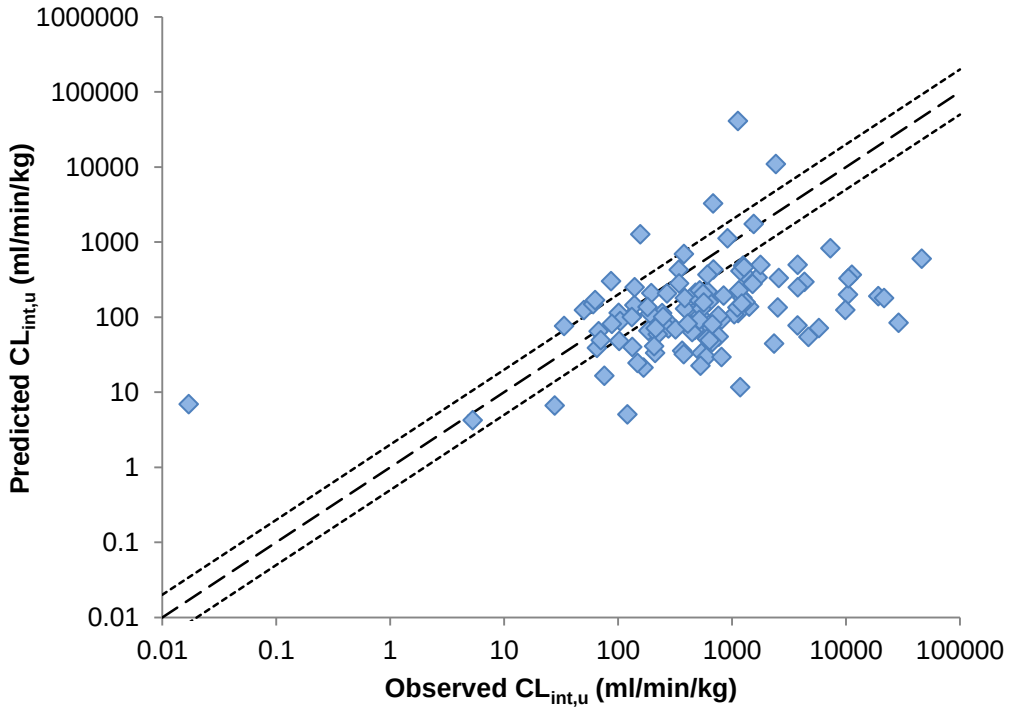


Figure 2-6. Comparison of predicted $CL_{int,u}$ in rat hepatocytes with observed *in vivo* $CL_{int,u}$. Dashed line represents unity and dotted lines a two-fold margin of error.

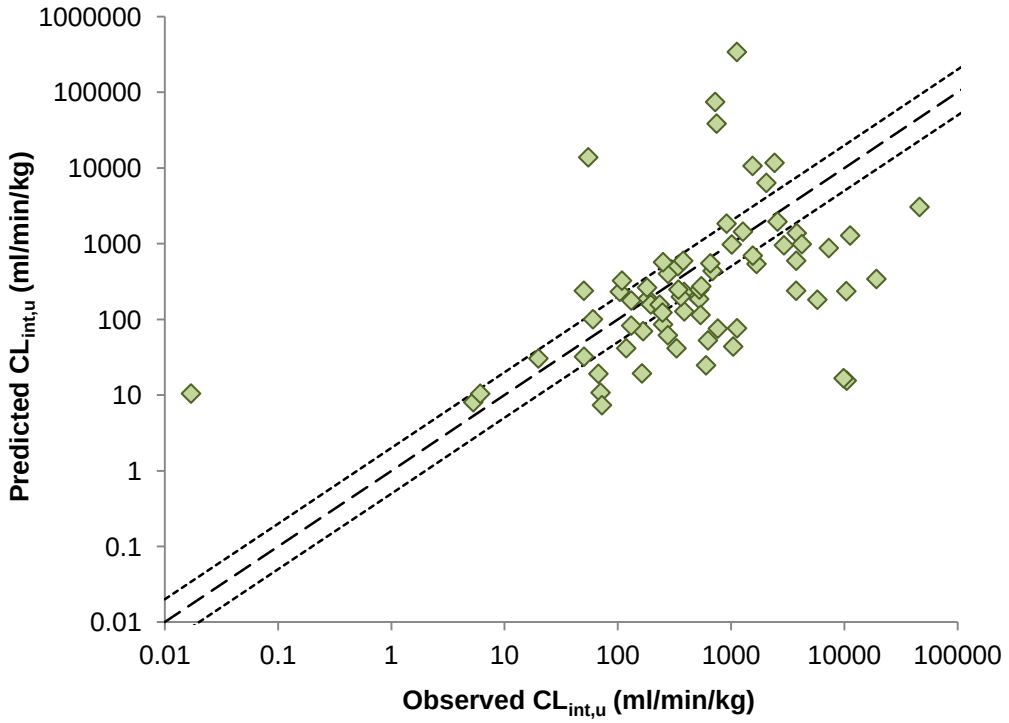


Figure 2-7. Comparison of predicted $CL_{int,u}$ in RLM with observed *in vivo* $CL_{int,u}$. Dashed line represents unity and dotted lines a two-fold margin of error.

As for human, *in vivo* $CL_{int,u}$ was also underpredicted by rat *in vitro* data (Figure 2-6 and Figure 2-7). The average fold-underprediction was 4.7 and 2.3 for hepatocytes and microsomes, representing predictions 21% and 43% of observed values respectively (Table 2-4). Whilst greater precision was observed for predictions in hepatocytes (RMSE = 36203 compared to 63280 in microsomes), an increased percentage of predictions within two-fold of observed was found for microsomes (30% compared to 20% in hepatocytes) (Table 2-4).

Table 2-4. Accuracy and precision of *in vivo* $CL_{int,u}$ predictions in rat hepatocytes and RLM as represented by AFE, average fold-underprediction, RMSE and percentage of predictions within-, above-, and below two-fold of observed *in vivo* $CL_{int,u}$, n = number of compounds.

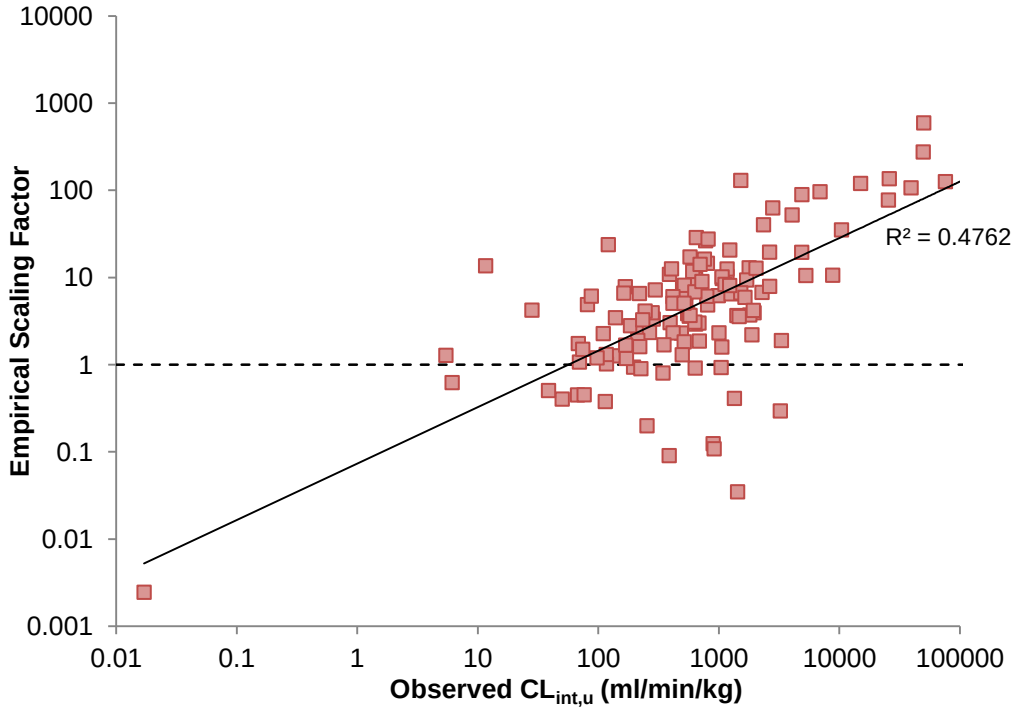
	Hepatocytes	Microsomes
n	128	71
AFE	0.21	0.43
Average fold-underprediction	4.7	2.3
RMSE	36203	63280
% predictions within two-fold of observed	20	30
% predictions above two-fold of observed	9	14
% predictions below two-fold of observed	71	56

Graphs of ESF against observed *in vivo* $CL_{int,u}$ were plotted for rat hepatocytes and RLM (Figures 2-8A and 2-8B respectively). Similarly to human, a stronger trend of clearance-dependent underprediction was observed in hepatocytes (R^2 = 0.4762) than microsomes (R^2 = 0.3653). Also analogous to human, the average ESF for hepatocytes and microsomes was comparable up to 1000 ml/min/kg. However, whilst the average ESF was larger for hepatocytes between 1000-10,000 ml/min/kg, it was greater for RLM above 10,000 ml/min/kg (Table 2-5).

Table 2-5. Average empirical scaling factor (±SD) for predicted $CL_{int,u}$ across level of observed $CL_{int,u}$ for rat hepatocytes and RLM.

Observed $CL_{int,u}$ (ml/min/kg)	Empirical Scaling Factor	
	Hepatocytes	Microsomes
< 10	0.6 ± 0.6	0.4 ± 0.4
10-100	3.0 ± 3.9	3.2 ± 4.1
100-1000	5.9 ± 6.6	3.5 ± 4.8
1000-10000	19 ± 30	7.2 ± 9.6
> 10000	379 ± 611	717 ± 787

A



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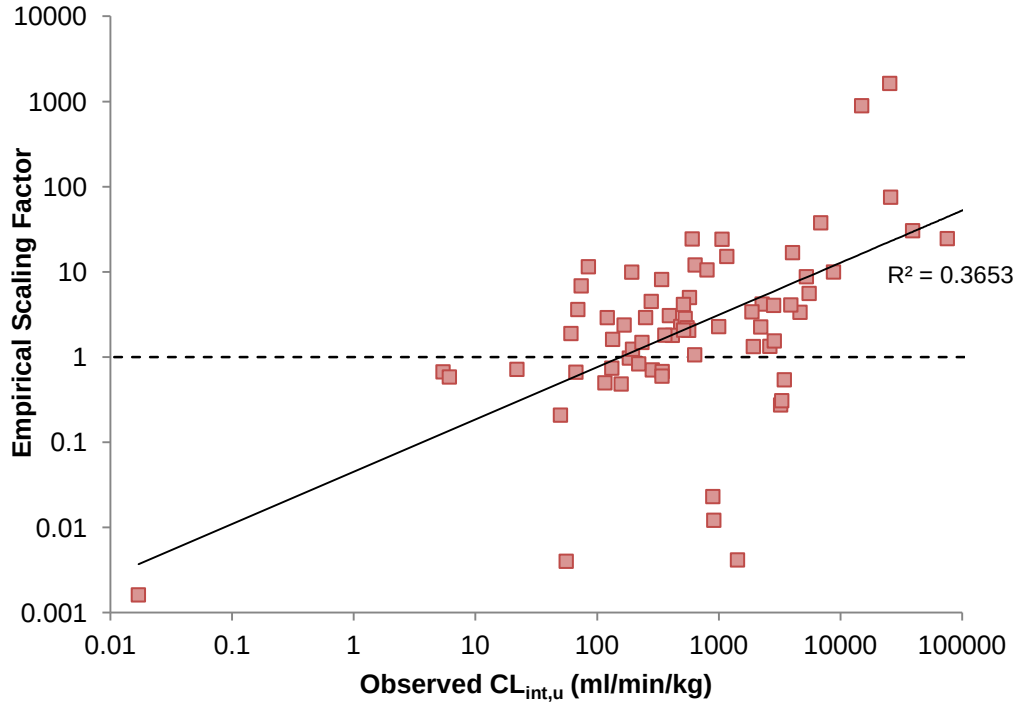


Figure 2-8. Relationship between empirical scaling factor and observed $CL_{int,u}$ for (A) rat hepatocytes and (B) RLM. Dashed lines represent exact predictions; solid lines represent fitted power functions.

2.5 Discussion

This chapter provides a comprehensive analysis of the literature regarding the prediction of *in vivo* clearance from hepatocyte and liver microsomal studies in human and rat.

Comparison of the WS and PT liver models using the human hepatocyte and microsome datasets revealed findings in agreement with Hallifax et al. (2010). Despite minor improvements in prediction bias (as represented by AFE) observed with use of the PT model (Table 2-1), use of the WS model is not considered to be a significant source of underprediction of *in vivo* clearance. The WS model was therefore selected for subsequent derivations of *in vivo* $CL_{int,u}$ from CL_h in this chapter and the remainder of this project.

The extent of underprediction of *in vivo* $CL_{int,u}$ in human has often been attributed to interindividual differences or donor mismatch between *in vitro* sources and clinical patients (Iwatsubo et al., 1996; Iwatsubo et al., 1997a; Wilson et al., 2003; Ito and Houston, 2005; Riley et al., 2005; Hallifax and Houston, 2009). However, predictions of *in vivo* $CL_{int,u}$ in rat exhibit a very similar degree of bias as the respective human systems (Table 2-6). This is unlikely to be representative of interindividual differences, as both genetic variation and environmental influences are considered to be greatly reduced in animals used for such research compared to human. Consequently, an additional source of bias in rat and therefore human is implied.

Table 2-6. Average fold-underprediction of *in vivo* $CL_{int,u}$ in human and rat hepatocytes and microsomes. (Data reproduced from Tables 2-1 and 2-4).

	Hepatocytes	Microsomes
Human	4.1	2.8
Rat	4.7	2.3

Although the degree of underprediction of *in vivo* clearance in RLM is in relative agreement with previous investigations, the large fold-underprediction in rat hepatocytes is in contrast to these studies which reported reasonable accuracy (Houston, 1994; Ito and Houston, 2004). The use of an up-to-date, broader dataset in this study may partially explain the observed discrepancy for hepatocytes. In addition, it should be recognised that the methodology of hepatocyte CL_{int} determination is expected to have changed more in the last 10-20 years than that for microsomal studies. As such, the incorporation of data which is reflective of current practices including high throughput screening, may also contribute to this difference.

The observed underprediction of *in vivo* clearance from microsomal assays in both human and rat is likely to be due to a combination of factors relating to the nature of this subcellular fraction. In this study, only microsomal incubations with added NADPH were considered; drug metabolism by UGTs, as well as cytosolic metabolic enzymes, is consequently unaccounted for, potentially resulting in underprediction of clearance. The absence of the

plasma membrane and associated transporters also influences *in vivo* $CL_{int,u}$ prediction; the clearance of drugs actively transported into hepatocytes may therefore be under- or overpredicted in microsomes depending on the rate-determining process. Substrates of uptake transporters which accumulate within cells are likely to be underpredicted, whereas drugs which are permeability or uptake rate-limited may be overpredicted. The lower average fold-underprediction in microsomes compared to hepatocytes also observed by Stringer et al. (2008), is at least in part due to the higher incidence of overpredictions of *in vivo* $CL_{int,u}$ in microsomes, which to some degree counteracts underprediction in the calculation of AFE and therefore average fold-underprediction.

In comparison, the maintenance of cellular structure of hepatocytes, presence of enzymes and cofactors at physiological concentrations and relevant transporters, lends itself to the assumption that hepatocytes would be better predictors of *in vivo* clearance. The low percentage of *in vivo* $CL_{int,u}$ predictions within two-fold of observed values in both human and rat hepatocytes (24% and 20% respectively) (Tables 2-1 and 2-4) is therefore discouraging. It is apparent from both previous data collation (Foster et al., 2009; Hallifax et al., 2010) and the current analysis that the accuracy of *in vivo* $CL_{int,u}$ predictions in hepatocytes is correlated with *in vivo* $CL_{int,u}$; the degree of underprediction or ESF increases with increasing *in vivo* $CL_{int,u}$. In this study however, in contrast to the findings of Hallifax et al. (2010), a similar trend was also observed for microsomal predictions (Figures 2-3B and 2-8B). In addition, predictions of *in vivo* $CL_{int,u}$ in human hepatocytes appear to be limited to 1000 ml/min/kg (Figure 2-1A), whereas predictions of up to 10,000 ml/min/kg are observed in HLM (Figure 2-2A). A similar scenario is evident in rat in which predictions in hepatocytes appear limited to approximately 10,000 ml/min/kg (Figure 2-6), and in RLM approximately 100,000 ml/min/kg (Figure 2-7). The restriction of maximal CL_{int} in hepatocytes relative to microsomes for both species may be indicative of an artificial *in vitro* limitation, for example loss of enzymatic activity, cofactor depletion or a permeability limitation (Hallifax et al., 2010; Foster et al., 2011). A loss of enzymatic activity during the time between removal of the liver and hepatocyte isolation would seem unlikely as microsomal preparation would also be subject to the same practical restrictions. The dataset in Hallifax et al. (2010) was re-examined to investigate the relationship between underprediction and permeability, however little correlation was found between passive permeability and *in vivo* $CL_{int,u}$ prediction accuracy (Hallifax et al., 2012). Some researchers have suggested cofactor loss or depletion as an explanation for clearance-dependent underprediction of *in vivo* clearance in hepatocytes (Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Wang et al., 2005; Hallifax et al., 2010; Foster et al., 2011). For lower clearance drugs, any cofactor depletion may not be sufficient to affect the *in vitro* CL_{int}, however for higher clearance drugs, supply of cofactor may become rate-limiting and therefore significantly impact upon measured *in vitro* CL_{int}. The hypothesis of cofactor depletion and supporting evidence is discussed in detail in Section 1.7.

Differentiation of *in vivo* $CL_{int,u}$ predictions from fresh and cryopreserved human hepatocytes revealed little difference between prediction accuracy or precision across both the whole dataset and a reduced dataset consisting only of drugs common to both preparations (Table 2-3, Figure 2-4). This substantial comparison is in agreement with previous studies which reported no differences in CL_{int} between freshly isolated and cryopreserved hepatocytes (Diener et al., 1995; Li et al., 1999b; Hewitt et al., 2000; Lau et al., 2002; Naritomi et al., 2003; McGinnity and Riley, 2004; Blanchard et al., 2005; Floby et al., 2009). An equivalent comparison was not performed using rat hepatocyte data as the CL_{int} of only eight drugs was determined in cryopreserved rat hepatocytes. Drugs subject to glucuronidation were distinguished from those metabolised via other pathways in human hepatocytes to identify any trends in underprediction relating to metabolic pathway. This analysis revealed no apparent differences in prediction accuracy between substrates of UGTs and other metabolic enzymes. Additionally, the categorisation of glucuronidated drugs into high, medium-high, medium-low and low fm_{UGT} also revealed no correlation with prediction accuracy (Figure 2-5). Since the rat dataset includes many unidentified proprietary compounds for which little information regarding the metabolic pathways represented is known, an equivalent analysis was not performed for this species.

As the data from hepatocyte and microsomal predictions differed in source for both human and rat, and the absolute inclusion of substrates also differed between datasets, the findings of this study may be considered limited to some extent. However, a study by Foster et al. (2011) which compared CL_{int} in hepatocytes and HLM from the same human donors found a similar trend regarding bias in hepatocyte CL_{int}; microsomal CL_{int} was underpredicted in hepatocytes in a clearance-dependent manner. This provides further support of the need to investigate an apparent CL_{int}-dependent limitation in the hepatocyte system.

2.6 Conclusion

The literature analysis in this chapter confirms the previously reported underprediction of *in vivo* clearance in both human hepatocytes and liver microsomes (Shibata et al., 2002; Hallifax et al., 2005; Riley et al., 2005; Brown et al., 2007b; Stringer et al., 2008) and the applicability of the WS model of hepatic drug clearance (Hallifax et al., 2010). The absence of a significant difference between the prediction accuracy of freshly isolated and cryopreserved human hepatocytes further supports the use of the latter as a suitable alternative in which to study drug metabolism. Predictions of *in vivo* clearance of UGT substrates were indistinguishable from predictions of drugs metabolised by other metabolic enzymes (predominantly CYP), indicating no apparent bias towards this metabolic pathway. Of particular interest, the prediction accuracy of rat hepatocytes was found to be very similar to that of human hepatocytes providing evidence of a fundamental system bias rather than interindividual variability. Freshly isolated rat hepatocytes are therefore indicated as a suitable *in vitro* system in which to study this underprediction; further investigation of both cofactor depletion and permeability rate-limitation will be undertaken in subsequent chapters.

Chapter 3. Optimisation of *in vitro* methods: assay format, cofactor supplementation and substrate concentration

3.1 Introduction

The *in vitro* determination of hepatic clearance processes is based upon the premise that the *in vivo* activity of metabolic enzymes and transporters, as well as basic cellular structure, is retained *in vitro*. Accounting for the difference in the quantity of enzyme or transporter present through scaling should theoretically yield quantitative estimates of *in vivo* clearance. However, accurate prediction of CL_h by IVIVE remains challenging. This project is predominantly focussed on the prediction of metabolic clearance in hepatocytes; the similar fold-underprediction and trend of clearance-dependent underprediction in human and rat appears to indicate an *in vitro* system limitation which is independent of species. Investigation is required to elucidate the underlying source of this underprediction.

As discussed previously (Section 1.6.2), reported conditions for *in vitro* metabolic clearance assays vary extensively and therefore the true effects of many incubation variables are undefined. The advancement of high throughput screening programmes in recent years has resulted in widespread use of multi-well plate formats for such assays. Notably however, a comparison of CL_{int} determinations between these and previous suspension formats does not appear to have been published. Although the use of monolayer cultured hepatocytes for determinations of metabolic clearance is infrequent (most likely because CYP enzymes are known to decrease in long-term cultured hepatocytes (Lake and Paine, 1982; Steward et al., 1985; Binda et al., 2003)), short-term cultured hepatocytes have been used for characterisation of transporter-metabolism interplay (Menochet et al., 2012a; Menochet et al., 2012b). Studies investigating the metabolic activity of short-term cultured hepatocytes have reported both significantly reduced (Blanchard et al., 2004; Griffin and Houston, 2005) and comparable (Blanchard et al., 2005; Jouin et al., 2006; Lundquist et al., 2014) CL_{int} to that in suspended hepatocytes.

As illustrated in Chapter 2, in both human and rat, the maximal predicted $CL_{int,u}$ in hepatocytes appears restricted by approximately 10-fold compared to microsomes (Figures 2-1 and 2-2, 2-6 and 2-7 respectively). Several researchers have suggested that hepatocytes may suffer cofactor loss or depletion (Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Wang et al., 2005; Hallifax et al., 2010; Foster et al., 2011) and that this may be the cause of clearance-dependent underprediction in hepatocytes, which previous to this study, was unobserved in microsomes (Hallifax et al., 2010; Foster et al., 2011). Should cofactor depletion occur, it may not be sufficient to affect the measured CL_{int} of a slowly metabolised drug, but theoretically could restrict metabolism of a high clearance drug.

3.2 Aim

The primary aim of this work was to assess the impact of assay format on CL_{int} determinations. Suspended hepatocytes in microcentrifuge tubes, 24-well plates, 96-well plates and hepatocytes in short-term monolayer culture were evaluated. Midazolam, a well-characterised CYP3A substrate (Ghosal et al., 1996; Shaw et al., 2002) was selected for this purpose.

Based on speculative explanations of clearance-dependent underprediction of *in vivo* $CL_{int,u}$ in hepatocytes, the effect of cofactor supplementation on CL_{int} in isolated hepatocytes was also investigated. Midazolam was used as a probe substrate to assess the effects of NADPH and its precursor nicotinamide on CYP oxidation. Gemfibrozil was chosen as a probe to examine the effects of supplemental UDPGA and glucose on glucuronidation. Unfortunately, it was subsequently discovered that phenol red, a constituent of the incubation medium in which these experiments were performed, inhibits glucuronidation (Driscoll et al., 1982) and therefore these results are not presented.

Subsequent to selection of an assay format, the effect of substrate concentration on CL_{int} determinations using the substrate depletion over time approach was evaluated in freshly isolated rat hepatocytes. CL_{int} determinations were performed at substrate concentrations of 0.1 µM and 1 µM for nine drugs (chlorpromazine, dextromethorphan, diclofenac, metoprolol, midazolam, naloxone, propranolol, triazolam and verapamil) spanning a wide range of *in vivo* $CL_{int,u}$ and metabolic pathways (Table 3-1). The accuracy of *in vivo* $CL_{int,u}$ predictions determined at these concentrations was also assessed. In addition, the incubation concentrations of these drugs in literature studies and reported K_m values were researched.

Table 3-1. Drug metabolising enzymes responsible for metabolism of the selected drugs in rat and human and reported *in vivo* $CL_{int,u}$ in rat. $CL_{int,u}$ values were derived from published CL_h and fu_b data using the WS model; please refer to Appendix 8.3 for references. Blank spaces represent instances where information regarding the enzymatic isoforms responsible for drug metabolism was unavailable. Note: not all drugs were used in each study.

Drug	Human		Rat		*In vivo* $CL_{int,u}$ ml/min/kg
	Metabolic Enzymes		Metabolic Enzymes		
	CYP	UGT	CYP	UGT	
Chlorpromazine	2D6[a], 1A2[a]	1A3[b] 1A4[c]			2264
Dextromethorphan	2D6[d], 2C9[d] 2C19[d], 3A4[d]		2D1[e], 2C11[f], 2C6[f], 3A2[g]		635
Diclofenac	2C9[h]	1A6[i], 1A9[i], 2B7[i], 2B15[i]	2C6[f], 2C11[f], 3A1[f]	2B1[i]	687
Metoprolol	2D6[d,j]		2D1[k], 2D2[rl]		513
Midazolam	3A4[m], 3A5[m]	1A4[n], 2B4[n], 2B7[n]	3A1[f], 3A2[f]		1925
Naloxone		2B7[o]		2B1[p]	253
Propafenone	1A2[q], 2D6[q], 3A4[q]				3227
Propranolol	1A2[d], 2C19[d], 2D6[d],	1A9[r], 2B4[r], 2B7[r]	2D2[l,s], 2D4[s], 2D6[s]		3295
Saquinavir	3A4[t]				911
Tolbutamide	2C8[u], 2C9[d,u], 2C19[d,v]		2C11[w]		6
Triazolam	3A4[m], 3A5[m]		3A1[f], 3A2[f]		1875
Verapamil	1A2[x], 2C8[y], 2C9[y] 3A4[d,x]				1056

[a] Yoshii et al. (2000), [b] Green et al. (1998), [c] Green and Tephly (1998), [d] McGinnity et al. (2000), [e] Kerry et al. (1993), [f] Chovan et al. (2007), [g] Witherow and Houston (1999), [h] Bort et al. (1999), [i] King et al. (2001), [j] Rowland et al. (1994), [k] Barham et al. (1994), [l] Komura and Iwaki (2005), [m] Patki et al. (2003), [n] Zhu et al. (2008), [o] Di Marco et al. (2005), [p] King et al. (1997), [q] Rendic (2002), [r] Sten et al. (2006), [s] Hiroi et al. (2002), [t] Eagling et al. (2002), [u] Relling et al. (1990), [v] Wester et al. (2000), [w] Choi et al. (2014b), [x] Kroemer et al. (1993), [y] Busse et al. (1995).

3.3 Materials and methods

3.3.1 Materials

Midazolam was kindly supplied by F. Hoffman-La Roche (Basel, Switzerland). Collagenase was purchased from Roche Applied Sciences (West Sussex, UK), WME was purchased from Scientific Lab Supplies Ltd. (Nottingham, UK) and 100 µm nylon mesh was purchased from Plastok Associates Ltd. (Merseyside, UK). Dulbecco's phosphate buffered saline (DPBS), Earl's balanced salt solution (EBSS), foetal bovine serum and penicillin-streptomycin solution were from Life Technologies (Renfrewshire, UK). All solvents were obtained from BDH Laboratory Supplies (VWR International (Leicestershire, UK)). All other drugs and reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK).

3.3.2 Animal source, housing and diet

Male Sprague Dawley rats of weight 250-300g were supplied by Charles River Laboratories (Margate, UK). Animals were housed in groups of two to four in individually ventilated cages with aspen chipping and Sizzle-Pet bedding. Accommodation was maintained at 20 ± 3°C with a humidity of 55 ± 10% and a 12 hour light/dark cycle. Animals were given free access to filtered drinking water and standard pelleted rodent diet.

3.3.3 Hepatocyte isolation and preparation

HEPES (final concentration 24 mM) was added to EBSS and WME; 5 M sodium hydroxide was used to adjust the pH to 7.4. Animals were culled with rising CO_2 and death confirmed by cervical dislocation. Hepatocytes were prepared using the collagenase perfusion method described by Griffin and Houston (2004). Briefly, this involves *ex vivo* perfusion of the liver lobes firstly with EBSS containing ethylene glycol tetraacetic acid (EGTA) followed by a wash-out period (EBSS only), and secondly with EBSS containing $CaCl_2$, trypsin inhibitor and collagenase to facilitate digestion of the tissue. Hepatocytes are dissociated by gentle agitation of the perfused liver lobe in ice cold WME, followed by filtration through 100 µm nylon mesh before washing via centrifugation and resuspension in WME. Viability was assessed using the trypan blue exclusion method: 50 µl of cell suspension is diluted into 400 µl of DPBS and 50 µl of 0.4% trypan blue; aliquots are loaded into a haemocytometer and counted under a light microscope to determine cell number and viability. Hepatocytes were diluted to the required concentration in WME accordingly. All cell preparations used were of 80% viability or greater; this was slightly lower than the typically accepted criteria throughout the remainder of this project due to training of the isolation procedure.

3.3.4 Optimisation of cell density and incubation time

Preliminary studies were undertaken to assess appropriate cell density and incubation time; hepatocytes were incubated at 0.25, 0.5 and 1 x 10^6 cells/ml for up to 60 minutes. Drugs were prepared as 10 mM stocks in dimethyl sulfoxide (DMSO) before dilution to 500 µM in

methanol and subsequent dilution to the desired concentration in WME. The final concentrations of DMSO and methanol in the incubation did not exceed 0.025% and 0.5% v/v respectively. Incubations were conducted in 1.5 ml microcentrifuge tubes, in an Eppendorf Thermomixer Compact at a temperature of 37°C and an agitation rate of 900 rpm as described by Griffin and Houston (2005). 125 µl of hepatocytes at twice the desired final cell concentration were pre-incubated for 5 minutes prior to the addition of 125 µl of pre-warmed midazolam solution (5 µM in WME). Individual reactions were terminated at selected time points (0, 5, 10, 20, 40 and 60 minutes) by addition of 250 µl of ice-cold methanol containing internal standard (2 µM diazepam). Details of manufacturers and models for the equipment utilised can be found in Appendix 8.4.

3.3.5 Effect of assay format and exogenous cofactor addition on CL_{int} determinations

Substrate depletion assays were performed in four different formats: suspension in 1.5 ml microcentrifuge tubes, 24-well plates, 96-well plates and short-term monolayer culture. Hepatocytes in suspension were incubated at 0.5 x 10^6 cells/ml; hepatocytes in monolayer culture were seeded at 0.24 x 10^6 cells/well. In all formats, hepatocytes were pre-incubated with either WME (control) or WME containing NADPH for 5 minutes prior to initiation of the reaction by addition of substrate (final concentration 2.5 µM). Final NADPH concentrations were 1 mM and 5 mM. Reactions were terminated by the addition of a volume equal to the incubation volume of ice-cold methanol containing internal standard (final concentration 1 µM) at 0, 1, 2.5, 5, 7.5, 10, 12.5 and 15 minutes. Details of manufacturers and models for the equipment utilised can be found in Appendix 8.4.

3.3.5.1 Microcentrifuge tubes

Incubations were conducted in 1.5 ml microcentrifuge tubes, in an Eppendorf Thermomixer Compact at a temperature of 37°C and an agitation rate of 900 rpm as described by Griffin and Houston (2005). 125 µl of hepatocytes were pre-incubated with 50 µl of WME alone or WME containing NADPH; 75 µl of substrate in WME was added to initiate the reaction.

3.3.5.2 24-well plates

Incubations were conducted in uncoated 24-well polystyrene plates on a Heidolph microtiterplate shaker at 37°C and 900 rpm. 250 µl of hepatocytes were pre-incubated with 100 µl of WME alone or WME containing NADPH; 150 µl of substrate in WME was added to initiate the reaction.

3.3.5.3 96-well plates

Incubations were conducted in round-bottomed 0.5 ml 96-well polypropylene plates on a Heidolph microtiterplate shaker at 37°C and 900 rpm. 60 µl of hepatocytes were pre-incubated with 24 µl of WME alone or WME containing NADPH; 36 µl of substrate in WME was added to initiate the reaction.

3.3.5.4 Short-term monolayer culture

Following determination of viability, freshly isolated rat hepatocytes were re-suspended at the required density in plating medium (WME (pH 7.4) supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and 0.01% insulin) and seeded onto 24-well collagen-coated plates at a density of 0.24 x 10^6 cells/well. Plates were incubated at 37°C in an atmosphere containing 5% CO_2 for two hours to allow cells to adhere to the collagen. Following this, the plating medium was removed and each well was rinsed twice with 0.5 ml of pre-warmed WME. Hepatocytes were pre-incubated for 5 minutes on a Heidolph microtiterplate shaker at 37°C and 300 rpm with 150 µl of pre-warmed WME alone or WME containing NADPH. Reactions were initiated by the addition of 150 µl of substrate in WME and terminated as described above.

3.3.6 Effect of substrate concentration on CL_{int} determinations

Substrate depletion assays were performed in 96-well plates as described in Section 3.3.5.3 according to the optimised incubation conditions given in Table 3-2. Drugs were incubated at 0.1 µM and 1 µM; individual wells provided depletion data for a single time point and eight time points in duplicate were utilised to produce a substrate depletion time profile.

Table 3-2. Incubation conditions of nine drugs used to examine the effect of substrate concentration on CL_{int} determination in substrate depletion assays.

Drug	Hepatocyte Density (10^6 cells/ml)	Incubation Time (min)
Chlorpromazine	0.125	7
Dextromethorphan	0.25	7
Diclofenac	0.5	15
Metoprolol	0.5	15
Midazolam	0.5	15
Naloxone	0.125	7
Propranolol	0.125	7
Triazolam	0.5	15
Verapamil	0.25	7

3.3.7 Sample preparation and liquid chromatography-mass spectrometry analysis

Terminated incubations were stored in a -20°C freezer for at least one hour before centrifugation at 2500 rpm for 10 minutes. Terminated incubate from suspension assays in 24-well plates and monolayer studies was transferred to a 96 deep-well plate prior to centrifugation. 120 µl of supernatant from each sample was removed for analysis by liquid

chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS methods for each drug (and internal standard) are given in Appendix 8.5. An 11 point calibration curve ranging from 0.005 µM to 5 µM including a solvent blank was prepared from stock spiking solutions and used to quantify the substrate remaining in each sample. The composition of calibration standards was tailored to contain the same final concentration of hepatocytes, cofactor and internal standard as the incubation samples. The standards were analysed before the experimental samples and again afterwards to ensure analytical consistency. Samples were quantified based on the standard curve using the software QuanLynx (v4.1, Waters, Watford, UK).

3.3.8 Data analysis

3.3.8.1 Calculation of *in vitro* CL_{int}

In vitro data was fitted to a nonlinear single exponential model using the software GraFit (v7.0.03, Erithacus Software Ltd., Horley, UK) to determine the elimination rate constant (k). Equation 3.1 was used to calculate $CL_{int.}$

Equation 3.1

$$CL_{int} = \frac{V \cdot k}{\text{No. of cells}}$$

where CL_{int} is the intrinsic clearance (µl/min/10^6 cells), V is the incubation volume (µl) and No. of cells is the number of cells in the incubation/10^6.

3.3.8.2 Scaling of *in vitro* CL_{int} to *in vivo* $CL_{int,u}$

For comparison with literature *in vivo* $CL_{int,u}$ data, hepatocyte CL_{int} (µl/min/10^6 cells) was scaled to $CL_{int,u}$ (ml/min/kg) using Equation 2.3. Where possible, fu_{heps} values from published experimental data were used and if necessary corrected for cell concentration using Equation 3.2 (Gertz et al., 2008; Kilford et al., 2008) and Equation 3.3 (rearrangement of Equation 3.2 to obtain K_p). Where experimental data was unavailable, fu_{heps} was calculated using Equation 2.2.

Equation 3.2

$$fu_{heps} = \frac{1}{1 + K_p \cdot V_R}$$

Equation 3.3

$$K_p = \frac{1 - fu_{heps}}{fu_{heps} \cdot V_R}$$

where K_p is the hepatocyte/medium concentration ratio and V_R is the volume ratio of hepatocytes to medium (0.005 for 10^6 cells/ml) (Brown et al., 2007b).

3.3.8.3 Evaluation of the accuracy of $CL_{int,u}$ predictions

Predicted $CL_{int,u}$ (ml/min/kg) determined at 0.1 µM and 1 µM was compared to observed *in vivo* $CL_{int,u}$ (ml/min/kg) derived from CL_h literature data. AFE (Equation 2.6) was used as measure of prediction bias.

3.3.9 Statistical analysis

The arithmetic mean and standard deviation were calculated for the CL_{int} in each condition, and where deemed appropriate a coefficient of variation (CV) was also calculated. One-way analysis of variance (ANOVA) was used to test for a statistically significant difference between midazolam CL_{int} across assay formats (microcentrifuge tubes, 24-well plates, 96-well plates and monolayer). A two-tailed, paired Student's *t*-test was used to determine the existence of a statistically significant difference between two experimental conditions (e.g. presence and absence of NADPH) or substrate concentrations; values were reported as significant when $p < 0.05$.

3.4 Results

3.4.1 Optimisation of cell density and incubation time

Optimisation of cell density and incubation time was performed in microcentrifuge tubes; depletion of 2.5 µM midazolam was assessed at cell densities of 0.25 x 10^6 cells/ml, 0.5 x 10^6 cells/ml and 1 x 10^6 cells/ml over an incubation period of 60 minutes. An example plot of substrate depletion at these cell densities is given in Figure 3-1.

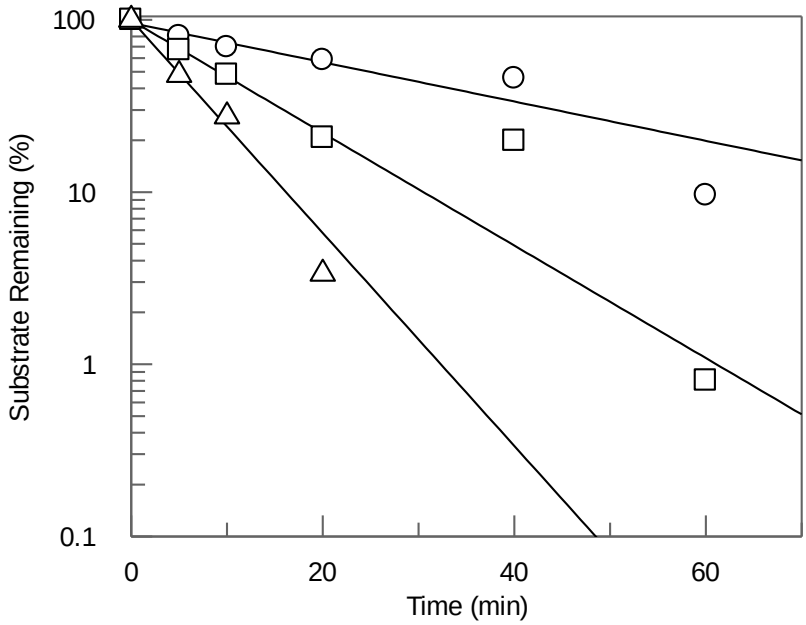


Figure 3-1. Depletion of midazolam in rat hepatocytes of density 0.25 x 10^6 cells/ml (○), 0.5 x 10^6 cells/ml (□) and 1 x 10^6 cells/ml (Δ).

A criterion of at least 50% linear depletion of substrate over a period in which eight time points could be feasibly sampled was used to select the cell density of 0.5 x 10^6 cells/ml and

incubation time of 15 minutes to be used for all further studies with midazolam. The depletion of midazolam in incubations of 1 x 10^6 cells/ml was considered too fast and for 0.25 x 10^6 cells/ml a longer incubation time would have been necessary to attain 50% depletion. A shorter incubation period was desired such that in the instance multiple sequential assays were performed, the period between isolation of cells and use was minimal. The cell densities and incubation times used for the other tested drugs were selected based on published data of reported *in vitro* CL_{int} relative to the *in vitro* CL_{int} of midazolam in these conditions. Linear depletion of substrate throughout the incubation period was verified for all drugs and incubations conditions modified as necessary.

3.4.2 Effect of assay format on CL_{int} determinations

Midazolam substrate depletion assays were conducted using suspended hepatocytes in microcentrifuge tubes, 24-well plates, 96-well plates and short-term monolayer cultured hepatocytes (24-well format). The CL_{int} of midazolam varied over three-fold between different formats; the highest CL_{int} was in microcentrifuge tubes, followed by 96-well plates, 24-well plates and short-term monolayer culture (Table 3-3, Figure 3-2). One-way ANOVA identified a statistical difference between CL_{int} across assay formats ($p < 0.001$). As illustrated by the CVs, the variability of the CL_{int} estimates also noticeably differed between formats, with higher variability observed in microcentrifuge tubes and 96-well plates and lower variability in 24-well plates and short-term monolayer culture (Table 3-3).

Table 3-3. CL_{int} of midazolam in suspended hepatocytes in microcentrifuge tubes, 24-well plates, 96-well plates and in short-term monolayer cultured hepatocytes in the absence and presence of 1 mM and 5 mM NADPH. Data represent mean ± SD (%CV) of at least three separate preparations.

Treatment	CL_{int} (µl/min/10^6 cells)			
	Microcentrifuge tubes	24-well plates	96-well plates	Monolayer
Control	229 ± 113 (49)	116 ± 20 (17)	178 ± 83 (47)	62 ± 10 (16)
1 mM NADPH	340 ± 79* (23)	176 ± 36* (20)	264 ± 86 (33)	74 ± 5 (7)
5 mM NADPH	321 ± 36 (11)	135 ± 47 (35)	223 ± 123 (55)	72 ± 3 (4)

* $p < 0.05$ using Student's paired *t*-test in comparison to paired incubations in the absence of NADPH (control).

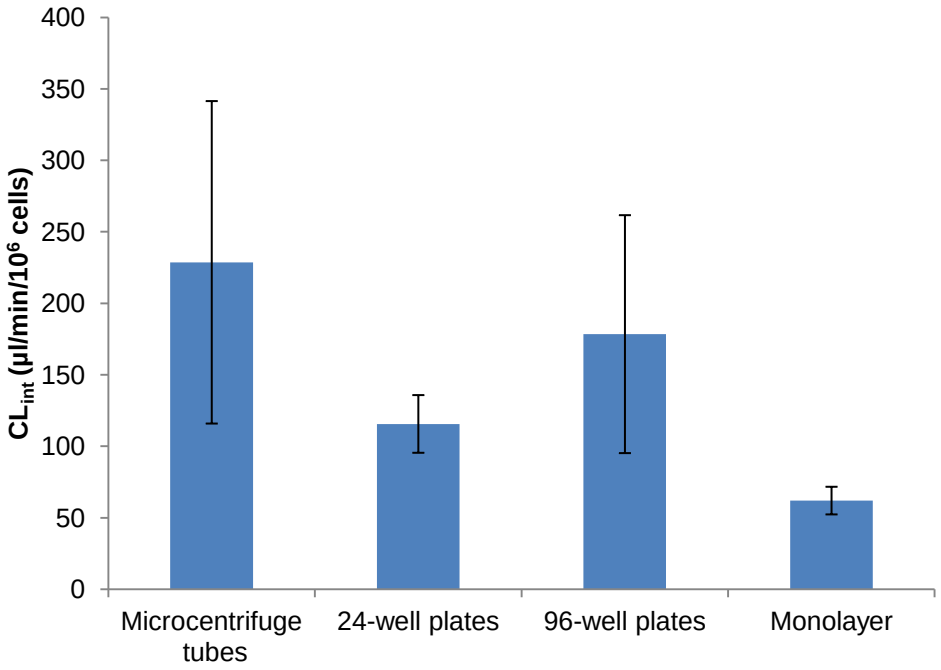


Figure 3-2. CL_{int} of midazolam in suspended hepatocytes in microcentrifuge tubes, 24-well plates, 96-well plates and in short-term monolayer cultured hepatocytes. Data represent mean ± SD of at least three separate preparations.

3.4.3 Incubation with exogenous cofactors

Based on the notion that the underprediction of CL_{int} from *in vitro* assays may be due to depletion of essential cofactors (Section 1.7), the addition of NADPH and nicotinamide (a metabolic precursor of NADPH) to hepatocyte incubations was investigated.

3.4.3.1 NADPH

Due to large differences in the CL_{int} of midazolam between assay formats in unsupplemented incubations (Figure 3-2), the effect of exogenous NADPH addition was also investigated across all four assay formats. Hepatocytes were supplemented at the point of pre-incubation (5 minutes prior to substrate addition) with WME (control), or WME containing NADPH (1 mM and 5 mM final concentrations). The intracellular concentration of NADPH is reported to be between 150-300 µM (Di, 2014), therefore in both conditions this cofactor was supplemented in excess relative to physiological concentrations. Significant ($p < 0.05$) increases in midazolam CL_{int} were observed with supplementation of 1 mM, but not 5 mM NADPH (Table 3-3). Analogous to the CL_{int} of midazolam, the magnitude of the effect of NADPH supplementation was also assay-format specific; significant ($p < 0.05$) increases in midazolam CL_{int} were only observed in microcentrifuge tubes (average increase 79%) and 24-well plates (average increase 48%) (Figure 3-3).

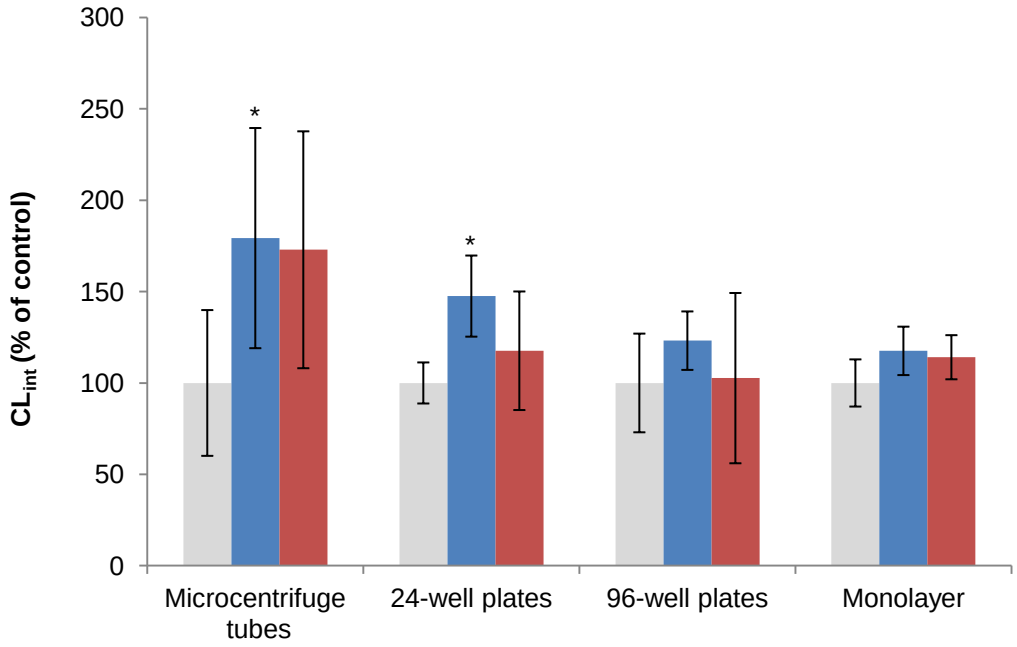


Figure 3-3. CL_{int} of midazolam as a percentage of control (▫) in the presence of 1 mM (▪) and 5 mM (▪) NADPH in suspended hepatocytes in microcentrifuge tubes, 24-well plates, 96-well plates, and short-term monolayer cultured hepatocytes. Data for each format represent mean ± SD of at least three separate preparations, * $p < 0.05$ using Student's paired *t*-test in comparison to paired incubations in the absence of NADPH (control).

3.4.3.2 Nicotinamide

On account of controversy over whether NAD+ and therefore NADPH is able to traverse the plasma membrane intact (Section 1.7.2), the addition of nicotinamide (a basic metabolic precursor of NADPH) to hepatocyte incubations was also investigated. A 96-well plate format was chosen for this study based on the higher CL_{int} of midazolam relative to that in 24-well plates and monolayer (Table 3-3) and capacity to perform much higher throughput experiments than in microcentrifuge tubes.

Table 3-4. CL_{int} of midazolam in suspended hepatocytes in the absence and presence of 1 mM and 5 mM nicotinamide. Data represent mean ± SD of three separate preparations.

Treatment	CL_{int} (µl/min/10^6 cells)
Control	212 ± 73
1 mM nicotinamide	208 ± 49
5 mM nicotinamide	141 ± 19

There was observed to be no statistically significant ($p < 0.05$) effect of either 1 mM or 5 mM nicotinamide supplementation on the CL_{int} of midazolam (Table 3-4).

3.4.4 Effect of substrate concentration on CL_{int} determinations

3.4.4.1 Incubation of substrates at 0.1 µM and 1 µM

The CL_{int} of each of a panel of nine well-characterised drugs was determined in freshly isolated rat hepatocytes at substrate concentrations of 0.1 µM and 1 µM. For seven of the drugs (chlorpromazine, dextromethorphan, metoprolol, midazolam, naloxone, propranolol and verapamil), the CL_{int} was statistically significantly ($p < 0.05$) higher when determined at a substrate concentration of 0.1 µM compared to 1 µM (Table 3-5). For diclofenac and triazolam, the same trend was apparent but was not found to be statistically significant (Figure 3-4). Of the seven drugs, the average fold increase in *in vitro* CL_{int} determined at 0.1 µM compared to 1 µM ranged from 1.4-fold (metoprolol and naloxone) to 9.8-fold (propranolol) (Figure 3-5).

Table 3-5. CL_{int} of nine drugs incubated at 0.1 µM and 1 µM in freshly isolated rat hepatocytes. Data represent mean ± SD of at least three separate preparations.

Drug	CL_{int} (µl/min/10^6 cells)	
	0.1 µM	1 µM
Chlorpromazine	1705 ± 782	517 ± 182*
Dextromethorphan	887 ± 120	317 ± 68*
Diclofenac	128 ± 26	116 ± 27
Metoprolol	129 ± 28	94 ± 26**
Midazolam	920 ± 303	447 ± 124*
Naloxone	1360 ± 97	950 ± 187*
Propranolol	1798 ± 272	184 ± 39*
Triazolam	301 ± 31	215 ± 44
Verapamil	752 ± 271	334 ± 131*

* $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

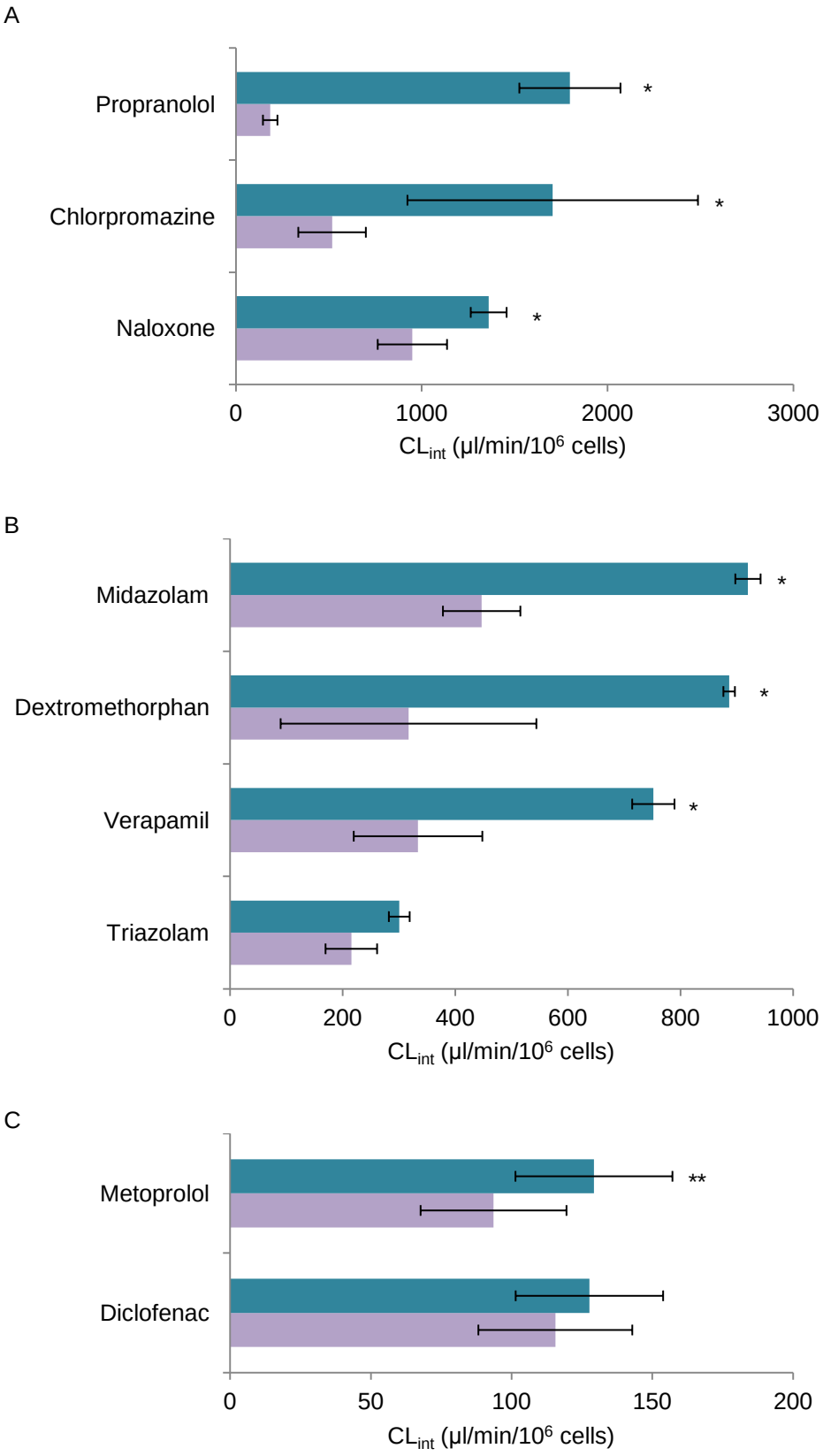


Figure 3-4. CL_{int} of 0.1 µM (■) and 1 µM (■) (A) propranolol, chlorpromazine, and naloxone, (B) midazolam, dextromethorphan, verapamil and triazolam and (C) metoprolol and diclofenac in freshly isolated rat hepatocytes. Data represent mean ± SD of at least three separate preparations, * $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

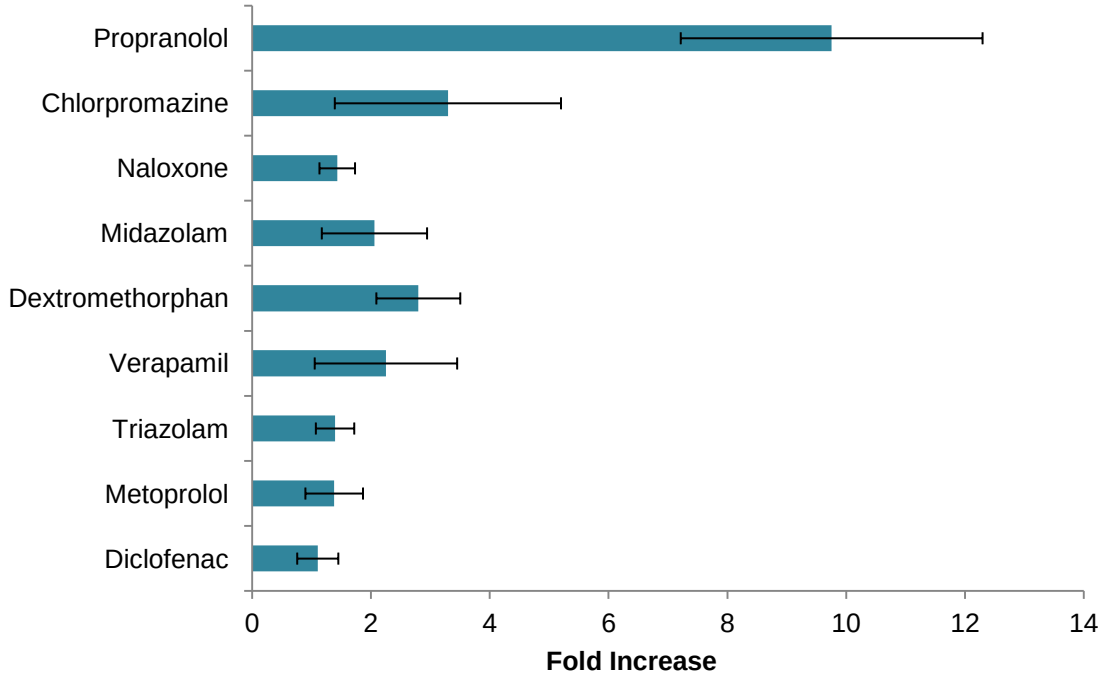


Figure 3-5. Average fold-increase (± SD) in *in vitro* CL_{int} determined at 0.1 µM compared to 1 µM. Data represent mean of at least three separate preparations; drugs are presented in descending order of *in vitro* CL_{int} determined at 0.1 µM.

3.4.4.2 Comparison with published literature data

The substrate concentrations of the selected drugs used in equivalent determinations of CL_{int} in the literature are given in Table 3-6. For the eight drugs for which literature data was available (*in vitro* data was not found for chlorpromazine), all except one study (Hallifax and Houston, 2006b) used an incubation concentration of 1 µM or above.

Table 3-6. Substrate concentration of the selected drugs in published studies of determinations of CL_{int} in rat using the substrate depletion approach.

Drug	Substrate Concentration (μM)	Reference
Chlorpromazine	-	-
Dextromethorphan	1	Huang et al. (2012)
	2.5	Jacobson et al. (2007)
Diclofenac	1	Huang et al. (2012)
	1	Sohlenius-Sternbeck et al. (2012)
	2	Lundquist et al. (2014)
Metoprolol	1	Jacobson et al. (2007)
Midazolam	1	Blanchard et al. (2004)
	1	Sohlenius-Sternbeck et al. (2012)
	2	Lu et al. (2006)
	2	Lundquist et al. (2014)
	2.5	Jones and Houston (2004)
Naloxone	1	Blanchard et al. (2004)
Propranolol	0.04	Hallifax and Houston (2006b)
	1	Huang et al. (2012)
	2	Lu et al. (2006)
	2.5	Jacobson et al. (2007)
	5	Griffin and Houston (2004)
Triazolam	2.5	Jones and Houston (2004)
Verapamil	1	Huang et al. (2012)
	2.5	Jacobson et al. (2007)

In response to these findings, literature data regarding the reported K_m values for either specific metabolic pathways or total metabolism of the selected drugs was researched (Table 3-7). For some drugs, for example propranolol, the observations in this study are in agreement with reported K_m values of below 1 μM. However for others, for example midazolam, a significant increase in CL_{int} determined at 0.1 μM compared to 1 μM was observed, yet the reported K_m values for individual metabolic pathways and total metabolism are considerably greater than 1 μM (Table 3-7).

Table 3-7. Reported K_m values (from literature) for the selected drugs.

Drug	Metabolic Pathway	K_m (µM)	Reference
Dextromethorphan	O-demethylation	2.80	Di Marco et al. (2003)
	O-demethylation[a]	0.15 ± 0.03	Brown et al. (2007a)
	O-demethylation[b]	10.0 ± 3.60	Brown et al. (2007a)
Midazolam	1'-hydroxylation	18.0 ± 6.00	Brown et al. (2007a)
	4'-hydroxylation	15.0 ± 7.00	Brown et al. (2007a)
	All	7.80 ± 2.30	Blanchard et al. (2004)
Naloxone	All	15.0 ± 0.40	Blanchard et al. (2004)
Propranolol	All	0.72 ± 0.28	Hallifax (1998)
Verapamil	All	4.6 ± 0.4 (fresh)	Nicolai et al. (2015)
		2.7 ± 0.3 (cryo)	Nicolai et al. (2015)

[a] Represents the 'high-affinity, low-capacity pathway', [b] Represents the 'low-affinity, high-capacity pathway'. Kinetic data was not found for chlorpromazine, diclofenac, metoprolol or triazolam. Fresh, freshly isolated hepatocytes; cryo, cryopreserved hepatocytes.

3.4.4.3 Effect of substrate concentration on the accuracy of *in vivo* $CL_{int,u}$ predictions in freshly isolated rat hepatocytes

To assess the effect of substrate incubation at 0.1 µM and 1 µM on the accuracy of *in vivo* $CL_{int,u}$ predictions, measured CL_{int} (µl/min/10^6 cells) was scaled to whole body $CL_{int,u}$ (ml/min/kg) and compared to observed $CL_{int,u}$ *in vivo* (ml/min/kg) calculated from published CL_h data (given in Appendix 8.3) using the WS liver model (Figure 3-6). On average, *in vivo* $CL_{int,u}$ was overpredicted by *in vitro* CL_{int} determinations performed at both 0.1 µM and 1 µM; the AFE (equivalent to fold-overprediction) for each dataset was 3.3 and 1.5 respectively.

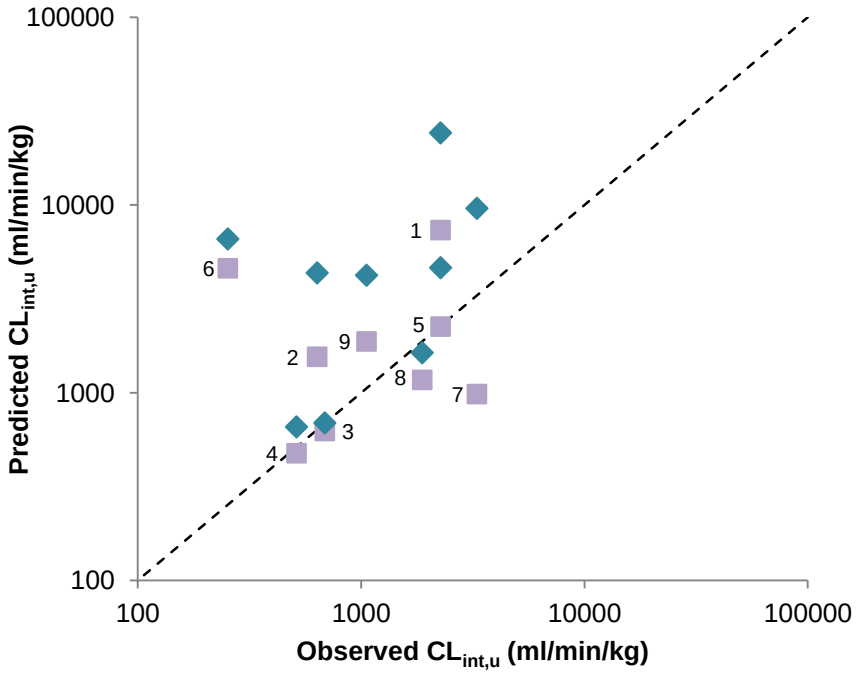


Figure 3-6. Predicted $CL_{int,u}$ determined in *in vitro* incubations of 0.1 µM (◆) and 1 µM (■) substrate in comparison to observed $CL_{int,u}$ *in vivo*. Dashed line represents unity. Data labels: 1. Chlorpromazine, 2. Dextromethorphan, 3. Diclofenac, 4. Metoprolol, 5. Midazolam, 6. Naloxone, 7. Propranolol, 8. Triazolam, 9. Verapamil.

3.5 Discussion

3.5.1 Effect of assay format on CL_{int}

Midazolam substrate depletion assays were performed in four commonly reported formats: suspended hepatocytes in microcentrifuge tubes, 24-well plates, 96-well plates, and short-term monolayer cultured hepatocytes (24-well). The CL_{int} of midazolam varied over three-fold between the different formats, from 62 ± 10 µl/min/10^6 cells in short-term monolayer cultured hepatocytes to 229 ± 113 µl/min/10^6 cells in suspended hepatocytes in microcentrifuge tubes. The lower clearances in monolayer are in relative agreement with reports of reduced metabolic activity in hepatocyte cultures compared to suspensions, previously attributed to either down-regulation of metabolising enzymes or a drug uptake rate-limitation in this system (Blanchard et al., 2004; Griffin and Houston, 2005). Additionally, it was assumed in this study that all viable cells seeded adhered to the collagen-coated plate, when in practice it is possible that this may not have occurred. If, in the experimental period, there were less than the assumed 240,000 cells/well, this could (partially) account for the reduced CL_{int} (µl/min/10^6 cells) measured. To circumvent this potential problem in future, the protein content of a control well in each assay could be measured and CL_{int} instead expressed as µl/min/mg protein.

The distinct lack of published data comparing CL_{int} in suspended hepatocytes across different formats is presumably due to an assumption of equivalence. However, in this study, up to two-fold differences in midazolam CL_{int} were observed between suspension formats. Although some variability between individual hepatocyte preparations is expected, it is unlikely that this would account for such observations. The most probable explanation is related to the degree of cell movement or mixing in each format. As centrifugal force is related to incubate volume, shaking frequency and shaking diameter amongst other parameters (Hermann et al., 2003), despite use of the same shaking speed, the force exerted upon the cells undoubtedly differed between formats. The potential effects of shaking on CL_{int} are discussed in greater depth in Chapter 5. Hepatocytes in short-term monolayer culture were also subject to mixing, although at a reduced speed (300 rpm) compared to suspended formats, in order to ensure movement of substrate/cofactor over the hepatocytes, but avoid cell detachment from the plate. The reduced mixing speed could be a further explanation for the lower clearances observed in this format, particularly as Griffin and Houston (2005) suggested an uptake rate-limitation in monolayer cultured hepatocytes.

3.5.2 Incubation with exogenous cofactors

Based on the proposition that clearance-dependent underprediction of *in vivo* $CL_{int,u}$ in hepatocytes may be due to cofactor depletion (Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Wang et al., 2005; Hallifax et al., 2010; Foster et al., 2011) (Section 1.7), the addition of NADPH (the cofactor for CYP-mediated oxidative metabolism) to hepatocyte incubations was investigated. Statistically significant ($p < 0.05$) effects on midazolam CL_{int} were observed with supplementation of 1 mM NADPH, but not 5 mM NADPH (Table 3-3, Figure 3-3). Interestingly, this is in accordance with the concentration of NADPH used in microsomal CL_{int} assays, which is typically 1 mM (Nomeir et al., 2004). Analogous to midazolam CL_{int}, the effects of 1 mM NADPH supplementation also differed between formats; significant ($p < 0.05$) increases were only observed for hepatocyte incubations in microcentrifuge tubes and 24-well plates (Table 3-3, Figure 3-3).

Due to claims that NAD+ must first be broken down into precursor molecules before it is able to enter cells (Nikiforov et al., 2011), the effect of supplementation of hepatocytes with nicotinamide (a basic precursor of NADPH) (see Figure 1-4) on midazolam CL_{int} was assessed. There was found to be no significant effect of nicotinamide at concentrations of 1 mM or 5 mM (Table 3-4), suggesting that the complete cofactor is required to affect metabolic activity in this situation.

3.5.3 Effect of substrate concentration on CL_{int} determinations

The requirement of a substrate concentration considerably below the K_m for determination of CL_{int} using the substrate depletion approach is well established (Obach et al., 1997), but in practice this principle may be overlooked. In an industry survey of experimental methodology,

Klopf and Worboys (2010) found that 8 out of 12 respondents (pharmaceutical companies) used a single substrate concentration of 1 µM in *in vitro* CL_{int} determinations using this approach. As it was not specified whether this related to animal or human hepatocytes, it is assumed to encompass both. The applicability of this generic incubation concentration was assessed using a panel of nine well-studied drugs; CL_{int} determinations were performed in freshly isolated rat hepatocytes at substrate concentrations of 0.1 µM and 1 µM. For seven of the nine studied drugs, CL_{int} determined at 0.1 µM was statistically significantly ($p < 0.05$) higher than CL_{int} determined at 1 µM, ranging from 1.4-fold (metoprolol and naloxone) to 9.8-fold (propranolol) (Table 3-5, Figure 3-5).

Following the findings of disparity between apparent CL_{int} at 0.1 µM and 1 µM for the majority of the tested drugs in this study, the substrate incubation concentrations of these drugs in published studies (which formed part of the database in Chapter 2) were researched. In all but one of the studies utilising the substrate depletion approach, the selected drugs were incubated at or above 1 µM (Table 3-6). Given the principles underlying the substrate depletion approach (Section 1.1), stipulations regarding substrate concentration should be followed to reduce avoidable underpredictions of *in vivo* $CL_{int,u}$. Adherence to these principles however, relies on the availability of such information. The K_m values (for either specific metabolic pathways or total metabolism) of the selected drugs were compiled from the literature (Table 3-7). Notably, kinetic data regarding metabolism of these drugs in rat hepatocytes was sparse, highlighting a distinct apparent preference for the substrate depletion approach of CL_{int} determination. The lack of such data may explain the use of generic incubation concentrations and the assumption that 1 µM is typically 'low enough'. In addition, the K_m values reported for midazolam and naloxone are relatively high in view of the differences in *in vitro* CL_{int} determined at 0.1 µM and 1 µM (Figure 3-4). Such likely inaccuracies in determined K_m values may be caused by use of a substrate concentration range which falls above the K_m for certain metabolic pathways, or obscuration of an alternative metabolic pathway involving a higher affinity enzyme component through measurement of kinetic parameters relating to total metabolism.

Calculation of the AFE of *in vivo* $CL_{int,u}$ predictions from *in vitro* determinations at 0.1 µM and 1 µM substrate revealed average overpredictions in both instances (Figure 3-6), which was unexpected given the general trend of underprediction from collated literature data (Figure 2-6). It should be recognised however, that this dataset represents only a small sub-set of drugs included in the literature analysis and that for some of the drugs the value of *in vivo* $CL_{int,u}$ is based upon a single literature study, thereby reducing the precision of such values. In addition, there is the possibility that *in vivo* assessments of clearance may have been performed at (partially) saturating drug concentrations.

The use of 1 µM as standard in *in vitro* CL_{int} determinations is likely historical, originating from a time when analytical sensitivity prevented the use of concentrations much lower this.

However nowadays, improvements to analytical instrumentation mean that such concentrations are achievable, and yet are still not implemented in practice. The findings of this work with a number of well-studied drugs which may be used as probe substrates for control metabolic incubations, as well as drug-drug interaction studies, highlights a need to re-evaluate current practices, at least for the use of rat hepatocytes. Whilst there may be methodological restrictions in drug discovery programmes due to generic LC-MS/MS methods and robotic execution of experiments, the importance of appropriate substrate concentration should be recognised. It is suggested that semi-specific optimisation of protocols for entire drug series or projects may be one route to achieve this (Klopf and Worboys, 2010).

3.6 Conclusion

The current study illustrated the influence of assay format on *in vitro* CL_{int}. Based on the high CL_{int} of midazolam (relative to other investigated formats) and the capacity to perform higher throughput experiments, hepatocyte suspension in 96-well plates will be used for all future studies in this project. As significant increases in *in vitro* CL_{int} were observed with the addition of NADPH to hepatocytes, the effect of supplemental NADPH on alternative hepatocyte preparations and the mechanism by which increases in CL_{int} are facilitated will be investigated further in Chapter 4. This work has also demonstrated the importance of appropriate substrate concentration for accurate determination of CL_{int} and highlighted the use of saturating substrate concentrations in published literature studies which may contribute to the general trend of underprediction of *in vivo* $CL_{int,u}$. The effect of substrate concentration on CL_{int} determinations in human hepatocytes will be investigated in Chapter 6.

Chapter 4. Measurements of viability in rat: cell membrane integrity and specific enzyme activity

4.1 Introduction

Whilst there are several measures of cell viability, plasma membrane integrity as determined by trypan blue exclusion is one of the oldest (Evans and Schulemann, 1914) and most frequently employed. However, the question arises of whether plasma membrane integrity is an accurate representation of viability, and indeed how viability should truly be defined. Many papers report a pre-specified limit of viability, below which hepatocyte preparations are not used, for example 75% (Blanchard et al., 2004), 80% (Lu et al., 2006; Paine et al., 2008; Nordell et al., 2013; Lundquist et al., 2014) or 85% (Ashforth et al., 1995; Jones and Houston, 2004; Jacobson et al., 2007; Parker and Houston, 2008; Jigorel and Houston, 2012). Despite the general acceptance of the necessity of such thresholds, the rationale used to define them is unclear. The only logical implication is that preparations less than this value are of lower quality, and therefore unsuitable for use. Berry et al. (1991) recommended no less than 90% viability, claiming that "a preparation with a high proportion of damaged cells is hardly suitable for metabolic studies"; however as evidenced above, this limit appears to have been somewhat lowered over the years. As more research is performed using cryopreserved hepatocytes of all species, especially human, a practical trade-off between desired cell viability and experimental output must be reached. Particularly expensive or limited samples such as human hepatocytes are unlikely to be discarded if the viability falls below a pre-defined cut-off. It is important therefore to examine whether cell viability is a true reflection of the cellular activity we are trying to measure (in this case metabolic activity), and to consider whether use of sub-optimal preparations could be a contributing factor to the underprediction of *in vivo* CL_{int}.

This chapter explores these themes within the concept of viability across three sections:

a) comparison of the metabolic activity of high and low viability preparations of the same pool of cryopreserved hepatocytes, achieved through the use of Percoll purification;
b) whether trypan blue exclusion is an accurate representation of the metabolic capacity of isolated hepatocytes, through the use of artificially low viability preparations created by ultra-sonication and permeabilisation techniques, and
c) the usefulness of ATP content as an alternative measure of viability.

4.2 Percoll purification

It is generally accepted that the processes of both hepatocyte isolation and cryopreservation are detrimental to cells, and therefore in some instances it can be difficult to obtain high viability preparations, especially of cryopreserved hepatocytes (Coundouris et al., 1993; Guillouzo et al., 1999; Blanchard et al., 2006; Richert et al., 2006). Should viability fall below the desired level, a technique frequently employed to enrich the viable cell population is Percoll purification; cells are centrifuged through a layer of silica particles and due to the differing densities, the intact cells sink to the bottom of the tube and the membrane-damaged cells remain in the supernatant (Berry et al., 1991). As cryopreserved preparations are generally of lower viability than freshly isolated hepatocytes, Percoll purification is most commonly, but not solely, restricted to this instance. Improved viability however, is acquired at the expense of yield (Kreamer et al., 1986; Diener et al., 1993), and therefore this technique may be deemed unfeasible for some studies.

Whilst Percoll purification evidently separates cells based on plasma membrane integrity (resulting in improved percentage viability), this technique has been additionally advocated by some researchers on the basis that the remaining viable cells are of higher quality, exhibiting a longer lifetime, increased maintenance of CYP content during culture, plasma membrane integrity and DNA repair capability (Dalet et al., 1982; Kreamer et al., 1986; Utesch et al., 1992; Coundouris et al., 1993). Another research group has even reported increased GST and SULT activity in cryopreserved human and rat hepatocytes respectively, after Percoll centrifugation (Diener et al., 1993; Diener et al., 1994; Hengstler et al., 2000). With regard to plasma membrane integrity, Dalet et al. (1982) showed that Percoll-purified rat hepatocyte suspensions were able to retain viability (as determined by trypan blue exclusion) for much longer than conventional preparations (exhibiting a half-life of 30-35 h compared to 4-6 h). The accompanying explanation was that loss of cell viability arises from the action of proteolytic enzymes released from damaged cells and therefore the effects of Percoll are attributed purely to separation of intact cells from these destructive enzymes (Dalet et al., 1982). Alternatively, Berry et al. (1991) stated that of all cells excluding trypan blue during initial assessment at 0-4°C, approximately 10% will lose plasma membrane integrity upon incubation at 37°C, and consequently attributed the effects of Percoll purification to early removal of this population of cells. Additionally, another report claimed that Percoll centrifugation decreases the proportion of non-parenchymal liver cells contaminating the hepatocyte preparation (Ostrowska et al., 2000); removal of non-metabolising cells could therefore theoretically result in an increase in the metabolic rate per 10^6 cells. In practice, the use of Percoll purification, like many other hepatocyte assay variables, appears inconsistent and unstandardised.

4.2.1 Aims

The primary aim of this section was to assess the effect of Percoll purification on the metabolic activity of cryopreserved rat hepatocytes. A secondary aim was to investigate cofactor supplementation of both unpurified and Percoll-purified cryopreserved rat hepatocytes, following the observed increases in CL_{int} of midazolam with addition of exogenous NADPH to freshly isolated rat hepatocytes (Section 3.4.3.1).

Midazolam and propranolol, both predominantly metabolised by CYP enzymes, were chosen as model drugs; midazolam is a mid-high clearance substrate of the rat CYP3A subfamily (Ghosal et al., 1996; Shaw et al., 2002) and propranolol is a high clearance substrate of the rat CYP2D subfamily (Masubuchi et al., 1993; Hiroi et al., 2002).

4.2.2 Materials and methods

4.2.2.1 Materials

Cryopreserved male Sprague Dawley rat hepatocytes (pool of eight animals) were purchased from Tebu-Bio Ltd. (Cambridgeshire, UK) (supplied by Sekisui XenoTech LLC (Kansas, USA)). Hepatocyte thawing kits containing two tubes of supplemental Dulbecco's modified eagle medium (DMEM) with and without isotonic Percoll were also purchased from Tebu-Bio Ltd. (±) Propranolol was purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). All other materials and reagents were as detailed in Section 3.3.1.

4.2.2.2 Preparation of cryopreserved hepatocytes

Cells were stored in the vapour phase of liquid nitrogen and thawed as per the supplier's instructions (see Appendix 8.6). For each experiment two vials were thawed simultaneously, one was centrifuged at 100 x g for 5 minutes in the DMEM containing isotonic Percoll (as per the supplier's instructions) and the other centrifuged at 60 x g for 3 minutes in DMEM. As described in Section 3.3.3, cells were counted using a haemocytometer and light microscope and viability assessed using the trypan blue exclusion method; cells were subsequently diluted to the required concentration in WME buffered with HEPES (24 mM final concentration), pH 7.4.

4.2.2.3 Substrate depletion assays

Substrate depletion assays were performed in 96-well plates as described in Section 3.3.5.3. Briefly, unpurified and Percoll-purified hepatocytes were pre-incubated with either WME or WME containing NADPH (final concentration 1 mM) for 5 minutes in a microtiterplate shaker at 900 rpm and 37°C. Following this period, substrate was added to the hepatocytes to initiate the reaction. Individual wells were quenched (using a volume equal to the incubation volume of methanol containing internal standard) at appropriate time points (n = 8) to give a substrate depletion profile. Each condition was performed in duplicate on three separate

occasions. Details of manufacturers and models for the equipment utilised can be found in Appendix 8.4.

Table 4-1. Incubation conditions of midazolam and propranolol in substrate depletion assays using cryopreserved rat hepatocytes.

Drug	Hepatocyte Density (10^6 cells/ml)	Substrate Concentration (μM)	Incubation Time (min)
Midazolam	0.5	2.5	15
Propranolol	0.125	1	7

4.2.2.4 Sample preparation and liquid chromatography-mass spectrometry analysis

Incubation samples were treated and prepared for LC-MS/MS analysis in the same manner as described in Section 3.3.7. The LC-MS/MS systems and conditions used for individual drugs and the associated internal standards are given in Appendix 8.5.Procedures for sample quantification were also as given in Section 3.3.7.

4.2.2.5 Data analysis

Generated *in vitro* data was analysed in the same manner as described in Section 3.3.8.

4.2.2.6 Statistical analysis

The arithmetic mean and standard deviation were calculated for each condition for each substrate. A two-tailed, paired Student's *t*-test was used to determine the existence of a statistically significant difference between two conditions; values were reported as significant when $p < 0.05$.

4.2.3 Results

4.2.3.1 Effect of Percoll purification on CL_{int}

As described in Section 4.2.2.2, thawed cryopreserved rat hepatocytes were centrifuged either in DMEM following a typical protocol or in DMEM containing isotonic Percoll following the supplied protocol. Viability (as determined by trypan blue exclusion) of thawed cryopreserved hepatocytes in the unpurified and Percoll-purified conditions was 62 ± 3% and 86 ± 1% respectively for the midazolam assays and 67 ± 3% and 89 ± 3% respectively for the propranolol assays - a statistically significant ($p < 0.01$) difference in viability afforded by Percoll treatment in both instances (Table 4-2). Consequently, large differences were also apparent in the total cell concentrations of unpurified and Percoll-purified preparations (on average 28% and 25% fewer cells in Percoll-purified preparations for midazolam and propranolol assays respectively). Aside from a potential effect on non-specific incubational binding, the increased population of non-viable cells in unpurified preparations is typically

not expected to affect the CL_{int}. However, when normalised to viable cell number (as is standard practice), the average CL_{int} of both midazolam and propranolol was considerably higher in unpurified hepatocytes compared to Percoll-purified hepatocytes. The reduction in CL_{int} in Percoll-purified preparations was 33% and 34% for midazolam and propranolol respectively, yet when normalised to total cell number, the CL_{int} in unpurified and Percoll-purified hepatocytes became very similar (Table 4-2, Figure 4-1). This suggests that the extra 'non-viable' cells in the unpurified hepatocyte preparations may have contributed to the metabolism of both midazolam and propranolol.

Despite selection of midazolam and propranolol as representatives of mid-high clearance and high clearance drugs respectively, there appeared to be little difference between the determined *in vitro* CL_{int} of these two drugs.

Table 4-2. Viability of (as determined by trypan blue exclusion), total cell concentration and CL_{int} of midazolam and propranolol in unpurified and Percoll-purified cryopreserved rat hepatocytes. Data represent mean ± SD of three experiments.

Drug	Parameter	Unpurified	Percoll-purified
Midazolam	Initial viability (%)	62 ± 3	86 ± 1**
	Total cell concentration (10^6/ml)	0.81 ± 0.04	0.58 ± 0.01**
	CL_{int} (µl/min/10^6 cells) normalised to viable cell number	245 ± 57	187 ± 33
	CL_{int} (µl/min/10^6 cells) normalised to total cell number	150 ± 29	160 ± 27
Propranolol	Initial viability (%)	67 ± 3	89 ± 3**
	Total cell concentration (10^6/ml)	0.37 ± 0.02	0.28 ± 0.01**
	CL_{int} (µl/min/10^6 cells) normalised to viable cell number	217 ± 33	141 ± 26
	CL_{int} (µl/min/10^6 cells) normalised to total cell number	146 ± 18	126 ± 22

** $p < 0.01$ using Student's paired *t*-test.

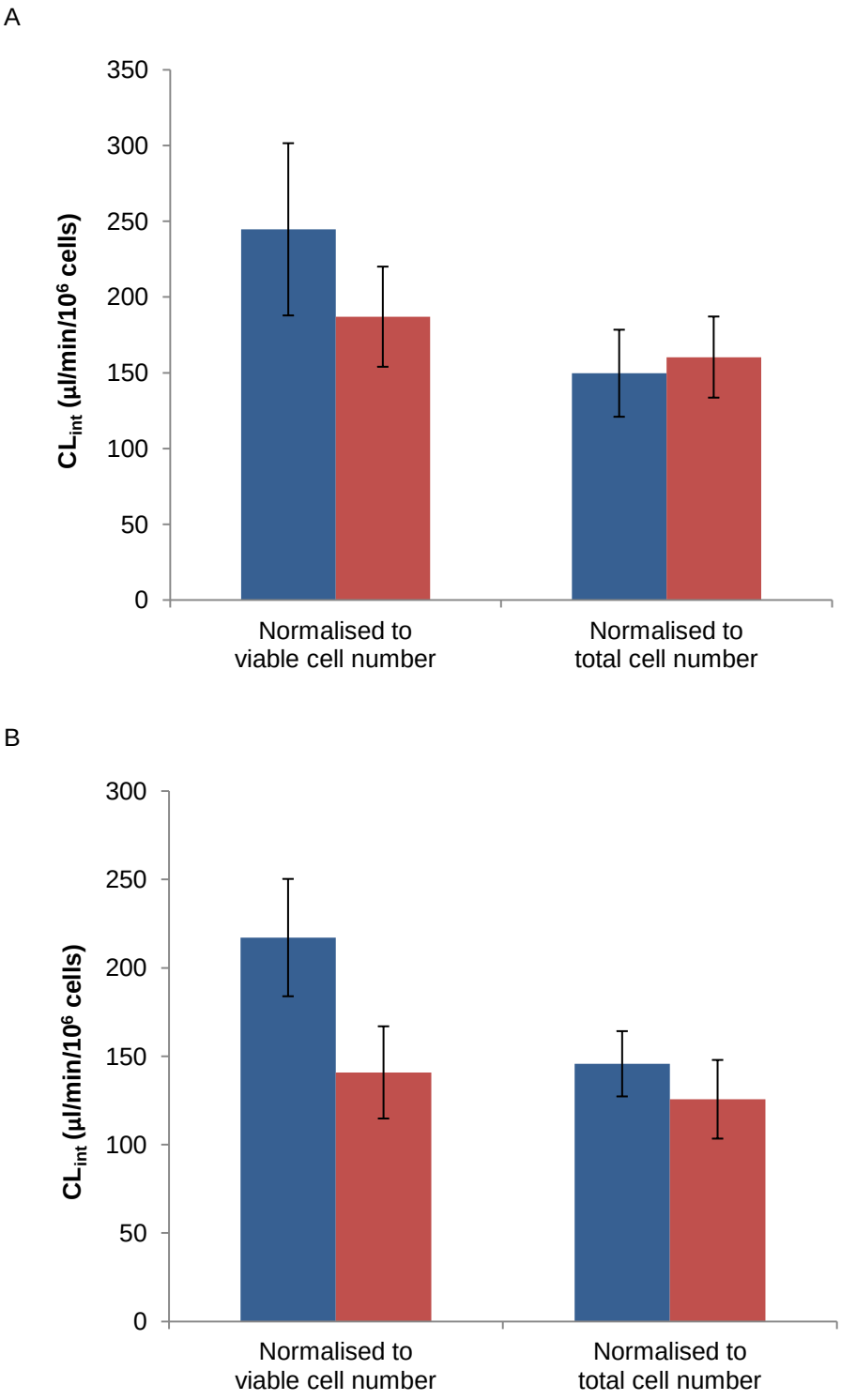


Figure 4-1. CL_{int} of (A) midazolam and (B) propranolol in unpurified (■) and Percoll-purified (■) cryopreserved rat hepatocytes, normalised to viable and total cell number. Data represent mean ± SD of three experiments.

4.2.3.2 Effect of cofactor supplementation on CL_{int}

The effect of exogenous NADPH addition on midazolam and propranolol CL_{int} in unpurified and Percoll-purified cryopreserved rat hepatocytes was assessed. To account for the apparent differences in viability and potential metabolic activity of 'non-viable' cells, CL_{int} values were normalised to total cell number. As recorded in Table 4-2, for both drugs, the CL_{int} in unpurified and Percoll-purified preparations was similar when normalised in this way. However, much larger increases in CL_{int} with the addition of NADPH were observed in unpurified hepatocytes (55% and 41% for midazolam and propranolol respectively), than in Percoll-purified hepatocytes (12% and -12% for midazolam and propranolol respectively) (Table 4-3, Figure 4-2). This indicates increased availability of NADPH to unpurified hepatocytes and suggests a permeability barrier to this cofactor in intact cells.

Table 4-3. CL_{int} of midazolam and propranolol in unpurified and Percoll-purified cryopreserved rat hepatocytes with and without NADPH supplementation. Data are normalised to total cell number and represent mean ± SD of three experiments.

Drug	Preparation	CL_{int} (µl/min/10^6 cells)	
		Unsupplemented	NADPH-supplemented
Midazolam	Unpurified	150 ± 29	229 ± 29*
	Percoll-purified	160 ± 27	179 ± 26*
Propranolol	Unpurified	146 ± 18	205 ± 24**
	Percoll-purified	126 ± 22	110 ± 22

* $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

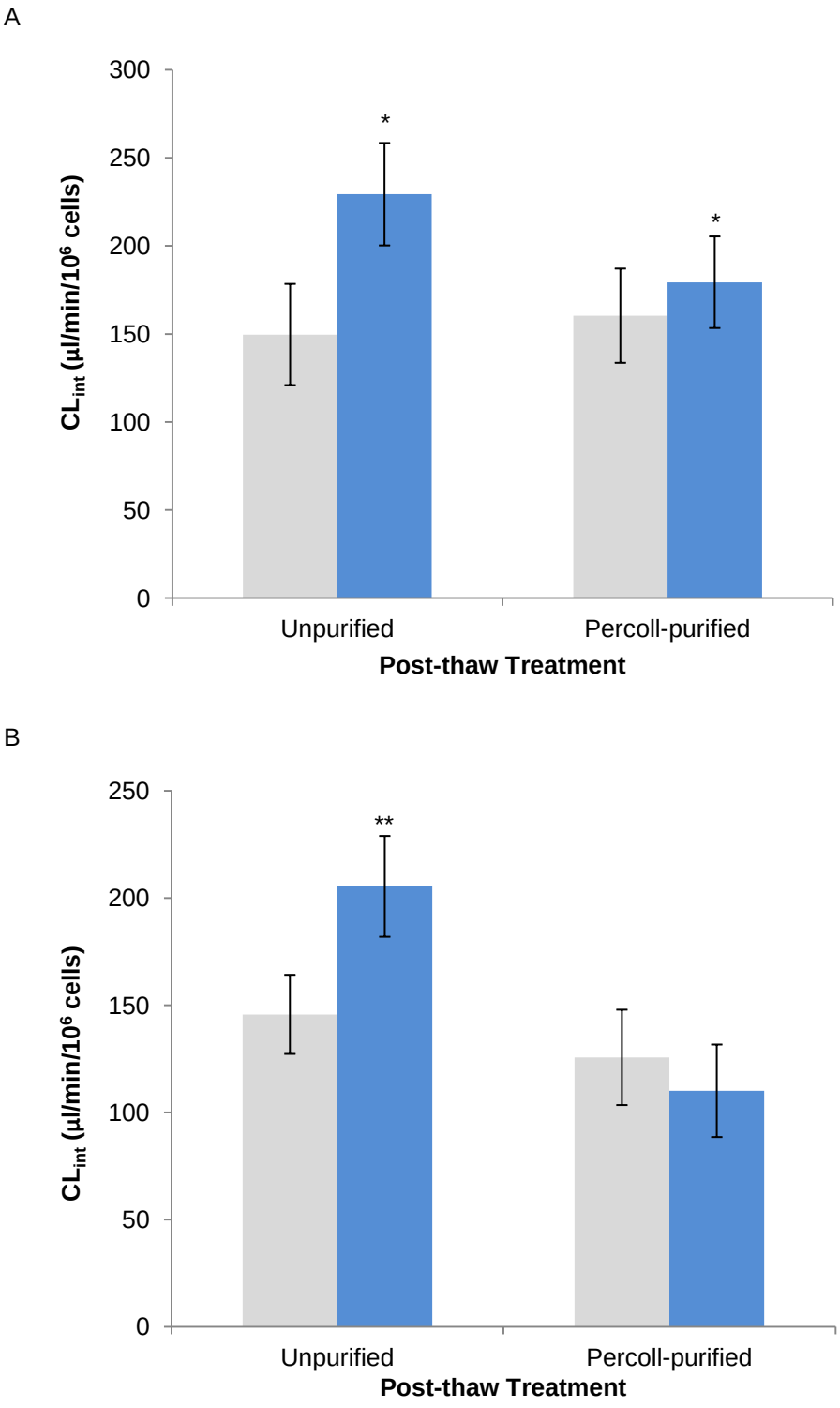


Figure 4-2. CL_{int} of (A) midazolam and (B) propranolol in unpurified and Percoll-purified cryopreserved rat hepatocytes in the absence (▫) and presence (■) of 1 mM NADPH. Data are normalised to total cell number and represent mean ± SD of three separate experiments, * $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

4.2.4 Discussion

The results of this work were somewhat unexpected as the literature suggested that Percoll-purified hepatocytes may exhibit a higher metabolic clearance than unpurified hepatocytes (Kreamer et al., 1986; Utesch et al., 1992), when indeed the opposite was observed (Figure 4-1). The virtual elimination of this considerable difference in CL_{int} between the two conditions by normalising to total cell number rather than viable cell number (Table 4-2, Figure 4-1) suggests that the cells determined 'non-viable' by trypan blue exclusion retained enzymatic activity. This is supported by the similar percent reduction in total cell concentration and CL_{int} between unpurified and Percoll-purified conditions. Furthermore, the 'non-viable' cells may offer unrestricted access of substrate (and exogenous cofactor) to metabolic enzymes through the compromised membrane (leaky to trypan blue). The similar *in vitro* CL_{int} observed for midazolam and propranolol (selected as mid-high and high clearance drugs respectively) is most likely a consequence of the use of a saturating substrate concentration for propranolol (as evidenced in Section 3.4.4.1). This study was undertaken prior to the investigation into substrate concentration and therefore selection of 1 µM propranolol was based upon previously published studies of the metabolic clearance of this drug in rat hepatocytes (Griffin and Houston, 2004; Lu et al., 2006; Huang et al., 2012).

The addition of 1 mM NADPH to incubations resulted in statistically significant ($p < 0.05$) increases of approximately 50% in the CL_{int} of both midazolam and propranolol in unpurified hepatocytes, but only marginal (if any) increases in CL_{int} in Percoll-purified hepatocytes (Figure 4-2). As the clearances in the incubations without additional NADPH were similar for unpurified and Percoll-purified preparations, this suggests that the greater effect of NADPH on unpurified hepatocytes was due to replenishment of this cofactor in the 'non-viable' cells (33-38% of the total population of the unpurified hepatocytes and 11-14% of the total population of the Percoll-purified hepatocytes). A permeability barrier to NADPH in intact cells is therefore implied, something which is a topic of debate (Bruzzone et al., 2001; Billington et al., 2008; Nikiforov et al., 2011).

The answer to whether Percoll purification should be used routinely is substrate dependent. For midazolam, which is not believed to be membrane-permeability rate-limited, there appears to be no difference in the CL_{int} in unpurified or Percoll-purified cells when normalised to total cell number. Indeed, for such drugs, one may avoid Percoll purification in order to retain a higher total cell yield and the same metabolic capacity. On the contrary, assuming that there is sufficient cofactor present in the medium or contained within the 'non-viable' cells to carry out metabolism, for uptake (passive or active) rate-limited compounds, an overestimation of *in vivo* clearance may result from use of unpurified hepatocytes, as a considerable proportion of these would allow unrestricted entry to substrate. Should little be known about the rate-determining processes of a compound's CL_{int}, Percoll purification of very low viability hepatocyte preparations would be advised to circumvent such issues.

4.3 Permeabilisation

To further explore the metabolic activity of cells deemed non-viable by trypan blue exclusion (as observed in the previous section of this chapter), permeabilisation techniques were applied to artificially create hepatocyte preparations with compromised membrane integrity and resultant low viability as determined by trypan blue exclusion.

Permeabilisation of cell membranes or the preparation of cell sonicates/lysates are techniques typically employed to allow entry or exit of molecules that would ordinarily be unable to pass across a cell membrane due to their size and/or charge (Geelen, 2005). The various documented methods utilised to both transiently and permanently permeabilise plasma membranes are summarised in a review by Geelen (2005). In pharmacokinetics, one applicable area of interest is the addition of exogenous cofactors to cells thought to be depleted of *in vivo* level reserves; these molecules are also assumed too large or charged to traverse the plasma membrane of intact cells (Swales and Utesch, 1998). Such investigations, usually in the context of applicability of cryopreserved hepatocytes as a substitute for fresh, appear to have favoured gross membrane destruction by sonication (Swales and Utesch, 1998; Bruni and Chang, 1999; Hewitt et al., 2000; Hewitt and Utesch, 2004; Dennison et al., 2008). In many instances, researchers aimed to test the theory of cofactor depletion by comparing the metabolic activity of intact cells with corresponding cell 'lysates' or 'homogenates' supplemented with exogenous cofactors (Swales and Utesch, 1998; Hewitt et al., 2000; Hewitt and Utesch, 2004). In other studies, the focus was directed towards understanding the specific contribution of plasma membrane permeability to overall CL_{int} determination (Bruni and Chang, 1999; Dennison et al., 2008). Plasma membrane permeabilisation techniques such as incubation with saponins or digitonin, as opposed to gross membrane destruction, have also been used for these purposes, but appear less commonly so (Banhegyi et al., 1993; Swales et al., 1996).

The use of the plant glycosides digitonin and saponin(s), as membrane permeabilising agents however, is far from a new concept, and the effects of these products on various cell and membrane types has been studied for many years (Seeman et al., 1973; Zuurendonk and Tager, 1974; Cook et al., 1983; Katz and Wals, 1985; Wassler et al., 1987; Niklas et al., 2011). Saponins and digitonin alike, permeabilise plasma membranes through formation of complexes with cholesterol (Melzig et al., 2001). It is on this basis, that at low concentrations (less than 0.1 mg/ml (0.01% w/v)), that treatment with saponin (or digitonin) is assumed to be specific to the plasma membrane, which has a much higher cholesterol content (cholesterol/phospholipid molar ratio: 0.76) than intracellular membranes (cholesterol/ phospholipid molar ratio: 0.27 and 0.06 for the Golgi apparatus and endoplasmic reticulum respectively) (Colbeau et al., 1971). The use of both electron microscopy (Seeman et al., 1973; Cook et al., 1983) and mitochondrial enzyme markers (Zuurendonk and Tager, 1974; Cook et al., 1983; Niklas et al., 2011) confirm this supposition and indeed, it was found that

a five-fold higher concentration of saponin was required to permeabilise the endoplasmic reticulum of rat hepatocytes compared to the plasma membrane (Wassler et al., 1987). The use of such agents therefore allows creation of an alternative system, described by Katz & Wals (1985) as “a stable organelle complex that is likely to have retained most of its enzymatic and metabolic capacity”, in which to study xenobiotic metabolism. Despite permeabilisation, when cytosol and exogenous cofactors are added to the incubation, these cells reportedly remain able to generate both ATP and NADH, as well as maintain glucose production (Katz and Wals, 1985). Although the pores created in the membrane may permit exit of intracellular soluble components such as cofactors which require replacement, they also allow unrestricted access of substrates and other constituents of up to at least 285 kDa contained within the extracellular medium into the cell (Bijleveld and Geelen, 1987). With the optimal incubation conditions, these ‘cell ghosts’ could represent a system closer to the intact cell than microsomes or sonicated cell preparations, but absent of the permeability barrier presented by the cell membrane.

4.3.1 Aim

The aim of this section was to further test the implication that cells determined non-viable by trypan blue exclusion can retain enzymatic activity, through the use of deliberate gross membrane destruction and plasma membrane permeabilisation by ultrasonication and saponin treatment respectively. These techniques were also used to investigate the cell membrane as a permeability barrier to both substrates and exogenous cofactors; their potential use as a means to identify rate-limiting processes in the determination of CL_{int} were explored.

Midazolam, propranolol and saquinavir were selected as probe substrates. Midazolam is categorised as Class 1 in the Biopharmaceutical Drug Disposition Classification System (BDDCS), its high permeability indicating that passive diffusion is likely to be the predominant mechanism of entry into the cell (Wu and Benet, 2005). Propranolol is also classified as BDDCS Class 1 and therefore assumed not to have a significant transporter contribution to its uptake (Wu and Benet, 2005). Propranolol (incubated at a lower substrate concentration than in the previous section) was chosen as a higher clearance comparison to midazolam to test the potential contribution of permeability to CL_{int} determination. The human immunodeficiency virus protease inhibitor, saquinavir, is a CYP3A4 substrate (Eagling et al., 2002), but has been shown to exhibit up to a 12-fold higher clearance in RLM than in isolated rat hepatocytes, indicating that its clearance is likely to be uptake rate-limited (Parker and Houston, 2008). One study reports the contribution of active uptake as 52% of total uptake for saquinavir (Yabe et al., 2011). Due to its low solubility but high rate of metabolism, saquinavir is classified as BDDCS Class 2 (Wu and Benet, 2005).

4.3.2 Materials and methods

4.3.2.1 Materials

Saponin and saquinavir were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). All other materials and reagents were as detailed in Section 3.3.1.

4.3.2.2 Hepatocyte source, isolation and preparation

Male Sprague Dawley rat hepatocytes were freshly sourced, isolated and prepared as described in Sections 3.3.2 and 3.3.3. Isolated hepatocyte preparations were of at least 85% viability.

4.3.2.3 Permeabilisation of hepatocytes by probe ultrasonication

Preliminary experiments were undertaken to define the sonication conditions (adapted from Dennison et al. (2008)) required to yield a preparation of hepatocytes of approximately 50% viability. Prior to use in substrate depletion assays, a proportion of isolated hepatocytes were diluted to a concentration of 5 x 10^6 viable cells/ml and ultrasonicated using an ultrasonic probe homogeniser (CamLab Ltd. (Cambridgeshire, UK)) for 5 x 1 second pulses at 30% power. Viability was estimated using trypan blue exclusion and the hepatocytes were subsequently diluted to give the equivalent number of total hepatocytes (using the original cell count) to that in the intact condition. Details of manufacturers and models for the equipment utilised can be found in Appendix 8.5.

4.3.2.4 Permeabilisation of hepatocytes by treatment with saponin

Preliminary experiments based on the previous investigation by Wassler et al. (1987) and study by Kazmi et al. (2014) were initially undertaken to determine the lowest saponin concentration required to permeabilise the plasma membrane of all cells in the preparation. Saponin was dissolved in WME to give a 0.5% w/v solution. Hepatocytes were diluted to 1 x 10^6 viable cells/ml in WME and pre-incubated with 0.01% w/v saponin for 5 minutes prior to use in the substrate depletion assay. An estimate of viability was obtained using the trypan blue exclusion method before use.

4.3.2.5 Substrate depletion assays

Substrate depletion assays were performed in the 96-well format as described in Section 3.3.5.3. Briefly, hepatocytes (intact or sonicated/saponin-treated) were pre-incubated for 5 minutes with either WME (control) or WME containing NADPH (final concentration 1 mM) in a microtiterplate shaker at 900 rpm and 37°C. The reaction was initiated by the addition of substrate. Individual wells were quenched at appropriate time points (n = 8) to give a substrate depletion profile. Each condition (with and without NADPH) was performed in duplicate and a further incubation in the absence of cells was performed as a non-specific loss control. The optimised incubation conditions for midazolam and propranolol are as given in Table 4-1, saquinavir was incubated at 1 μM with hepatocytes of concentration

0.125 x 10^6 cells/ml for 15 minutes. Experiments were performed on at least three separate occasions. Details of manufacturers and models for the equipment utilised can be found in Appendix 8.4.

4.3.2.6 Sample preparation and liquid chromatography-mass spectrometry analysis

Incubation samples were treated and prepared for LC-MS/MS analysis in the same manner as described in Section 3.3.7. The LC-MS/MS systems and conditions used for individual drugs and the associated internal standards are given in Appendix 8.5. Procedures for sample quantification were also as given in Section 3.3.7.

4.3.2.7 Data analysis

Generated *in vitro* data were analysed using the software GraFit (v7.0.03, Erithacus Software Ltd., Surrey, UK) in the same manner as described in Section 3.3.8.1. In addition, any apparent loss of substrate due to mechanisms other than metabolism by hepatocytes (for example non-specific binding) was corrected for by the non-specific loss control. An elimination rate constant was calculated in the same way as that for the hepatocyte depletion data and this value of k was subtracted from the k values for the hepatocyte incubations. CL_{int} was then calculated using Equation 3.1 as described in Section 3.3.8.1.

4.3.2.8 Statistical analysis

The arithmetic mean and standard deviation were calculated for each condition for each drug. A two-tailed, paired Student's *t*-test was used to determine the existence of a statistically significant difference between two conditions; values were reported as significant when $p < 0.05$.

4.3.3 Results

4.3.3.1 Permeabilisation of hepatocytes using probe ultrasonication

To further investigate the proposition that 'non-viable' cells may possess metabolic enzymatic activity, preparations of low percentage viability were artificially created by ultrasonication. The sonication conditions chosen yielded a population of hepatocytes which were estimated to be between 45-55% viable as determined by trypan blue exclusion. When midazolam CL_{int} was normalised to original total cell number, the clearance in unsupplemented sonicated hepatocytes (64 ± 5 µl/min/10^6 cells) was approximately one quarter of the clearance in intact hepatocytes (250 ± 58 µl/min/10^6 cells) (Table 4-4). This clearly indicates that the cells rendered 'non-viable' by sonication do not retain full enzymatic activity. Even when normalised to viable cell number, the CL_{int} of midazolam in unsupplemented sonicated cells was only approximately half of that in the intact preparation (Table 4-4). Such values imply either that the estimate of the proportion of viable cells remaining in the preparation was incorrect, or that sonication somehow reduces the metabolic capacity of the remaining viable cells. As completely fragmented cells are unable

to be visualised under a light microscope in a standard trypan blue cell count, it is most likely the former.

The addition of 1 mM NADPH to both intact and sonicated hepatocytes resulted in statistically significant ($p < 0.05$) increases in midazolam CL_{int}, although a greater effect was observed in the sonicated preparations (Table 4-4, Figure 4-3). NADPH-supplementation elevated midazolam CL_{int} in sonicated hepatocytes to a level comparable to that in intact unsupplemented hepatocytes (normalised to total cell number) (Figure 4-3).

Table 4-4. Initial viability of and CL_{int} of midazolam in intact and sonicated freshly isolated rat hepatocyte preparations with and without NADPH supplementation. Data represent mean ± SD of three separate preparations.

Parameter	Intact		Sonicated	
	Unsupplemented	NADPH-supplemented	Unsupplemented	NADPH-supplemented
Initial 'viability' (%)	88 ± 2		52 ± 6**	
Midazolam CL_{int} normalised to <u>viable</u> cell number (µl/min/10^6 cells)	284 ± 68	369 ± 90*	127 ± 19	381 ± 28*
Midazolam CL_{int} normalised to <u>total</u> cell number (µl/min/10^6 cells)	250 ± 58	325 ± 78*	65 ± 5§	198 ± 34*

* $p < 0.05$ using Student's paired *t*-test in comparison to paired unsupplemented condition, ** $p < 0.01$ using Student's paired *t*-test in comparison to intact hepatocytes, § $p < 0.05$ using Student's paired *t*-test in comparison to unsupplemented intact hepatocytes.

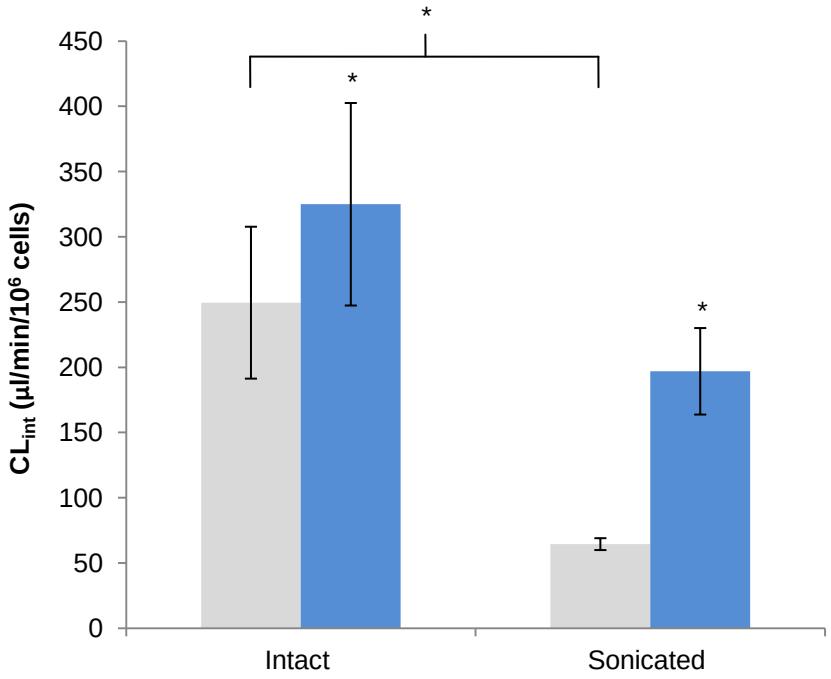


Figure 4-3. CL_{int} of midazolam in intact and sonicated rat hepatocytes in the absence (▫) and presence (■) of 1 mM NADPH. Data are normalised to total cell number and represent mean ± SD of three separate preparations, * $p < 0.05$ using Student's paired *t*-test.

4.3.3.2 Permeabilisation of hepatocytes using saponin

Following the work with sonication, saponin treatment was chosen as a more controlled technique by which to generate 'non-viable' cells. Using the conditions described in Section 4.3.2.4, the entire population of subjected cells were rendered 'non-viable' according to trypan blue exclusion, whilst the overall cell structure was assumed to be left largely intact (cells remained visible). Reports of maintained cell density with 100% trypan blue permeability after digitonin treatment provide additional support for this assumption (Niklas et al., 2011). CL_{int} of drugs was normalised to initial total cell number for consistency and as normalising to viable cell number for saponin-treated cells would be unrealistic or even impossible.

Saponin treatment resulted in complete abolishment of midazolam, propranolol and saquinavir clearance (Table 4-5, Figures 4-4, 4-5 and 4-6 respectively). However, addition of 1 mM NADPH to these preparations restored substrate metabolism to levels either consistent with (midazolam and propranolol), or greater than (saquinavir), that of intact hepatocytes, demonstrating that the CYP enzymes responsible for each drug's metabolism remained functional.

Table 4-5. CL_{int} of midazolam, propranolol and saquinavir in intact and permeabilised freshly isolated rat hepatocytes with and without NADPH-supplementation. Data are normalised to total cell number and represent the mean ± SD of at least three preparations.

Drug	CL_{int} (µl/min/10^6 cells)			
	Intact		Permeabilised	
	Unsupplemented	NADPH-supplemented	Unsupplemented	NADPH-supplemented
Midazolam	235 ± 18	315 ± 28*	6 ± 6	203 ± 48*
Propranolol	982 ± 303	1294 ± 391	0 ± 0[§]	1553 ± 294*
Saquinavir	299 ± 98	499 ± 102*	0 ± 0[§]	753 ± 232**

* $p < 0.05$, $p < 0.01$ using Student's paired *t*-test in comparison to paired unsupplemented condition. [§]No detectable metabolism was observed for propranolol or saquinavir in any of the tested preparations.

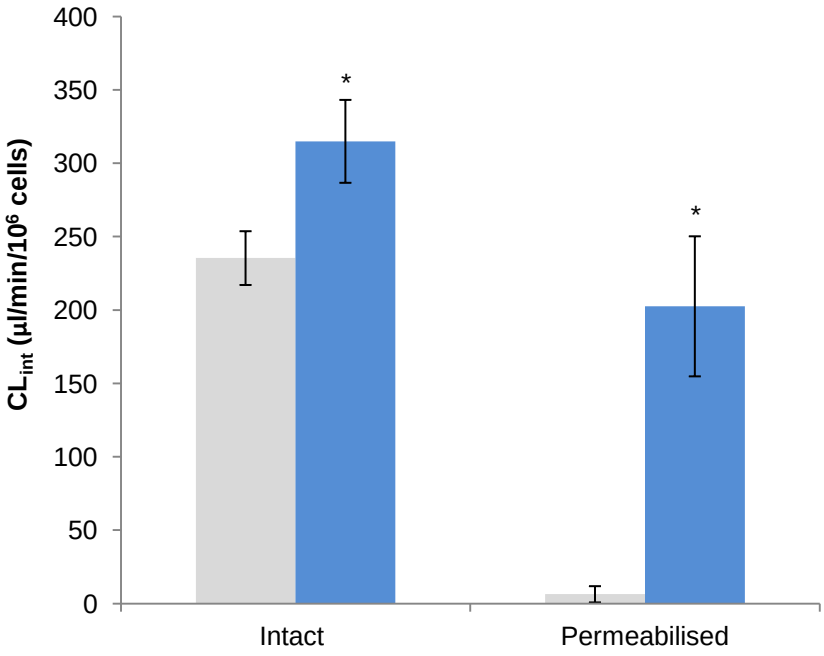


Figure 4-4. CL_{int} of midazolam in intact and permeabilised rat hepatocytes in the absence (□) and presence (■) of 1 mM NADPH. Data are normalised to total cell number and represent mean ± SD of three separate preparations; * $p < 0.05$ using Student's paired *t*-test.

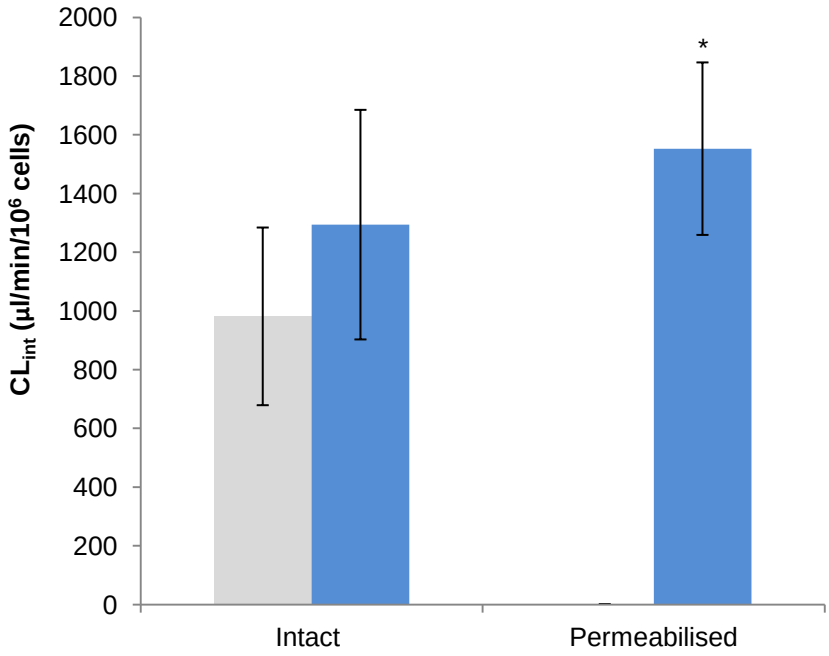


Figure 4-5. CL_{int} of propranolol in intact and permeabilised rat hepatocytes in the absence (▫) and presence (■) of 1 mM NADPH. Data are normalised to total cell number and represent mean ± SD of three separate preparations; * $p < 0.05$ using Student's paired *t*-test.

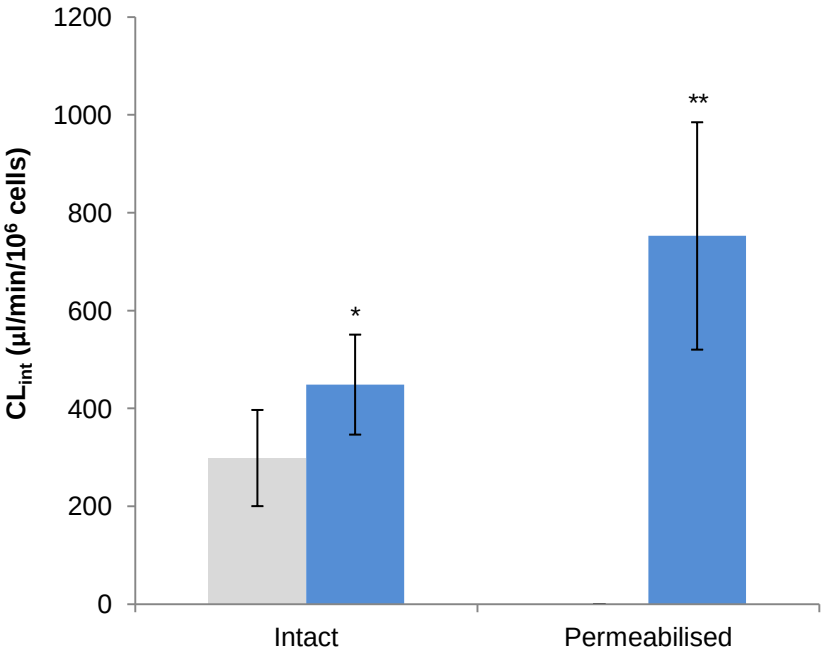


Figure 4-6. CL_{int} of saquinavir in intact and permeabilised rat hepatocytes in the absence (▫) and presence (■) of 1 mM NADPH. Data are normalised to total cell number and represent mean ± SD of at least three separate preparations; * $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

4.3.4 Discussion

Following from the work on Percoll purification of cryopreserved rat hepatocytes which revealed the potential for retention of enzymatic activity by cells deemed non-viable by trypan blue exclusion, it was decided to further explore this phenomenon through the use of deliberate cell disruption and membrane permeabilisation. Intact cells of at least 85% viability were compared with those that had undergone either probe sonication or saponin treatment.

The viability of all cell preparations was assessed using trypan blue exclusion, however, for sonicated hepatocytes this estimate was not necessarily accurate since there were likely to have been cells which fragmented during sonication that were therefore unaccounted for. As saponin treatment causes pore formation (Melzig et al., 2001) rather than fragmentation and the entire population of cells were rendered 'non-viable', estimation of viability did not pose a problem for this method.

4.3.4.1 Metabolic activity of 'non-viable' hepatocytes

Consistent with previously published studies (Swales et al., 1996; Swales and Utesch, 1998), these results confirm that severe damage to the cell membrane, whether caused by sonication or permeabilisation, results in a marked loss of metabolic activity, and further demonstrate that this loss is principally attributable to the dissipation of internal cofactor gradients. When normalised to initial total cell number, the CL_{int} of midazolam in estimated 45-55% viability sonicated preparations was approximately one quarter of that in the equivalent intact cell preparations (Table 4-4), and the CL_{int} of midazolam, propranolol and saquinavir in saponin-permeabilised hepatocytes was negligible (Table 4-5). The large increases in clearance with the addition of 1 mM NADPH to such preparations (Tables 4-4 and 4-5, Figures 4-3, 4-4, 4-5 and 4-6) signal a loss or considerable dilution of this cofactor subsequent to disruption/permeabilisation, but demonstrate that the CYP enzymes responsible for metabolism of these drugs remain functional. The complete lack of clearance by saponin-treated hepatocytes (which were 100% 'non-viable') without exogenous NADPH supplementation, compared with reduced activity in unsupplemented sonicated hepatocytes (estimated 45-55% viable), suggests that the remaining activity is likely to derive only from the population of intact 'viable' cells.

These findings differ to those of Kazmi et al. (2014), who found no effect of either sonication or saponin treatment, with or without exogenous NADPH on 1'-hydroxy-midazolam formation in cryopreserved human hepatocytes. It is possible that any effect of NADPH was not detectable as a concentration of only 0.1 mM was used; preliminary work in this project (data not shown) indicated that concentrations of 0.2 mM NADPH were not sufficient to show the marked effect on midazolam clearance achieved with 1 mM. Of greater interest however, is that these researchers observed no decrease in clearance with sonication or saponin

treatment when the resulting preparations were incubated in the absence of supplemental NADPH (Kazmi et al., 2014). This may be due to the fact that midazolam is metabolised more slowly in human than rat and therefore even the vastly diluted NADPH concentration is sufficient to continue metabolism at the usual rate, but given the results in this section, it seems unlikely.

4.3.4.2 Comparison of CL_{int} in intact and disrupted hepatocytes

A secondary objective of this work was to explore the use of disrupted hepatocyte preparations (either sonicated or permeabilised) as a means to identify rate-limiting processes in CL_{int} determination; comparison of intact hepatocytes with either sonicated or permeabilised hepatocytes supplemented with metabolic cofactors in excess could theoretically identify compounds which are active uptake or permeability rate-limited in their disposition.

Although there are examples in the literature where researchers have compared the metabolic activity of intact cells with corresponding cell 'lysates', 'homogenates' or permeabilised cells (Banhegyi et al., 1993; Swales et al., 1996; Swales and Utesch, 1998; Bruni and Chang, 1999; Hewitt et al., 2000; Hewitt and Utesch, 2004; Dennison et al., 2008), the majority of these comparisons have been in the context of cofactor depletion (Swales et al., 1996; Swales and Utesch, 1998; Hewitt et al., 2000; Hewitt and Utesch, 2004). Interestingly, across and even within papers from the same group, the outcome of sonication/permeabilisation and supplementation of exogenous cofactors was mixed, appearing not only substrate-, but also species-specific. For example, in one study, total midazolam hydroxylation and p-nitrophenol glucuronidation did not differ between intact cells and homogenates supplemented with cofactor in either rat, dog or monkey, but dextromethorphan demethylation was significantly higher in homogenates of monkey only (Hewitt and Utesch, 2004). A higher CL_{int} in homogenates appears to indicate a permeability rate-limitation, rather than cofactor depletion. Indeed, Dennison et al. (2008) investigated the kinetics of vincristine metabolite formation and found cell lysates exhibited both a three-fold higher V_{max}, and a three-fold lower K_m; the smaller K_m implying a lower intracellular concentration in the intact hepatocytes relative to the external medium and suggesting that permeability may be rate-limiting in the metabolic clearance of vincristine.

In this study, although the CL_{int} of propranolol in NADPH-supplemented permeabilised hepatocytes was slightly higher on average than in intact hepatocytes both in the absence and presence of exogenous NADPH (Figure 4-5), the difference was not large enough to suggest such a permeability-rate limitation. In the case of midazolam, the CL_{int} in both sonicated and permeabilised NADPH-supplemented hepatocytes was slightly lower than that observed in intact NADPH-supplemented hepatocytes (Figures 4-3 and 4-4). Since CL_{int} values are normalised to total cell number, it would be expected that if NADPH were the only soluble intracellular constituent requiring replacement in disrupted/permeabilised cells, CL_{int}

across all NADPH supplemented preparations would be equal. It should be recognised however, that the actions of both cell disruption and membrane permeabilisation allow not only unrestricted access to substrate, but also unobstructed release of soluble components of the cell. It is possible that not only is NADPH lost as a result of cell disruption/membrane permeabilisation, but also other cytosolic coenzymes, messengers and metabolic intermediates needed for optimal cell functionality. The very similar clearances between the NADPH-supplemented condition in sonicated (197 ± 33 µl/min/10^6 cells) and permeabilised hepatocytes (203 ± 48 µl/min/10^6 cells) is noteworthy and implies that there may be a rate-limiting factor other than NADPH in these preparations. Notably, Swales et al. (1996) added both NADPH and ATP to digitonin-permeabilised rat hepatocyte incubations and in a later paper, Swales & Utesch (1998) noted the need for 'a balance' of cofactors in the case of phase II metabolism of hydroxycoumarin. Indeed, a lack of phase II conjugation could also reduce phase I metabolism via end product inhibition as described in microsomes (Jones et al., 2005). For saquinavir, a clearer distinction was evident between CL_{int} in intact and NADPH-supplemented permeabilised hepatocytes (Figure 4-6). The higher clearances in permeabilised cells (with replenishment of cofactor) are consistent with a permeability/uptake rate-limitation and are therefore in agreement with previous reports of saquinavir as an uptake rate-limited drug in rat hepatocytes (Parker and Houston, 2008).

4.3.4.3 Addition of NADPH to intact hepatocytes

The question of whether NAD+ is able to directly pass into intact cells is controversial (Bruzzone et al., 2001; Billington et al., 2008; Nikiforov et al., 2011), however, consistent with the perspective that NADPH is unable to traverse the intact plasma membrane (Jones et al., 1977; Swales and Utesch, 1998), the earlier work in the present study with cryopreserved hepatocytes (Section 4.2) appeared to indicate that exogenously added NADPH was acting only on the 'non-viable' cells. This is also supported by larger percentage increases in CL_{int} of midazolam in sonicated hepatocytes compared to intact hepatocytes (Table 4-4). The observed increases in CL_{int} with addition of NADPH to intact cell preparations are therefore likely to be due to the activity of the 'non-viable' cells which are responsive to exogenous NADPH. Although Swales et al. (1996) found that addition of NADPH and ATP to intact cells resulted in an increased rate of testosterone metabolism, to over two-fold of control values, this increase is likely to at least partially reflect the high percentage of 'non-viable' cells in the preparation (28%). It may also represent the effect of exogenous ATP on both membrane-damaged and intact cells, as this cofactor is believed to be able to cross the plasma membrane (Chaudry, 1982).

Lastly, the similar average clearances of midazolam in intact unsupplemented hepatocytes between the two separate experiments (284 ± 68 µl/min/10^6 cells and 235 ± 18 µl/min/10^6 cells (Tables 4-4 and 4-5 respectively)) confirms that despite some inherent variability, clearance predictions from this system are reproducible.

4.4 Examination of ATP content as an alternative measure of cell viability

Despite being much-favoured, determination of cell viability based purely on plasma membrane integrity has come under criticism, as such a measure doesn't necessarily reflect metabolic status or capabilities (Cook and Mitchell, 1989; Page et al., 1992; Sandker et al., 1993). Cells with a damaged or temporarily compromised plasma membrane deemed 'non-viable' using trypan blue staining may be functionally competent (Tran et al., 2011; Husmann, 2013). Alternatively, cells may have an intact membrane and appear 'viable', but are in fact metabolically compromised (Page et al., 1992), or even apoptotic (Elmore, 2007).

Aside from altered membrane integrity, there may be undetected metabolic and morphological changes which have occurred as a result of stress induced by isolation and/or cryopreservation of cells (Pang et al., 1996; Loven et al., 2005). Reversal of oxidative stress-induced changes is likely to require energy and therefore could leave hepatocytes with depleted carbohydrate reserves and/or reduced ATP levels (Loven et al., 2005). Indeed, Berry et al. (1991) noted that following a period of hypoxia, ATP levels can be reduced by between 20-40%. Pang et al. (1996) observed that cryopreserved hepatocytes, despite exhibiting normal ATP levels, displayed a higher oxygen uptake compared to freshly isolated hepatocytes. The authors suggest this may represent need for a greater energy source to replenish metabolic intermediates affected by hypoxia and to repair membrane alterations caused by cryopreservation (Pang et al., 1996). In addition, decreased glycogenolysis and gluconeogenesis, which may reflect depleted glycogen stores and a potential imbalance of metabolic cofactors, has also been reported (Loven et al., 2005). Whilst some cellular processes appear to closely correlate with plasma membrane integrity, others may decrease prematurely, possibly as a result of changes to the energy status of the cell (Dalet et al., 1982).

As there are a number of properties which may give an indication as to a cell's condition, some researchers advocate using a measure of viability which is specific to the assay purpose (Cook and Mitchell, 1989; Sandker et al., 1993), in this instance for example, metabolic activity. Page et al. (1992) claimed that trypan blue exclusion is a poor test of viability for metabolic studies using hepatocytes and suggested ATP content as an alternative, more appropriate measure of metabolic status. Berry et al. (1991) reported that the anabolic activities of cell preparations of less than 80% viability are typically reduced to a higher degree than would be assumed from the trypan blue exclusion test and suggested use of cellular ATP levels as a suitable indicator of hypoxic insult. In addition, Page et al. (1992) demonstrated that viabilities determined by trypan blue exclusion did not correlate with ATP content; only cell preparations of 99% viability or greater (as determined by trypan blue exclusion) were consistently found to have an ATP content above the study's inclusion threshold of 2.0 μmol/g wet weight. Preparations of less than 85% viability are recommended to be discarded on the basis that they are extremely unlikely to possess such

levels and between these two cut-off points there appeared to be no obvious correlation of trypan blue exclusion and ATP content (Page et al., 1992). Furthermore, the authors report anecdotal evidence of 'anomalous metabolic rates' with sub-threshold ATP levels, including unusually high clearance of ethanol (Page et al., 1992).

4.4.1 Aims

The aims of this section were to investigate whether viability as determined by trypan blue exclusion or ATP content reflects metabolic activity of isolated hepatocyte preparations. The relationships between viability and ATP content, and the CL_{int} of the selected substrates were examined.

A panel of nine drugs (chlorpromazine, diclofenac, metoprolol, midazolam, naloxone, propranolol, tolbutamide, triazolam and verapamil) spanning a wide range of clearances and metabolic pathways was utilised in order to increase the chance of identifying any trends, regarding for example specific metabolic pathways or clearances. Further information regarding drug metabolising enzymes and *in vivo* clearances for the selected drugs is given in Table 3-1.

4.4.2 Materials and methods

4.4.2.1 Materials

An ATP assay kit (colorimetric/fluorometric) containing ATP assay buffer, ATP probe, ATP converter, developer mix, and 1 µmol ATP standard was purchased from Abcam (Cambridge, UK). Black clear-bottomed 96-well plates were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). Perchloric acid, potassium hydroxide and tolbutamide were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). All other materials and reagents were as detailed in Section 3.3.1.

4.4.2.2 Hepatocyte source, isolation and preparation

Male Sprague Dawley rat hepatocytes were freshly isolated and prepared as described in Sections 3.3.2 and 3.3.3.

4.4.2.3 Trypan blue viability measurement

Viability was determined using the standard trypan blue exclusion method as described in Section 3.3.3.

4.4.2.4 ATP measurement

ATP measurement assays were performed according to the supplier's protocol for product ab83355 ATP assay kit (colorimetric/fluorometric) (see Appendix 8.7).

4.4.2.4.1 Hepatocyte samples

Specific hepatocyte preparations were selected to span a range of viabilities representative of those used or accepted in the literature; 75% viability as determined by trypan blue

exclusion was defined as the lower limit. ATP content in hepatocytes was measured according to the supplier's protocol. Briefly, following cell density estimation by use of a haemocytometer and light microscope, 10^6 viable hepatocytes were transferred into a 1.5 ml microcentrifuge tube and washed with cold DPBS before resuspension in 100 µl of ATP assay buffer. Cell suspensions were vigorously pipetted and centrifuged for 2 minutes at 13,000 rpm in a microcentrifuge to remove any insoluble material; the resulting supernatant was transferred to a clean 1.5 ml microcentrifuge tube. ATP assay buffer was added to the supernatant to make up to 550 µl, to which 100 µl of ice cold 4 M perchloric acid was added. Samples were vortexed briefly and incubated on ice for 5 minutes before centrifugation at 13,000 rpm for 2 minutes. Excess perchloric acid was precipitated by adding an equal volume (to the obtained supernatant) of 2 M potassium hydroxide. Samples were vortexed and then centrifuged for 15 minutes at 13,000 rpm. The acquired supernatant was removed to a separate 1.5 ml microcentrifuge tube and the volume measured. Details of manufacturers and models for the equipment utilised can be found in Appendix 8.4.

4.4.2.4.2 Standard curve preparation

The supplied ATP standard was reconstituted in 100 µl of MilliQ double distilled water to create a 10 mM ATP standard stock solution which was diluted 1 in 10 to generate a 1 mM standard and a further 1 in 10 to generate a 0.1 mM standard. This was used to create a standard curve ranging from 0-20 µM (final concentration).

4.4.2.4.3 Fluorometric measurement

An ATP reaction master mix consisting of 91.6% ATP assay buffer, 4% ATP converter, 4% developer mix and 0.4% ATP probe was prepared fresh on each occasion. 50 µl of reaction master mix was added to each of the required wells in a black, clear-bottomed 96-well plate. 50 µl of standard or sample was added, briefly mixed and the plate was incubated at room temperature for 30 minutes protected from light. Standards and samples were measured in duplicate on a Tecan Safire fluorescence microplate reader (Tecan Group Ltd., Männedorf, Switzerland) using excitation and emission wavelengths of 535 nm and 587 nm respectively, within 2 hours of completing the assay. Data acquisition was performed using Magellan software (v7.1, Tecan Group Ltd., Männedorf, Switzerland).

4.4.2.4.4 Data analysis

Data analysis was performed as directed in the supplier's protocol. The average absorbance was determined for each duplicate standard; corrected absorbance readings were generated by subtracting the absorbance value of the blank from itself and all other standards and samples. A plot of corrected absorbance against ATP concentration was generated for the standards and a linear trendline fitted. ATP concentration in each sample was calculated using Equation 4.1 and Equation 4.2.

Equation 4.1

$$\text{ATP (nmol)} = \frac{\text{(corrected absorbance – y-intercept)}}{\text{linear trendline gradient}}$$

Equation 4.2

$$[\text{ATP}]\ (\text{nmol}/\mu\text{l or mM}) = \frac{\text{ATP (nmol)}}{V_s}$$

where V_s is the assayed sample volume (µl).

ATP concentration was normalised to 10^6 cells by multiplying the ATP concentration by the total volume of the final supernatant and dividing by the total number of cells initially transferred to the microcentrifuge tube (Equation 4.3). ATP concentration in µmol/g liver was calculated using a PBSF of 120 x 10^6 cells/g liver (Bayliss et al., 1999) (Equation 4.4).

Equation 4.3

$$[\text{ATP}]\ (\text{nmol}/10^6\ \text{cells}) = \frac{[\text{ATP}]\ (\text{nmol}/\mu\text{l}) \cdot V_t}{\text{Total number of cells } (10^6)}$$

where V_t is the total volume (µl).

Equation 4.4

$$[\text{ATP}](\mu\text{mol/g liver}) = \frac{[\text{ATP}](\text{nmol}/10^6\ \text{cells})}{1000} \cdot \text{PBSF}$$

where PSBF is the physiologically based scaling factor of 120 x 10^6 cells/g liver.

4.4.2.5 Intrinsic clearance assays

4.4.2.5.1 Incubation conditions

With the exception of tolbutamide, all CL_{int} determinations were performed using the substrate depletion approach as described in Section 3.3.5.3 (in the absence of cofactor). Tolbutamide CL_{int} was determined by measurement of the formation of the 4'-hydroxy metabolite across four substrate concentrations, as the very low CL_{int} of this drug prevented accurate measurement of substrate depletion. All drugs were incubated in 96-well format; individual incubation conditions are given in Table 4-6.

Table 4-6. Incubation conditions of drugs selected to represent metabolic activity of isolated rat hepatocytes in ATP study.

Drug	Hepatocyte Density (10^6 cells/ml)	Substrate Concentration (µM)	Incubation Time (min)
Chlorpromazine	0.125	0.1	7
Diclofenac	0.5	1	15
Metoprolol	0.5	0.1	15
Midazolam	0.5	0.1	15
Naloxone	0.125	0.1	7
Propranolol	0.125	0.1	7
Tolbutamide	1	1, 2.5, 5, 10	30
Triazolam	0.5	0.1	15
Verapamil	0.25	0.1	7

4.4.2.5.2 Sample preparation and liquid-chromatography-mass spectrometry analysis

Incubation samples were treated and prepared for LC-MS/MS analysis in the same manner as described in Section 3.3.7. The LC-MS/MS systems and conditions used for individual drugs and the associated internal standards are given in Appendix 8.5.Procedures for sample quantification were also as given in Section 3.3.7.

4.4.2.5.3 Data analysis

Substrate depletion data were analysed in the same manner as described in Section 3.3.8.1. For tolbutamide, 4'-hydroxy-tolbutamide formation was quantified over time to calculate a rate of formation at each substrate concentration. As the K_m of 4'-hydroxy-tolbutamide formation in rat hepatocytes was reported to be 1390 ± 490 µM (Griffin and Houston, 2004) and 650 µM (Ashforth et al., 1995), it is assumed that at concentrations of 10 µM and below, the rate of metabolite formation is linear with respect to substrate concentration. Based on this assumption, CL_{int} was determined using Equation 4.5.

Equation 4.5

$$CL_{int} = \frac{\Delta v}{\Delta [S]}$$

where CL_{int} is the intrinsic clearance (µl/min/10^6 cells), v is reaction velocity (pmol/min/10^6 cells) and [S] is the substrate concentration (µM).

4.4.2.6 Statistical analysis

The arithmetic mean and standard deviation was calculated for the CL_{int} of each drug. For each assay, the percentage deviation from average CL_{int} was also calculated for each drug. This was compared to viability and ATP content of the associated hepatocyte preparation to allow an overall comparison and identification of possible trends.

Pearson product-moment correlation coefficients (r) and coefficients of determination (r^2) were computed using GraphPad Prism software, (v6.04, California, USA) to assess the relationship between ATP content and viability. Equivalent analyses were also performed to assess the relationship between the CL_{int} of individual drugs and viability, and the CL_{int} of individual drugs and ATP content. In addition, to examine the existence of any overall relationship between metabolic activity and viability or ATP content, linear regression was performed using the deviation from average CL_{int} data of all drugs combined into a single group.

A correlation matrix of r values was generated to identify any potential trends or correlations between the CL_{int} of individual drugs.

4.4.3 Results

Viability as determined by trypan blue exclusion, ATP content and metabolic capacity as represented by the CL_{int} of nine model substrates (chlorpromazine, diclofenac, metoprolol, midazolam, naloxone, propranolol, tolbutamide, triazolam and verapamil) were assessed for individual hepatocyte preparations. For one of the eight preparations metabolic clearance data was not collected and for another, the measurement of ATP content was excluded due to poor agreement between duplicate measurements of the standard curve. The viability of hepatocyte preparations ranged from 75-93% (average 84 ± 6%). The ATP content ranged between 1.24 and 2.50 µmol/g liver (average 1.62 ± 0.41 µmol/g liver); only one of the preparations had an ATP content above 2.00 µmol/g liver. The average CL_{int} across the nine drugs selected to represent metabolic capacity spanned six orders of magnitude (

Table 4-7).

Table 4-7. CL_{int} of selected drugs in suspended rat hepatocytes in metabolic assays of ATP study. Data represent mean ± SD of seven separate preparations; drugs are presented in descending order of *in vitro* CL_{int}.

Drug	CL_{int} (µl/min/10^6 cells)
Chlorpromazine	2361 ± 610
Propranolol	2169 ± 754
Naloxone	1510 ± 252
Verapamil	949 ± 192
Midazolam	758 ± 914
Triazolam	391 ± 87
Metoprolol	144 ± 56
Diclofenac	129 ± 22
Tolbutamide	0.08 ± 0.03

4.4.3.1 Correlation of ATP content with viability

The ATP content was determined for seven out of eight hepatocyte preparations. There was found to be no significant correlation between ATP content and viability; six preparations ranging from 75-90% viability were found to have very similar ATP contents (1.24-1.65 µmol/g liver). The preparation of 93% viability did however show an elevated ATP content of 2.50 µmol/g liver (Figure 4-7).

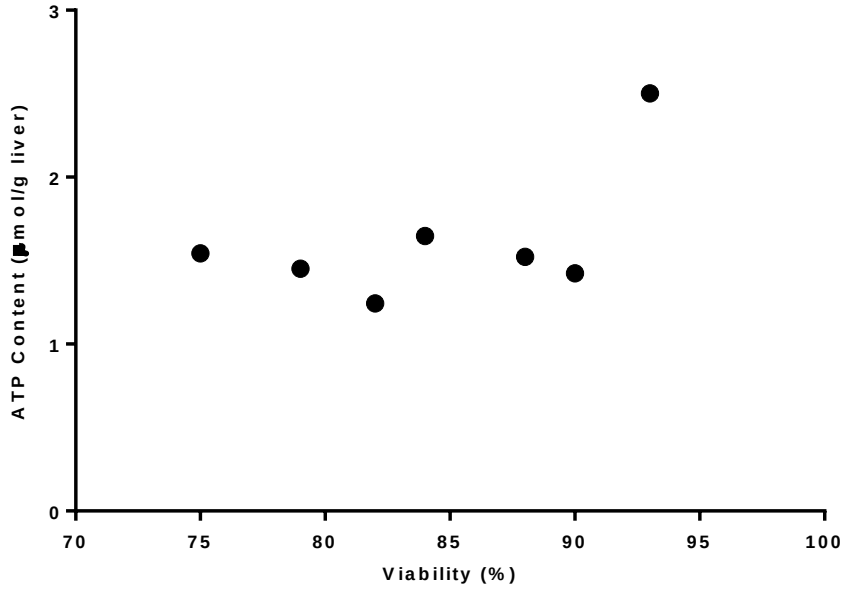


Figure 4-7. Relationship between ATP content and viability (determined by trypan blue exclusion) of isolated rat hepatocyte preparations.

4.4.3.2 Correlation of metabolic activity with viability

The CL_{int} of the selected drugs was determined in seven out of eight hepatocyte preparations. To enable comparison of trends across the panel of drugs, the percentage deviation from average CL_{int} was calculated for each drug, for each assay, and is presented as a function of viability in Figure 4-8. Across the assayed preparations, there was no clear trend of CL_{int} (relative to average) with viability (Figure 4-8). The Pearson product-moment correlation coefficient, r, was calculated for the measured CL_{int} against viability for individual drugs; no significant correlations were found (Table 4-8). A correlation analysis was also performed for the deviation from average CL_{int} of all drugs (grouped) against viability; no significant correlation was found (Table 4-8, Figure 4-9).

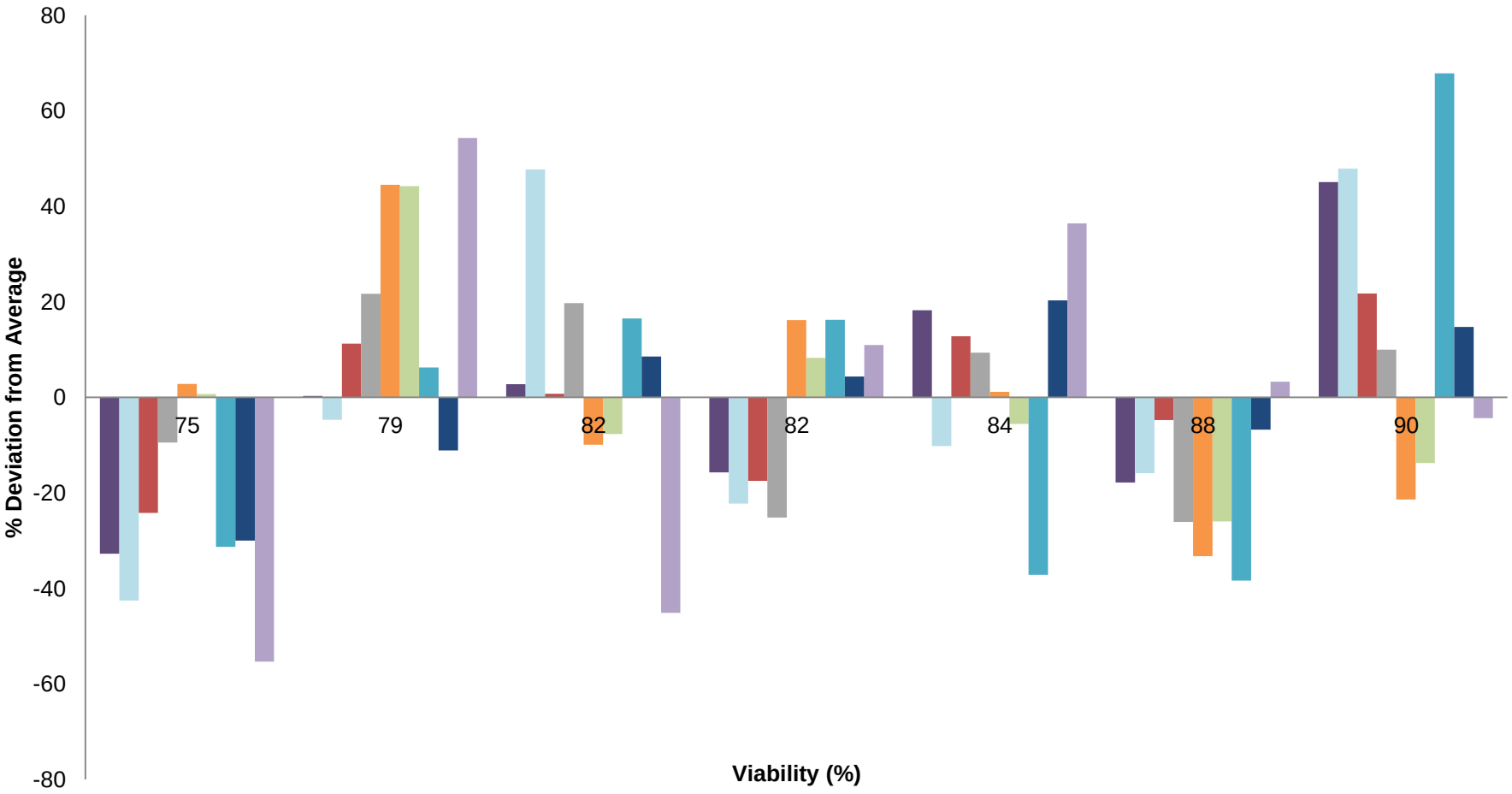


Figure 4-8. Percentage deviation from average metabolic CL_{int} across viability (as determined by trypan blue exclusion) for chlorpromazine (■), propranolol (■), naloxone (■), verapamil, (■), midazolam (■), triazolam (■), metoprolol (■), diclofenac (■) and tolbutamide (■).

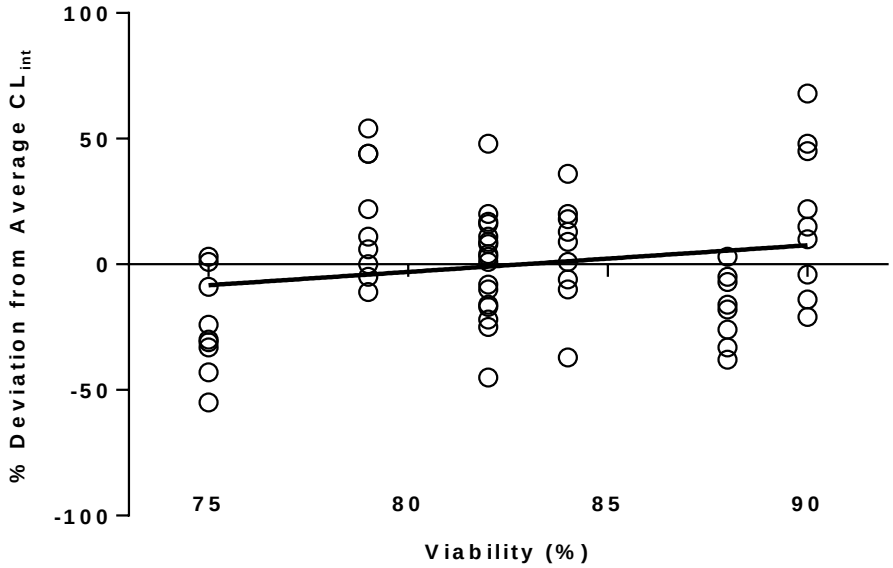


Figure 4-9. Correlation of percentage deviation from average CL_{int} of all drugs with viability of hepatocyte preparations. Solid line represents linear regression line.

Table 4-8. Correlational analyses of viability with CL_{int}.

Drug	r	p
Chlorpromazine	0.654	0.11
Propranolol	0.543	0.21
Naloxone	0.598	0.16
Verapamil	-0.091	0.85
Midazolam	-0.666	0.10
Triazolam	-0.607	0.15
Metoprolol	0.369	0.42
Diclofenac	0.681	0.09
Tolbutamide	0.231	0.62
All[§]	0.324	0.13

n = 7, [§] The deviation from average CL_{int} for each compound in each preparation analysed as a single group.

4.4.3.3 Correlation of metabolic capacity and ATP content

An equivalent analysis was performed regarding ATP content and CL_{int} for six of the hepatocyte preparations. There appeared no obvious trend between these two measures; the preparations with the highest and lowest ATP contents showed a similar number of drugs with CL_{int} above and below average (Figure 4-10). Correlation analysis revealed no significant correlation between ATP content and CL_{int} for any of the investigated drugs individually or when analysed as a single group (Table 4-9, Figure 4-11).

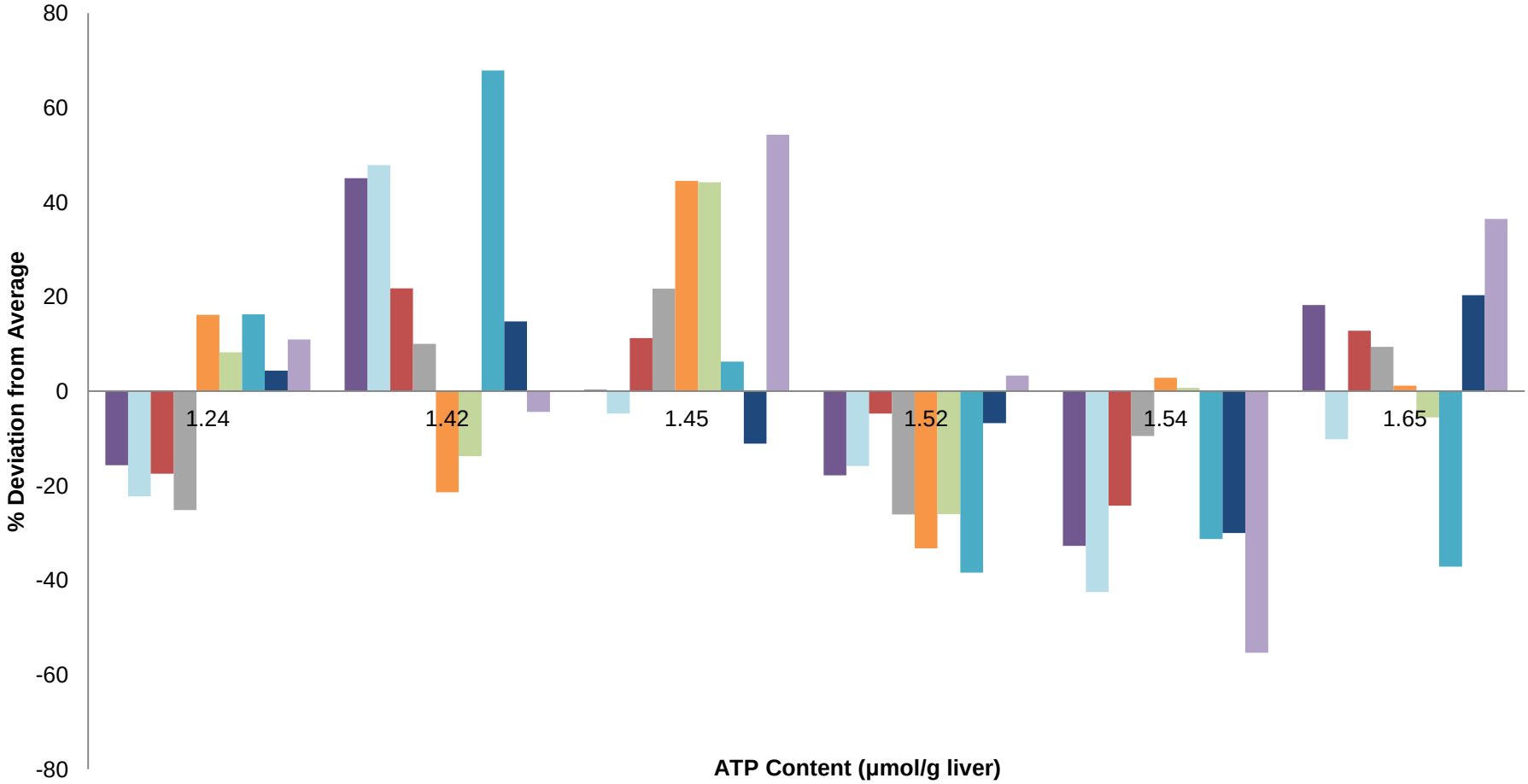


Figure 4-10. Percentage deviation from average metabolic CL_{int} across ATP content for chlorpromazine (■), propranolol (■), naloxone (■), verapamil, (■), midazolam (■), triazolam (■), metoprolol (■), diclofenac (■) and tolbutamide (■).

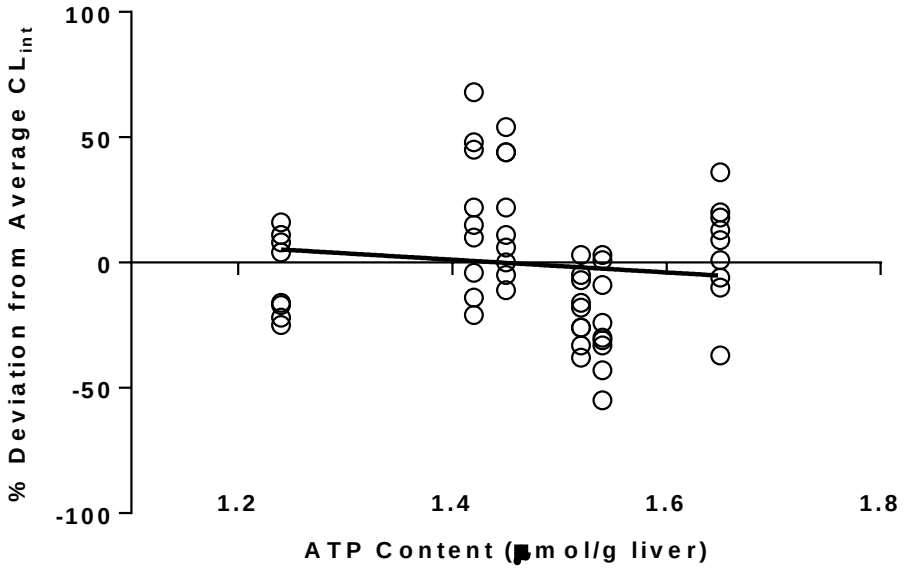


Figure 4-11. Correlation of percentage deviation from average CL_{int} of all drugs with ATP content of hepatocyte preparations. Solid line represents linear regression line.

Table 4-9. Correlational analyses of ATP content with CL_{int}.

Drug	r	p
Chlorpromazine	0.070	0.90
Propranolol	-0.133	0.80
Naloxone	0.238	0.65
Verapamil	0.325	0.53
Midazolam	-0.261	0.62
Triazolam	-0.268	0.61
Metoprolol	-0.625	0.18
Diclofenac	-0.033	0.95
Tolbutamide	-0.035	0.95
All[§]	-0.193	0.38

n = 6, [§] The deviation from average CL_{int} for each compound in each preparation was analysed as a single group.

4.4.3.4 Correlation of CL_{int} of individual drugs

A Pearson correlation matrix was generated to identify the presence of any correlations between the CL_{int} of individual drugs (Table 4-10). Strong correlations were observed between the CL_{int} of midazolam and triazolam ($r = 0.976$, $n = 7$, $p = 0.0002$), and the CL_{int} of chlorpromazine and naloxone ($r = 0.914$, $n = 7$, $p = 0.004$); moderate-strong significant correlations were also found between the CL_{int} of diclofenac and chlorpromazine ($r = 0.780$, $n = 7$, $p = 0.038$), and the CL_{int} of propranolol and chlorpromazine ($r = 0.756$, $n = 7$, $p = 0.045$).

Table 4-10. Pearson correlation matrix for the CL_{int} of selected drugs in suspended rat hepatocytes across seven incubations within the ATP study. Yellow shading indicates a strong correlation (r > 0.9), green shading indicates a moderate-strong correlation (0.9 > r > 0.6) and blue shading indicates a moderate correlation (0.6 > r > 0.4); * p < 0.05, ** p < 0.01, *** p < 0.001 using Student's paired *t*-test.

	Chlorpromazine	Propranolol	Naloxone	Verapamil	Midazolam	Triazolam	Metoprolol	Diclofenac	Tolbutamide
Chlorpromazine		0.765*	0.914**	0.591	-0.180	-0.121	0.646	0.780*	0.320
Propranolol			0.685	0.617	-0.314	-0.211	0.738	0.623	-0.110
Naloxone				0.685	-0.059	0.037	0.447	0.661	0.522
Verapamil					0.323	0.400	0.358	0.296	0.157
Midazolam						0.976***	0.012	-0.247	0.478
Triazolam							0.102	-0.292	0.482
Metoprolol								0.396	-0.025
Diclofenac									0.343
Tolbutamide									

4.4.4 Discussion

Following the results of the previous sections in this chapter which highlighted that plasma membrane integrity does not necessarily reflect the metabolic capability of a cell, the suggested use of ATP content as a more appropriate indicator of metabolic status/capability (Page et al., 1992) and its correlation with viability, as determined by trypan blue exclusion, was investigated. Metabolic activity was represented by the metabolic clearance of nine drugs encompassing observed *in vivo* $CL_{int,u}$ over five orders of magnitude and multiple metabolic pathways including both CYP oxidation and glucuronidation (Table 3-1).

4.4.4.1 Correlation of ATP content with viability

To investigate the relationship between ATP content and viability (trypan blue exclusion), the ATP content of seven hepatocyte preparations spanning a range of viabilities (75-93%) was determined. Based on the results of this study, in which no statistically significant correlation was found between these two measures, trypan blue exclusion does not appear to be a good indicator of metabolic status. Although the sample size was small, the results are in agreement with Page et al. (1992) who conducted 289 paired assessments of viability (by trypan blue exclusion) and ATP content and also found no correlation. It should be acknowledged however, that Page et al. (1992) focussed specifically on the correlation of ATP content and viability in preparations of between 86-100%, and as such only three of the preparations tested in this study are suitable for equivalent comparison. Notably, the authors of this paper regarded an ATP content of 2 µmol/g wet weight of cells as the minimum value of a viable preparation (Page et al., 1992); in this study, only a single tested preparation (that of the highest viability) was found to have an ATP content above this cut-off. Of the five preparations below 85% viability, none had an ATP content above 2 µmol/g liver (Figure 4-7), which is consistent with their reports that preparations of less than 85% viability are extremely unlikely to meet the ATP content criterion (Page et al., 1992).

4.4.4.2 Correlation of metabolic activity with viability

As lower viability hepatocyte preparations are generally not recommended for use in metabolic studies (Berry et al., 1991), yet the lower viability preparations gave equivalent CL_{int} of midazolam (when normalised to total cell number) in Section 4.2.3.1, it was decided to compare the CL_{int} of the afore-mentioned model substrates in hepatocytes of varying viabilities (ranging from 75-90%). Correlational analyses were used to examine the relationships between viability and the CL_{int} of individual drugs, as well as between viability and the CL_{int} of all drugs relative to their average (Table 4-8). If it is assumed that preparations of greater viability have suffered less oxidative stress and are more metabolically competent (Berry et al., 1991), a positive correlation between the two might be expected. Alternatively, if it is assumed that the 'non-viable' cells in the incubation may contribute some metabolic activity which is unaccounted for when normalised to viable cell number, higher clearances in the lower viability preparations would be expected, resulting in a negative correlation. In fact, moderate to strong correlations were observed in both the

positive (diclofenac, chlorpromazine, naloxone, propranolol) and negative (midazolam, triazolam) directions (although none reached statistical significance) (Table 4-8). As correlations of the CL_{int} of individual drugs with viability were both positive and negative, unsurprisingly there was no significant trend between viability and the relative CL_{int} of the drugs as a combined group (Table 4-8, Figure 4-9). A simple explanation for this would be that there is no correlation between viability and metabolic activity and the observed trends are coincidental. It may also be that the complex combination of factors and processes which viability represents is reflected in substrate dependence; many more replicates and a better understanding of the rate-limiting processes of CL_{int} of individual drugs would be required before any firm conclusions could be drawn. These results however, do give an indication that metabolic capacity is dependent on more than simply viability (as determined by plasma membrane integrity). The usefulness of trypan blue exclusion as a measure of viability can be further questioned as for some drugs the CL_{int} in lower viability preparations is equal to or greater than that in higher viability preparations.

Also worthy of consideration is the accuracy of manual cell counting and viability determination, which is particularly pertinent when CL_{int} or any other cellular function is to be normalised to hepatocyte number (as in this study) rather than protein concentration (Wigg et al., 2003). The inherent error in manual counting using a haemocytometer is reportedly 8-10% (Biggs and Macmillan, 1948) and is both area- and concentration-dependent (Hsiung, 2013). However, the 'technical error' arising from misuse or inter-user variability can be up to 16% in addition to this (Hsiung, 2013). Automated systems which use propidium iodide and fluorescein diacetate have been advocated as a more accurate means of cell number estimation (Jones and Senft, 1985; Wigg et al., 2003; Hsiung, 2013). Additionally, Wigg et al. (2003) found that whilst viability as assessed by trypan blue exclusion correlated only with CYP content, viability assessed by automated methods using propidium iodide correlated, in addition to CYP content, with lactate dehydrogenase leakage, ATP concentration, urea synthesis, ammonia removal, lactate removal and albumin synthesis. Incorrect determination of cell number and/or viability could at least partially account for some of the variability observed in *in vitro* CL_{int} measurements.

4.4.4.3 Correlation of metabolic activity with ATP content

Following the recommendation of Page et al. (1992) that ATP content be used an alternative index of viability which more reliably reflects metabolic activity, correlational analyses were also performed to assess the relationships between ATP content and the CL_{int} of individual drugs, as well as between ATP content and the CL_{int} of all drugs relative to their average. Should ATP content truly reflect the metabolic activity of isolated hepatocytes, one would expect to see positive correlations between ATP content and the CL_{int} of all tested drugs. However, analogous to the comparison with viability, no statistically significant correlations were found between ATP content and the CL_{int} of individual drugs (Table 4-9). Predictably, no significant correlation was found between ATP content and the CL_{int} relative to average of

the drugs as a single group also (Table 4-9). In fact, the linear regression model fit was negative (Figure 4-11). In general, the correlations between ATP content and CL_{int} were weaker than those between viability and CL_{int}. ATP levels were also largely similar across preparations, suggesting that measurement of ATP using this method may not be sensitive enough to be useful. Despite the relatively small sample size, these results appear to suggest that ATP content is no better marker of metabolic capability than plasma membrane integrity.

4.4.4.4 Correlation of CL_{int} of individual drugs

Whilst no significant correlation was found between the CL_{int} of individual drugs or all drugs and either viability or ATP content, significant correlations were found between the CL_{int} of individual drugs. Strong correlations were found between the CL_{int} of midazolam and triazolam ($r = 0.976$, $n = 7$, $p = 0.0002$) and the CL_{int} of chlorpromazine and naloxone ($r = 0.914$, $n = 7$, $p = 0.004$). The correlation between the two benzodiazepines is interesting, but perhaps not unexpected since both have a very similar structure and are predominantly metabolised by CYP3A4 (Table 3-1) by the same pathways. The correlation between chlorpromazine and naloxone is also of interest, as the common route of metabolism of these drugs is glucuronidation. Such a correlation indicates that both chlorpromazine and naloxone may be suitable probe substrates for UGT activity. Other moderate-strong significant correlations were identified between the CL_{int} of diclofenac and chlorpromazine ($r = 0.780$, $n = 7$, $p = 0.038$) and the CL_{int} of propranolol and chlorpromazine ($r = 0.756$, $n = 7$, $p = 0.045$). Like chlorpromazine, diclofenac and propranolol also undergo both CYP oxidation and glucuronidation; propranolol is a substrate of human CYP isoforms 1A2 and 2D6, in common with chlorpromazine (Table 3-1). Whilst identification of correlations between the CL_{int} of different drugs was not the primary aim of this study, such results have demonstrated that rather than viability or ATP content, a more accurate indication of the metabolic activity of a hepatocyte preparation could be the metabolism of a metabolically relevant probe substrate or positive control.

4.5 Conclusion

The experimental work presented in this chapter questions the use of the popular viability measurement, trypan blue exclusion. Contrary to previous assumptions, the viability of a hepatocyte preparation (as determined by trypan blue exclusion) does not appear to influence the metabolic activity of the viable cells within in it. In fact, the results of this study indicate that cells considered ‘non-viable’ by trypan blue exclusion may retain enzymatic activity. Consequently, failure to account for these cells could lead to an overestimation of CL_{int}. The use of low viability preparations is not recommended for determinations of CL_{int} of membrane-permeability or active-uptake rate-limited drugs to avoid such complications. Investigation of ATP content as an alternative measure of viability revealed this to be no better indicator of metabolic activity than trypan blue exclusion. The employment of either measure as a determinant of the use of a preparation is effectively redundant. Instead, the

CL_{int} of a metabolically similar probe substrate or positive control is proposed as a more accurate representation of the metabolic activity of hepatocyte preparation and means of identification of anomalous results.

Chapter 5. Effect of shaking on CL_{int} determinations in rat hepatocytes

5.1 Introduction

As indicated previously, a general consensus is lacking in regard to several variables of hepatocyte CL_{int} assays; notably, there is very little literature which examines the effect of shaking on metabolic clearance predictions. Whilst Berry et al. (1991) commented on the need to adapt shaking conditions to maintain both even suspension and viability of hepatocytes, no indication as to how this may impact the outcome of the assay was provided. Later, Bruni and Chang (1999) briefly reported shaking of hepatocytes at 240 rpm because below this speed the metabolism of bilirubin was negligible, but also gave no explanation regarding these observations. In general, most authors either do not report shaking conditions or do not detail the optimisation of them. Since it is generally presumed that vigorous shaking could have detrimental effects on the structural integrity of hepatocytes (Seglen, 2013), this may explain why many researchers avoid the use of shaking altogether. As illustrated in the previous chapter, damage to the plasma membrane of hepatocytes could facilitate unrestricted access of substrate into cells which are able to maintain metabolic enzyme functionality. However, as cells quickly settle out in unshaken incubations, there may be advantages to keeping hepatocytes in suspension. Prevention of excessive aggregation, as well as a potential reduction in the depth of the UWL surrounding cells, may facilitate increased oxygenation and access of both substrate and nutrients within the media.

The UWL is an undefined region adjacent to a cellular membrane in which the solvent travels markedly slower than in the bulk solution (Korjamo et al., 2009). It presents a diffusional barrier for solutes between the bulk medium and membrane and can therefore reduce the concentration of solute present at the cell surface relative to the surrounding medium. The depth of an UWL is dependent on a number of factors including the diffusion coefficient of the solute (partially a reflection of its size) and its permeability across the selected membrane (Pohl et al., 1998; Korjamo et al., 2009). Whilst the UWL occurs both *in vivo* and *in vitro*, the depth, and therefore contribution to overall compound permeability, of the UWL *in vivo*, appears to have been previously overestimated, at least with respect to intestinal permeability (Lennernas, 1998). On the contrary, the effects of its presence on *in vitro* assays have perhaps been underestimated (Winne, 1973; Naruhashi et al., 2003; Avdeef et al., 2004; Korjamo et al., 2009; Shibayama et al., 2015). Failure to take into account the UWL in *in vitro* assays can lead to false representations of membrane permeability in the parallel artificial membrane permeability assay (PAMPA), Caco-2 or Madin-Darby canine kidney (MDCK) cell-based assays (Avdeef et al., 2004; Korjamo et al., 2009; Ghosh et al., 2014); an overestimation of K_m values of multi-drug transporters (Winne, 1973; Barry and Diamond, 1984; Korjamo et al., 2009; Shibayama et al., 2015); or masking of efflux transporter involvement in a compound's distribution (Naruhashi et al., 2003). It has

been widely reported that the depth of the UWL may be reduced by shaking or agitation (Barry and Diamond, 1984; Williams et al., 1990; Avdeef et al., 2004; Ghosh et al., 2014; Shibayama et al., 2015), but the broader impact of shaking, or lack of it, on CL_{int} assays has not been demonstrated.

5.2 Aims

The aim of this work was to investigate the effect of shaking on *in vitro* metabolic clearance. Midazolam, a well-characterised CYP3A substrate (Ghosal et al., 1996; Shaw et al., 2002) was used as an initial probe for optimisation of shaking speed. A panel of 12 well-studied small molecule drugs (midazolam included) spanning a wide range of metabolic clearances and pathways (Table 3-1) were used to further characterise the effect of shaking on CL_{int} determinations in freshly isolated rat hepatocytes. Following work in Chapters 3 and 4, the effect of shaking in combination with exogenous NADPH supplementation was also studied.

Subsequently, further experiments were undertaken to determine the mechanism of the observed increases in CL_{int} with shaking. Firstly, the effect of an extended shaken pre-incubation period, prior to static incubation of hepatocytes with substrate, was examined using midazolam. Secondly, NADPH-supplemented saponin-permeabilised hepatocytes were used as a system in which the plasma membrane could not present a permeability barrier, but where an UWL may remain. Finally, hepatocyte sonicates (hepatocytes subject to gross membrane destruction by ultrasonication) provided a simpler comparative system in which neither a functional plasma membrane, nor an equivalent-sized UWL, would be expected to be present. As in Chapter 4, midazolam, propranolol and saquinavir were used as model substrates to represent a range of clearances and alternative rate-limiting processes. Tolbutamide was selected as a low clearance drug, almost exclusively metabolised by CYP enzymes (Miners and Birkett, 1996), the CL_{int} of which is likely to be metabolism rate-limited rather than uptake- or permeability rate-limited as the reported CL_{int} in hepatocytes and microsomes is similar (see Appendix 8.3).

5.3 Materials and Methods

5.3.1 Materials

All materials and reagents were as detailed in Sections 3.3.1 and 4.3.2.1 or purchased from Sigma-Aldrich Company Ltd. (Dorset, UK).

5.3.2 Hepatocyte source, isolation and preparation

Freshly isolated male Sprague Dawley rat hepatocytes were prepared as described in Section 3.3.3; viability was consistently above 85%.

5.3.3 Intrinsic clearance prediction assays

Details of manufacturers and models for the equipment utilised in the below studies can be found in Appendix 8.4.

5.3.3.1 Optimisation of shaking speed

Preliminary experiments were undertaken to assess the effect of shaking on CL_{int} in substrate depletion assays performed in 96-well plate format. Midazolam (2.5 µM) was used as a model substrate. Four shaking conditions were chosen for investigation: static, 450 rpm, 900 rpm and 1050 rpm. Incubations were performed on a microtiterplate shaker as described in Section 3.3.5.3 with the modification that incubation time was reduced to 10 minutes, with six time points at 0, 1, 2.5, 4, 6 and 10 minutes. Hepatocytes from each preparation were incubated in all four conditions and experiments were performed on three separate occasions.

5.3.3.2 Effect of shaking on CL_{int} determinations

A panel of 12 drugs spanning a range of metabolic clearances and pathways (Table 3-1) were used to further assess the effect of shaking on CL_{int}. Briefly, hepatocytes were pre-incubated for 5 minutes on a microtiterplate shaker either unshaken or at 900 rpm before the addition of substrate (and continuation of the selected shaking condition). Details of drug-specific incubation conditions are given in Table 5-1. A substrate concentration of 0.1 µM was typically employed based on the findings in Section 3.4.4.1. As the CL_{int} of diclofenac was shown not to differ between 0.1 µM and 1 µM (Section 3.4.4.1), the higher concentration was used due to limited LC-MS/MS sensitivity for this drug. A substrate concentration of 1 µM was also used for saquinavir due to the high degree of non-specific binding observed for this drug (Parker and Houston, 2008). 2.5 µM midazolam was utilised in order that comparisons could be made with CL_{int} determinations in other studies within this project undertaken at this concentration. Tolbutamide concentrations substantially lower than the reported $K_{m,}$ but from which metabolite formation was measurable, were selected to ensure that the rate of metabolite formation was linear with respect to substrate concentration and therefore CL_{int} could be calculated using Equation 4.5. Hepatocyte concentrations and incubation times were chosen in which at least 50% linear depletion of substrate was observed in preliminary studies. Substrate depletion was quantified at eight time points throughout the incubation to form a depletion profile from which a rate constant was derived and used for subsequent CL_{int} calculation (for further details, see Section 3.3.8.1). Incubations were performed in duplicate using at least three different hepatocyte preparations.

Table 5-1. Incubation conditions of 12 drugs selected to assess the effect of shaking on *in vitro* CL_{int}.

Drug	Hepatocyte Density (10^6 cells/ml)	Substrate Concentration (µM)	Incubation Time (min)
Chlorpromazine	0.125	0.1	7
Dextromethorphan	0.25	0.1	15
Diclofenac	0.5	1	15
Metoprolol	0.5	0.1	15
Midazolam	0.5	2.5	15
Naloxone	0.125	0.1	7
Propafenone	0.125	0.1	7
Propranolol	0.125	0.1	7
Saquinavir	0.125	1	15
Tolbutamide	1	1, 2.5, 5, 10	60
Triazolam	0.5	0.1	15
Verapamil	0.25	0.1	7

5.3.3.3 Interaction between shaking and exogenous NADPH addition on CL_{int} determinations

As previous experiments revealed moderate increases in the CL_{int} of midazolam with the addition of 1 mM NADPH to hepatocyte incubations (Section 3.4.3.1), the interaction between shaking speed and the effect of supplemental NADPH was investigated. Midazolam (2.5 µM), propranolol (0.1 µM) and saquinavir (1 µM) were used as probe substrates as described in Section 4.3.2.5. Assays were performed as described in Section 3.3.5.3 using the incubation conditions given in Table 5-1

.5.3.3.4 Effect of a shaken pre-incubation period on CL_{int} of midazolam in static hepatocyte incubations

To determine if shaking has an irreversible effect on the metabolic activity of rat hepatocytes, the effect of a 15 minute shaken pre-incubation period prior to a static incubation with substrate was assessed using midazolam (2.5 µM). Freshly isolated hepatocytes were diluted to 1 x 10^6 viable cells/ml and pre-incubated for 15 minutes (the duration of previous midazolam incubations) at 37°C with and without NADPH (final concentration 1 mM). Plates were either unshaken on a dual block dry bath heating system or shaken at 900 rpm on a microtiterplate shaker. Following the pre-incubation step, the 96-well plate containing the

shaken hepatocytes was transferred to a dual block dry bath heating system. The midazolam substrate depletion assay (as detailed in Section 3.3.5.3) was performed simultaneously on both plates (static).

5.3.3.5 Effect of shaking on CL_{int} determinations in saponin-permeabilised hepatocytes

Midazolam, propranolol, saquinavir and tolbutamide were used as probe substrates to assess the effect of shaking on saponin-permeabilised hepatocytes. Permeabilised hepatocytes were prepared as described in Section 4.3.2.4. Due to the higher cell concentration in tolbutamide assays, hepatocytes were incubated with 0.01% w/v saponin at 2 x 10^6 viable cells/ml. Total permeabilisation of preparations (0% viability as assessed by trypan blue exclusion) was confirmed before use. The incubation conditions were as given in Table 5-1 and assays were performed as detailed in Section 4.3.2.5, statically and at 900 rpm.

5.3.3.6 Effect of shaking on saquinavir CL_{int} in hepatocyte sonicates

Freshly isolated rat hepatocytes (of concentration 1 x 10^6 viable cells/ml) were sonicated for 8 x 1 second pulses at 50% power using an ultrasonic probe homogeniser. This sonication protocol was selected to give complete destruction of the plasma membrane of all cells in the suspension (no intact cells observed under a light microscope) at a low power and duration to minimise potential damage to metabolic enzymes. The resulting hepatocyte sonicates were subsequently diluted one in four to give the equivalent of 0.25 x 10^6 viable cells/ml for use in the substrate depletion assay. Assays were performed as detailed in Section 4.3.2.5, statically and at 900 rpm.

5.3.4 Sample preparation and liquid chromatography-mass spectrometry analysis

Incubation samples were treated and prepared for LC-MS/MS analysis in the same manner as described in Section 3.3.7. Details of the LC-MS/MS systems and analytical conditions used for individual drugs and the associated internal standards are given in Appendix 8.5. The procedures for sample quantification were also as given in Section 3.3.7.

5.3.5 Data analysis

Generated *in vitro* data were analysed and CL_{int} (µl/min/10^6 cells) calculated in the same manner as described in Section 3.3.8.1; non-specific loss of substrate was accounted for as described in Section 4.3.2.7.

5.3.5.1 Comparison to literature microsomal $CL_{int,u}$ data

To allow comparison of hepatocyte CL_{int} data generated in this study with literature microsomal and *in vivo* data, hepatocyte CL_{int} (µl/min/10^6 cells) and microsomal CL_{int} (µl/min/mg protein) were scaled to $CL_{int,u}$ (ml/min/kg) as described in Section 2.3.3.

Where possible, fu_{heps} values from published experimental data were used and if necessary corrected for cell concentration using Equations 3.2 and 3.3. Where experimental data were unavailable, fu_{heps} was calculated using Equation 2.2.

Where possible, fu_{mic} values from published experimental data were used and if necessary corrected for protein concentration using Equation 5.1 (Gertz et al., 2008; Kilford et al., 2008) and Equation 5.2 (rearrangement of Equation 5.1 to obtain K_a). Where experimental data were unavailable, fu_{mic} was calculated using Equation 2.1.

Equation 5.1

$$fu_{mic} = \frac{1}{1 + K_a \bullet P}$$

Equation 5.2

$$K_a = \frac{1 - fu_{mic}}{fu_{mic} \bullet P}$$

where K_a is the microsomal binding affinity equilibrium constant and P is the microsomal protein concentration (mg/ml).

5.3.5.2 Evaluation of the accuracy of $CL_{int,u}$ predictions

Predicted $CL_{int,u}$ (ml/min/kg) in static and shaken incubations was compared to observed *in vivo* $CL_{int,u}$ (ml/min/kg) derived from CL_h literature data. AFE (Equation 2.6) and number of predictions within-, above- and below two-fold of observed $CL_{int,u}$ were used as measures of bias and prediction accuracy, RMSE (Equation 2.7) was used as a measure of precision.

5.3.6. Statistical analysis

The arithmetic mean and standard deviation were calculated for each CL_{int} value and, where deemed appropriate, a CV was also calculated. A one-way repeated measures ANOVA was used to test for a statistically significant difference between the four shaking conditions (static, 450 rpm, 900 rpm and 1050 rpm) in optimisation of shaking speed. A two-tailed, paired Student's *t*-test was used to determine the existence of a statistically significant difference between two paired conditions (for example static and shaking); values were considered significant when $p < 0.05$. The average fold increase between static and shaken conditions was determined by dividing the average CL_{int} in shaken incubations by the average CL_{int} in static incubations for each drug. The percentage increase between unsupplemented and NADPH-supplemented conditions was determined by calculating the percentage increase between the two conditions for each hepatocyte preparation and taking a mean of these values. In some instances (Section 5.4.6 and 5.4.7) multiple comparisons were performed using Student's paired *t*-tests across the same dataset; it should be noted that the reported p values are not corrected for multiple comparisons.

5.4 Results

5.4.1 Optimisation of shaking speed

The effect of shaking speed on CL_{int} was initially investigated using midazolam as a probe substrate. Hepatocytes of three different preparations were used for substrate depletion assays performed unshaken (static), at 450 rpm, 900 rpm and 1050 rpm (Table 5-2, Figure 5-1). Upon visual inspection, in static incubations and at 450 rpm hepatocytes did not appear to be suspended, but rather aggregated at the bottom of each well; at 900 rpm and 1050 rpm hepatocytes appeared to be suspended throughout the incubation medium.

Table 5-2. CL_{int} of midazolam in rat hepatocytes at different shaking speeds. Data represent mean ± SD (CV (%)) of three separate preparations.

Static	450 rpm	900 rpm	1050 rpm
		µl/min/10^6 cells	
95 ± 19 (20)	78 ± 5 (6)	223 ± 79 (34)	225 ± 101 (45)

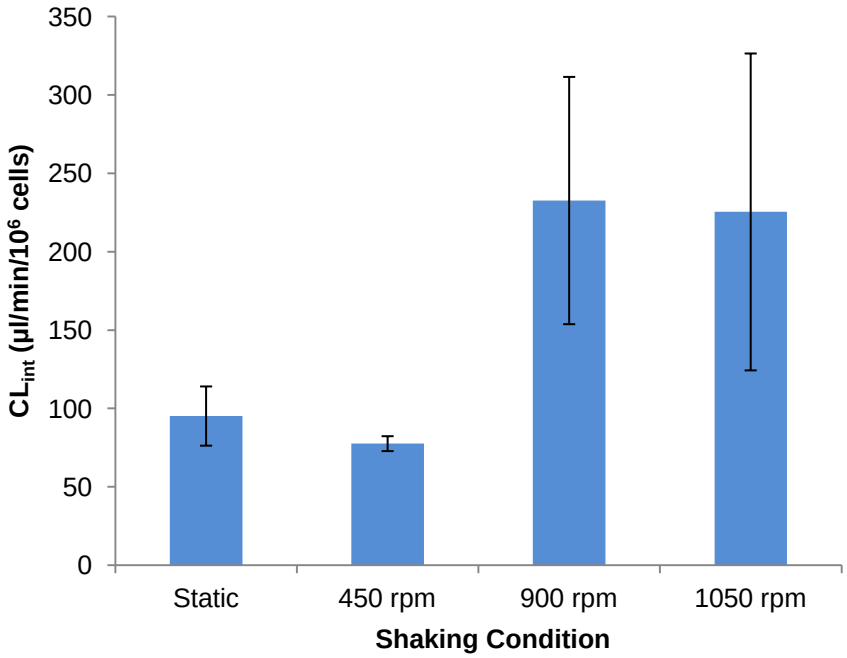


Figure 5-1. CL_{int} of midazolam in rat hepatocytes at different shaking speeds. Data represent mean ± SD of three separate preparations.

The determined CL_{int} of midazolam across these four shaking conditions was separated into two distinct groups: a lower CL_{int} with relatively low variability (as indicated by the percentage CVs) was observed in static incubations and when shaken at 450 rpm, and a higher CL_{int} with much greater variability was observed at shaking speeds of 900 rpm and 1050 rpm (Table 5-2, Figure 5-1). A one-way repeated measures ANOVA identified that the shaking conditions were statistically significantly ($p < 0.05$) different. As there appeared to be no difference between CL_{int} determined at 900 rpm and 1050 rpm, the former (henceforth

referred to as shaken) was chosen for subsequent assays consistent with previous experiments; the static condition was selected as an obvious comparison.

5.4.2 Effect of shaking on CL_{int} determinations in intact rat hepatocytes

Following the initial investigation, 12 drugs encompassing multiple metabolic pathways and potentially rate-limiting processes, and a wide range of *in vivo* clearance (Table 3-1) were selected for comparison of static and shaken assays. CL_{int} determined in shaken incubations was statistically significantly ($p < 0.05$) higher than that in static incubations for 10 of the 12 drugs, the observed increase ranging from an average of 2.7-fold (propranolol) to 5.5-fold (dextromethorphan) (Figures 5-2 and 5-3). A large increase between the mean CL_{int} of static and shaken incubations was also observed for triazolam, however CL_{int} in the shaken incubations showed high variability. In contrast to the other drugs, there was no observable effect of shaking on the CL_{int} of tolbutamide (Table 5-3, Figure 5-2).

Table 5-3. CL_{int} of selected drugs in rat hepatocytes in static and shaken (900 rpm) incubations. Data represent mean ± SD of at least three separate preparations; drugs are presented in descending order of *in vitro* CL_{int} in shaken assays.

Drug	CL_{int} (µl/min/10^6 cells)	
	Static	Shaken
Propafenone	453 ± 81	2015 ± 312**
Chlorpromazine	366 ± 40	1937 ± 509*
Propranolol	412 ± 150	1125 ± 345*
Naloxone	238 ± 104	1113 ± 145*
Dextromethorphan	168 ± 6	920 ± 222*
Verapamil	188 ± 22	755 ± 69**
Triazolam	73 ± 10	387 ± 227
Saquinavir	128 ± 62	282 ± 130**
Midazolam	101 ± 18	279 ± 46***
Diclofenac	60 ± 6	192 ± 17**
Metoprolol	33 ± 10	112 ± 33*
Tolbutamide	0.191 ± 0.104	0.123 ± 0.108

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Student's paired *t*-test.

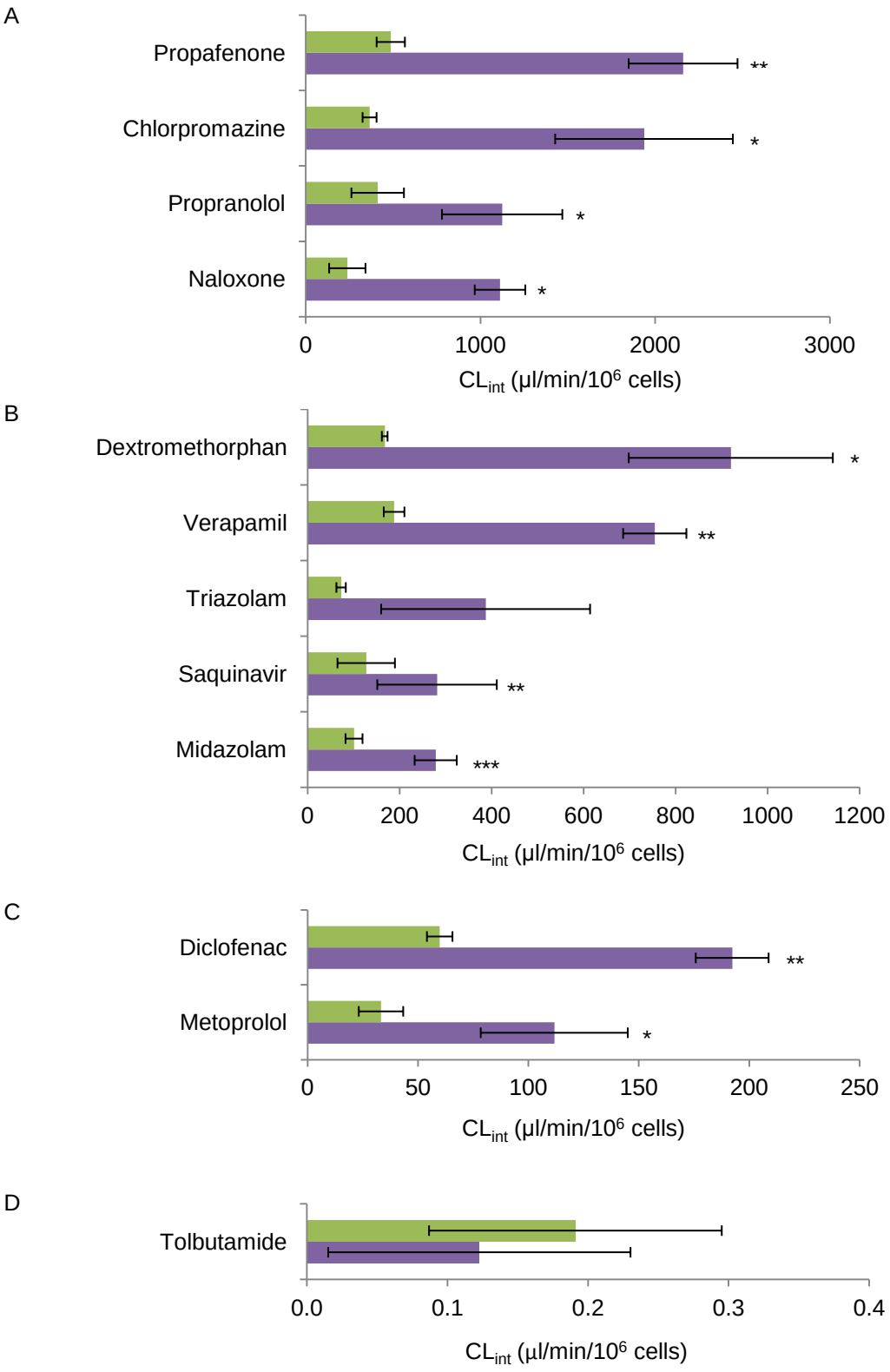


Figure 5-2. CL_{int} of (A) propafenone, chlorpromazine, propranolol and naloxone, (B) dextromethorphan, verapamil, triazolam, saquinavir and midazolam, (C) diclofenac and metoprolol and (D) tolbutamide in rat hepatocytes in static (■) and shaken (■) incubations. Data are normalised to viable cell number and represent mean ± SD of at least three separate preparations, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Student's paired *t*-test.

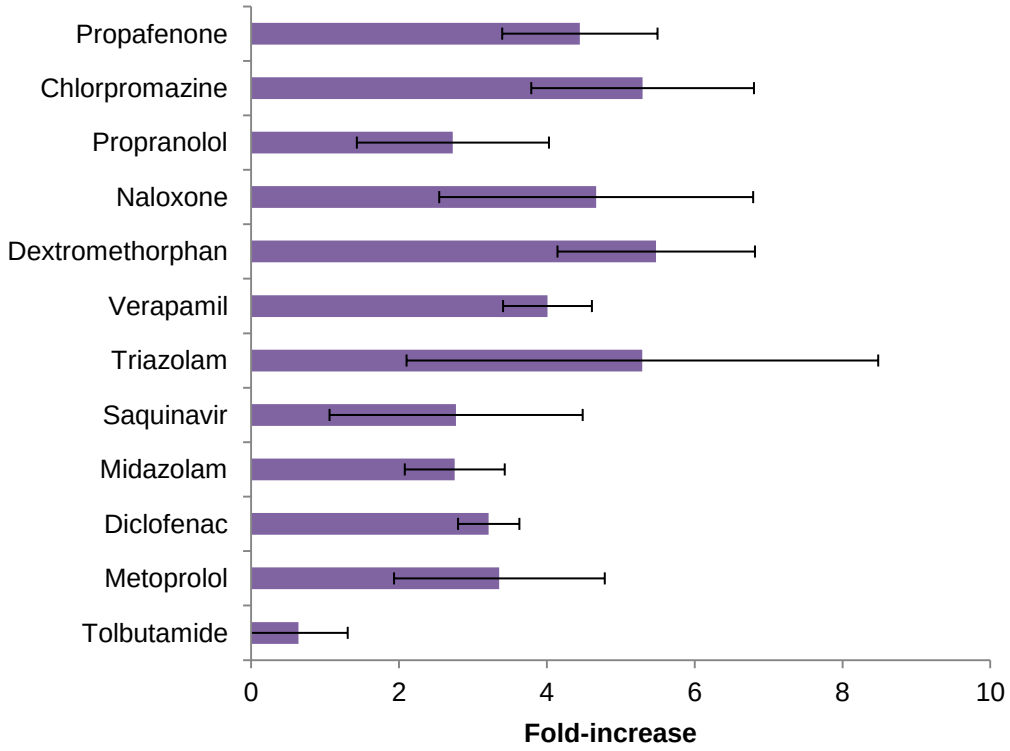


Figure 5-3. Average fold-increase (± SD) in CL_{int} determined in shaken incubations of rat hepatocytes compared to static. Data represent mean of at least three separate preparations; drugs are presented in descending order of *in vitro* CL_{int} in shaken assays.

There appeared to be no obvious relationship between the average fold-increase observed with shaking and *in vitro* CL_{int} (Figure 5-3). Notably however, tolbutamide, the lowest clearance drug by over 100-fold, did not exhibit an increase in CL_{int} with shaking.

5.4.3 Effect of shaking and exogenous NADPH addition on CL_{int} determinations in intact rat hepatocytes

To investigate the mechanism of both shaking-induced- and supplemental NADPH-induced increases in metabolic clearance observed in Section 5.4.2 and Section 3.4.3.1 respectively, midazolam, propranolol and saquinavir were incubated in the absence and presence of 1 mM NADPH in both static and shaken (900 rpm) conditions. Supplementation of hepatocytes with exogenous NADPH resulted in moderate increases in CL_{int} in both static and shaken incubations ranging from 32% (midazolam, shaken) to 114% (saquinavir, static) above control (Table 5-4). Shaking did not have a statistically significant ($p < 0.05$) effect (determined by performance of paired *t*-tests) on the magnitude of the increase afforded by exogenous NADPH addition to hepatocyte incubations for any of the three tested drugs.

Table 5-4. Average percentage increase in CL_{int} (above control) conferred by the addition of 1 mM NADPH to static and shaken incubations of intact rat hepatocytes. Data represent mean ± SD of at least three preparations.

Drug	% Increase Above Control	
	Static	Shaken
Midazolam	39 ± 10	32 ± 5
Propranolol	67 ± 21	34 ± 28
Saquinavir	114 ± 136	77 ± 56

5.4.4 Effect of a shaken pre-incubation period on CL_{int} of midazolam in static hepatocyte incubations

To further understand the mechanism of the observed shaking-induced increases in clearance, the CL_{int} of midazolam was assessed in a static incubation using hepatocytes that had been subject to either a static or shaken (900 rpm) 15 minute pre-incubation. No difference in either midazolam CL_{int} or the magnitude of the effect mediated by NADPH addition was observed between static and shaken pre-incubations (Figure 5-4).

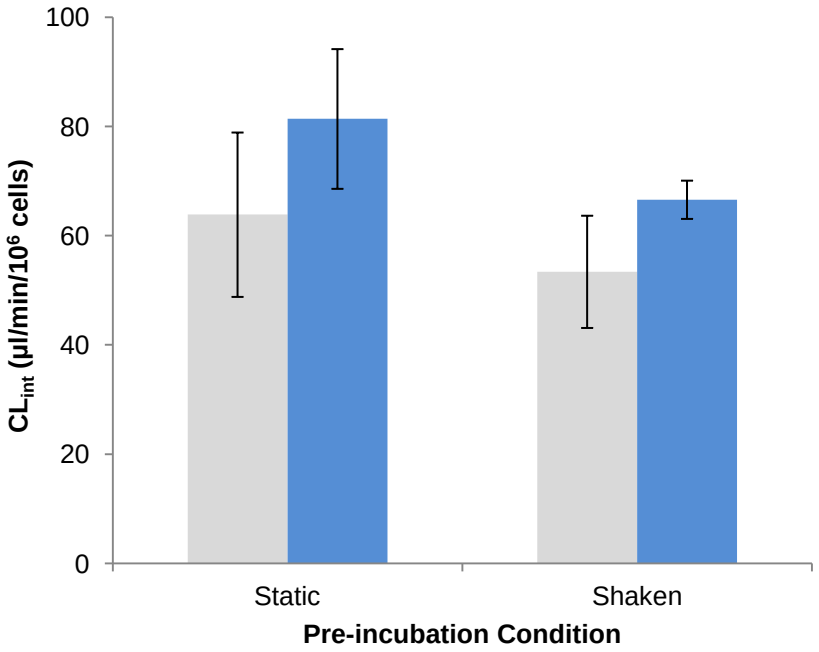


Figure 5-4. CL_{int} of midazolam in a static incubation of rat hepatocytes following a 15 minute static or shaken (900 rpm) pre-incubation in the absence (□) or presence (■) of supplemental NADPH. Data are normalised to viable cell number and represent mean ± SD of three separate preparations.

5.4.5 Effect of shaking on CL_{int} determinations in saponin-permeabilised rat hepatocytes

Further to the work detailed in Section 4.3 in which saponin treatment was used to investigate permeation through the plasma membrane as a rate-limiting factor of CL_{int}, the effect of shaking on permeabilised hepatocyte preparations was also examined. The results presented in this section represent saponin-permeabilised hepatocytes supplemented with 1 mM NADPH, as metabolic clearance was negligible in unsupplemented hepatocytes (some of this data has been presented previously in Section 4.3.3.2). Reported values of CL_{int} for all drugs are normalised to initial total cell number.

For midazolam there appeared to be little difference between CL_{int} determined in static and shaken incubations of NADPH-supplemented permeabilised hepatocytes. Both propranolol and saquinavir did show a trend of increased clearance in this system with shaking, but this was only statistically significant ($p < 0.05$) for saquinavir. For tolbutamide, CL_{int} in NADPH-supplemented permeabilised hepatocytes was higher on average in static incubations, however this was not statistically significant (Table 5-5, Figure 5-5).

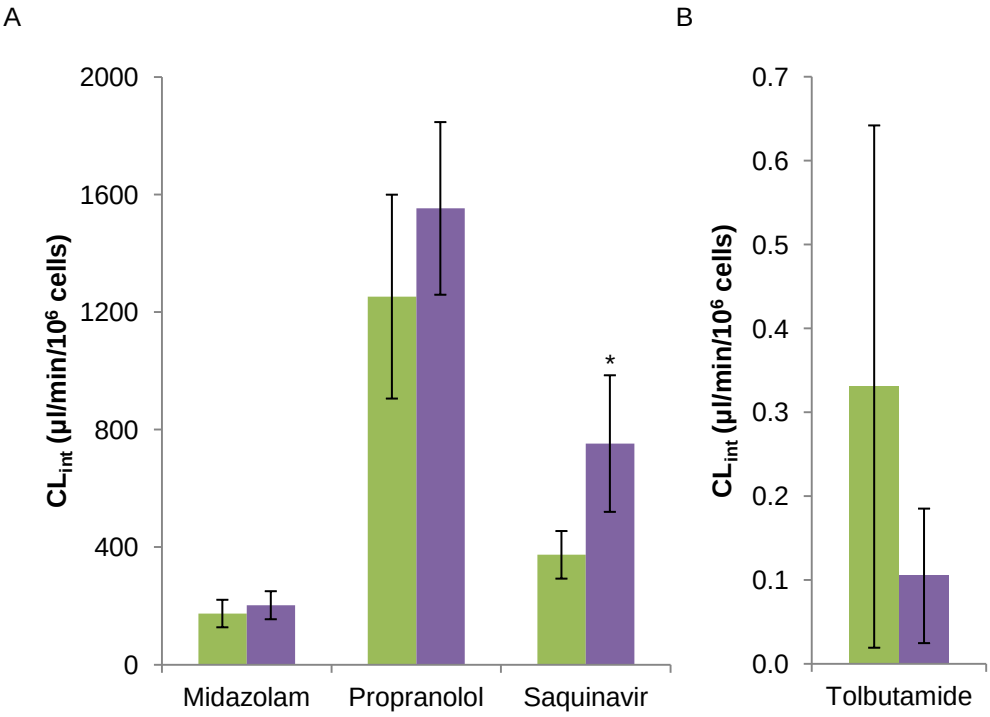


Figure 5-5. CL_{int} of (A) midazolam, propranolol and saquinavir and (B) tolbutamide in saponin-permeabilised rat hepatocytes supplemented with NADPH in static (■) and shaken (■) incubations. Data are normalised to total cell number and represent mean ± SD of at least three separate preparations, * $p < 0.05$ using Student's paired *t*-test.

To identify rate-limiting processes of CL_{int}, the CL_{int} of the selected drugs in saponin-permeabilised hepatocytes (with and without shaking) was compared to the CL_{int} in intact hepatocytes from the same preparations (with and without shaking). Data of individual experimental replicates is presented graphically to provide clarification and resolve a degree of the variability of mean CL_{int} values (Figures 5-6, 5-7, 5-8 and 5-9).

Table 5-5. CL_{int} in intact and saponin-permeabilised rat hepatocytes in static and shaken (900 rpm) incubations. Data are normalised to total cell number and represent mean ± SD of at least three separate preparations.

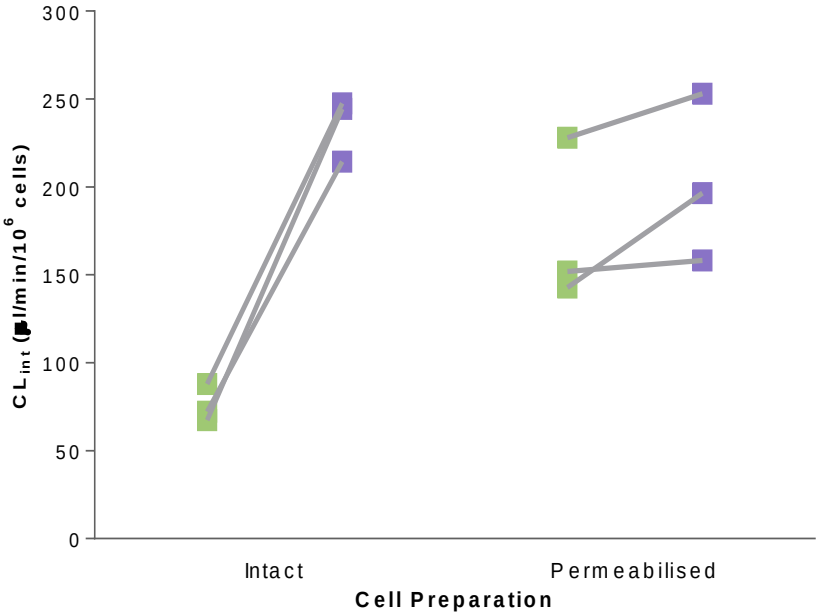
Drug	CL_{int} (µl/min/10^6 cells)			
	Intact Hepatocytes		Permeabilised Hepatocytes	
	Static	Shaken	Static	Shaken
Midazolam	76 ± 11	235 ± 18***	174 ± 47§	203 ± 48
Propranolol	359 ± 130	982 ± 303*	1253 ± 347§	1553 ± 294
Saquinavir	113 ± 34	299 ± 98*	374 ± 81§§	753 ± 232*§§
Tolbutamide	0.16 ± 0.09	0.11 ± 0.09	0.33 ± 0.31	0.11 ± 0.08

* $p < 0.05$, *** $p < 0.001$ using Student's paired *t*-test between static and shaken incubations of the same type of hepatocyte preparation (intact/permeabilised).

§ $p < 0.05$, §§ $p < 0.01$ using Student's paired *t*-test between intact and permeabilised hepatocytes in the same incubation condition (static/shaken).

5.4.5.1 Midazolam

A



B

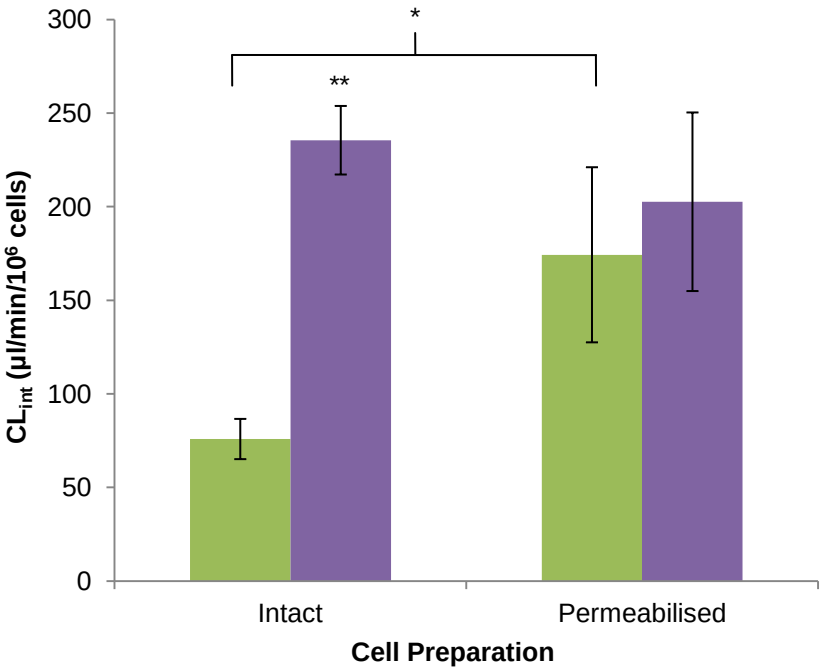


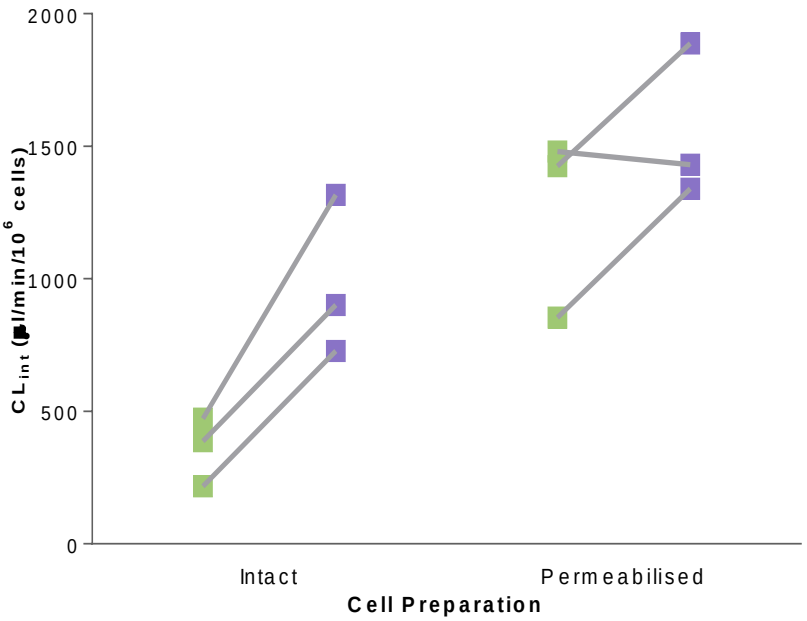
Figure 5-6. (A) individual replicates and (B) mean ± SD of CL_{int} of midazolam in unsupplemented intact and NADPH-supplemented saponin-permeabilised rat hepatocytes in static (■) and shaken (■) incubations. Data are normalised to total cell number, * $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

The CL_{int} of midazolam in unsupplemented intact hepatocytes was in very close agreement across the three preparations which showed large increases in CL_{int} with shaking (Figure 5-6A). In NADPH-supplemented permeabilised hepatocytes, the CL_{int} of midazolam showed greater variability and there was found to be no statistically significant difference between static and shaken incubations. A statistically significant ($p < 0.05$) increase in midazolam CL_{int} between unsupplemented intact and NADPH-supplemented permeabilised hepatocytes was observed in static incubations, but not in shaken incubations (Table 5-5, Figure 5-6).

5.4.5.2 Propranolol

The CL_{int} of propranolol in both static and shaken incubations of unsupplemented intact hepatocytes also showed close agreement between the three preparations (Figure 5-7A) and a statistically significant increase in CL_{int} with shaking of intact hepatocytes was observed (Table 5-5). The effect of shaking on NADPH-supplemented permeabilised hepatocytes was less discernible; for two of the three preparations there was a distinct increase in propranolol CL_{int} in shaken incubations compared to static, but this was not observed for the third (Figure 5-7A); overall this was found not to be statistically significant (Table 5-5). Analogous to midazolam, there was a significant ($p < 0.05$) effect of permeabilisation and NADPH-supplementation of hepatocytes on propranolol CL_{int} in static incubations, but the difference was not statistically significant in shaken incubations (Table 5-5, Figure 5-7).

A



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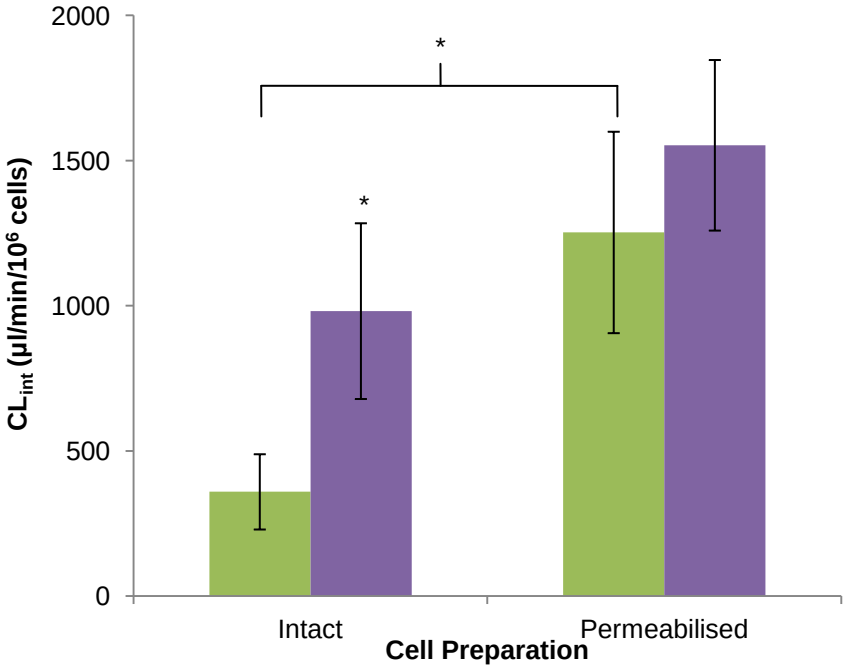
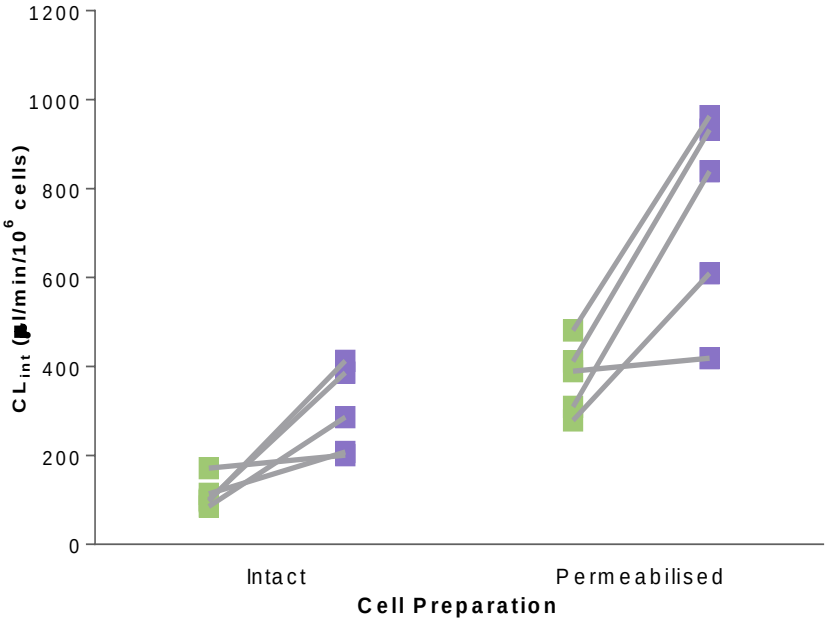


Figure 5-7. (A) individual replicates and (B) mean ± SD of CL_{int} of propranolol in unsupplemented intact and NADPH-supplemented saponin-permeabilised rat hepatocytes in static (■) and shaken (■) incubations. Data are normalised to total cell number, * $p < 0.05$ using Student's paired *t*-test.

5.4.5.3 Saquinavir

A



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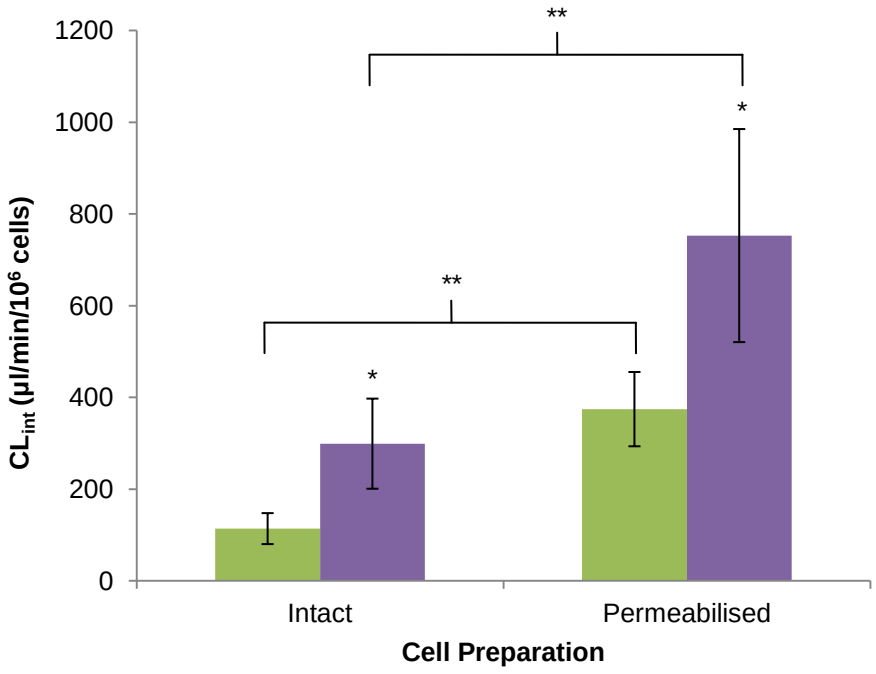


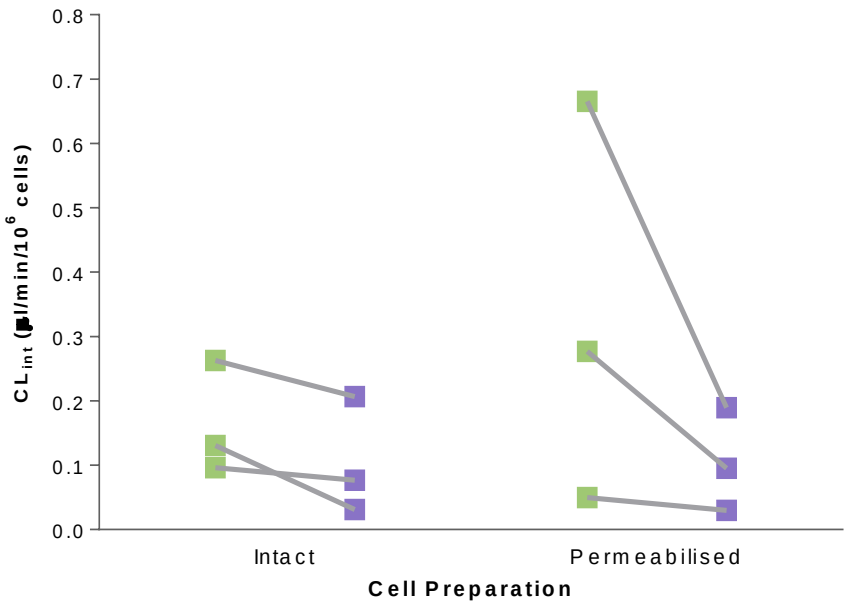
Figure 5-8. (A) individual replicates and (B) mean ± SD of CL_{int} of saquinavir in unsupplemented intact and NADPH-supplemented saponin-permeabilised rat hepatocytes in static (■) and shaken (■) incubations. Data are normalised to total cell number, * $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

Additional experimental replicates were performed for saquinavir as for one hepatocyte preparation there was observed to be almost no effect of shaking on CL_{int} in incubations of unsupplemented intact hepatocytes (Figure 5-8A) which was considered anomalous. In NADPH-supplemented permeabilised hepatocytes from the same preparation, there also appeared to be little effect of shaking. In the other four preparations however, a clear effect of shaking on intact hepatocytes was observed, resulting overall in a statistically significant ($p < 0.05$) increase in saquinavir CL_{int} (Table 5-5, Figure 5-8). Analogous to midazolam and propranolol, a significant ($p < 0.01$) increase in the CL_{int} of saquinavir was observed between unsupplemented intact and NADPH-supplemented permeabilised hepatocytes in static incubations. In contrast to the two previous drugs, a statistically significant ($p < 0.05$) increase in CL_{int} was also observed with shaking of NADPH-supplemented permeabilised hepatocytes and consequently a significant ($p < 0.01$) difference was found between unsupplemented intact and NADPH-supplemented permeabilised hepatocytes in shaken incubations (Table 5-5, Figure 5-8).

5.4.5.4 Tolbutamide

In contrast to the other drugs, for tolbutamide, CL_{int} in both unsupplemented intact and NADPH-supplemented permeabilised hepatocytes appeared to be reduced with shaking, however this was not found to be statistically significant for either system. The estimates of tolbutamide CL_{int} showed greater variability than the other drugs studied, although this is most likely a reflection of the very low concentrations of metabolite measured (Table 5-5, Figure 5-9).

A



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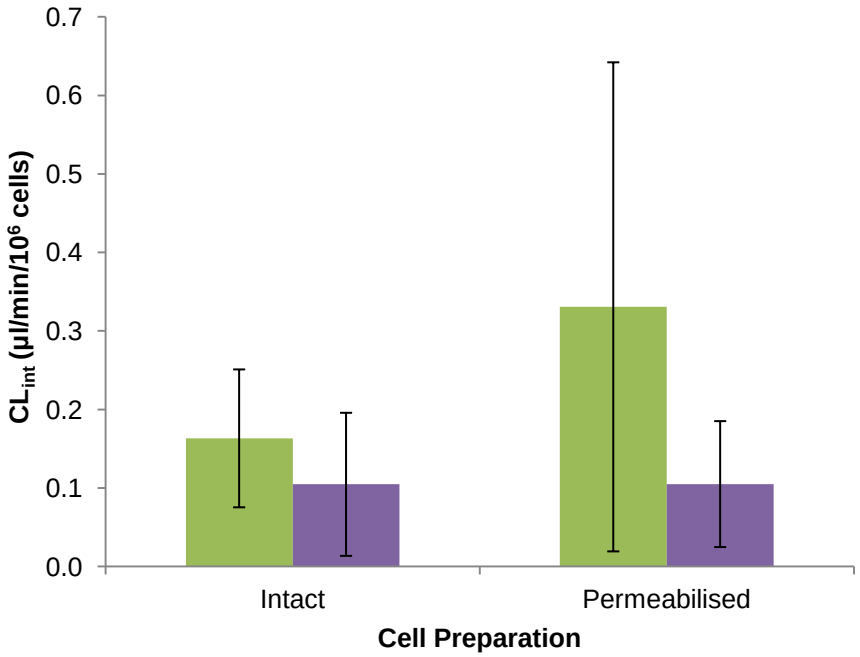


Figure 5-9. (A) individual replicates and (B) mean ± SD of CL_{int} of tolbutamide in unsupplemented intact and NADPH-supplemented saponin-permeabilised rat hepatocytes in static (■) and shaken (■) incubations. Data are normalised to total cell number.

5.4.5.5 Comparison of $CL_{int,u}$ in NADPH-supplemented permeabilised hepatocytes to microsomal $CL_{int,u}$ (obtained from literature)

The CL_{int} (µl/min/10^6 cells) of the four selected drugs in shaken NADPH-supplemented permeabilised hepatocytes scaled to *in vivo* $CL_{int,u}$ (ml/min/kg) was compared with literature microsomal data (also scaled to ml/min/kg and corrected for incubational binding) (Table 5-6). Where possible, studies using a similar substrate concentration to those used in this study were selected to preclude any differences in CL_{int} arising from differing substrate concentrations; however for tolbutamide, such data was unavailable. Given the variability of the literature microsomal data, it was deemed appropriate to present the data from individual studies rather than average values. Owing to the absence of permeability restrictions, microsomal $CL_{int,u}$ should represent metabolism rate-limited $CL_{int,u}$. Therefore, if shaken NADPH-supplemented permeabilised hepatocyte and microsomal $CL_{int,u}$ values are similar, the $CL_{int,u}$ in the former would be confirmed as determined by metabolic rate.

Table 5-6. Scaled $CL_{int,u}$ in microsomes and shaken NADPH-supplemented permeabilised hepatocytes (microsomal data obtained from literature).

Drug	Microsomes			Shaken NADPH-Supplemented Permeabilised Hepatocytes		
	Conc. (µM)	$CL_{int,u}$ (ml/min/kg)	Ref.	Conc. (µM)	$CL_{int,u}$ (ml/min/kg)	Ref.
Midazolam	2.5	238 ± 50	[1]	2.5	1017 ± 240	[2]
Propranolol	0.04 - 8	6600 ± 6000	[3]	0.1	8009 ± 1516	[2]
Saquinavir	0.1 - 10	74958 ± 49412	[4]	1	3845 ± 1185	[2]
	0.4 - 4.0	3515 ± 314[a]	[5]			
Tolbutamide	50 - 3000	5	[6]	1 - 10	0.51 ± 0.39	[2]
	130 - 2000	17	[7]			

Conc., concentration; ref., reference. [1] Jones and Houston (2004), [2] This study, [3] Hallifax (1998), [4] Parker and Houston (2008), [5] Shibata et al. (2002), [6] Veronese et al. (1990), [7] Ashforth et al. (1995). [a] fu_{mic} determined using K_a from [4].

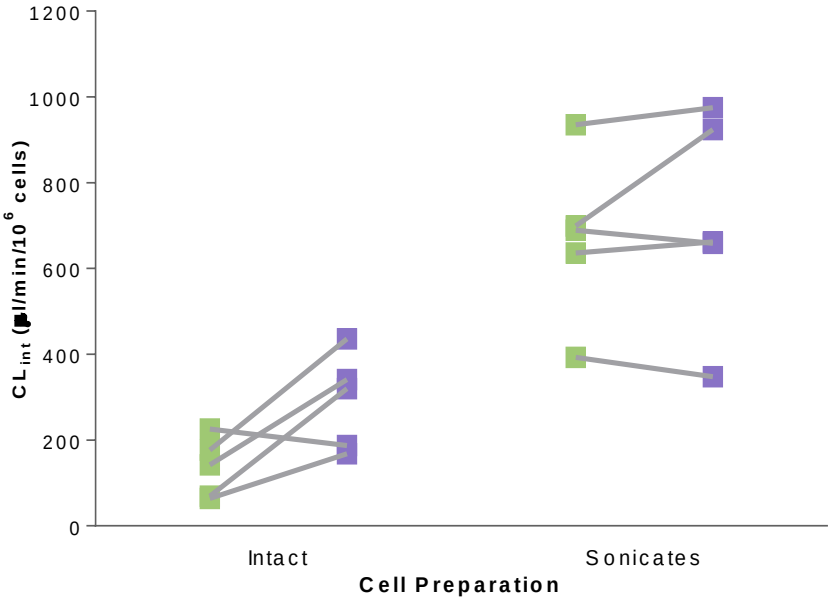
For midazolam, the $CL_{int,u}$ in shaken NADPH-supplemented permeabilised hepatocytes was approximately five-fold larger than the reported $CL_{int,u}$ of the same concentration substrate in microsomes. For propranolol, given the variability of the microsomal value, the $CL_{int,u}$ in shaken NADPH-supplemented permeabilised hepatocytes was comparable. The $CL_{int,u}$ of saquinavir in shaken NADPH-supplemented permeabilised hepatocytes was very similar to one estimate of $CL_{int,u}$ in microsomes, but vastly lower than the other (Table 5-6). This is likely to be due to the fact that the $CL_{int,u}$ of saquinavir reported by Parker and Houston

(2008) was calculated by back extrapolation to substrate concentrations below 0.1 µM. As the authors estimate that concentration-independent CL_{int} is only observed below 0.02 µM, use of 1 µM substrate in this study is expected to be at least partially saturating and therefore limiting the measured CL_{int}. The $CL_{int,u}$ of tolbutamide in shaken NADPH-supplemented permeabilised hepatocytes was 10-fold below the lowest estimate of $CL_{int,u}$ in microsomes and 100-fold below the highest (Table 5-6).

5.4.6 Effect of shaking on saquinavir CL_{int} in rat hepatocyte sonicates

To determine whether the observed increase in saquinavir CL_{int} with shaking of NADPH-supplemented permeabilised hepatocytes was mediated by increased distribution of substrate throughout the bulk medium or reduction in the depth of the UWL, hepatocytes subject to complete gross membrane destruction by sonication supplemented with NADPH were incubated in static and shaken conditions.

A



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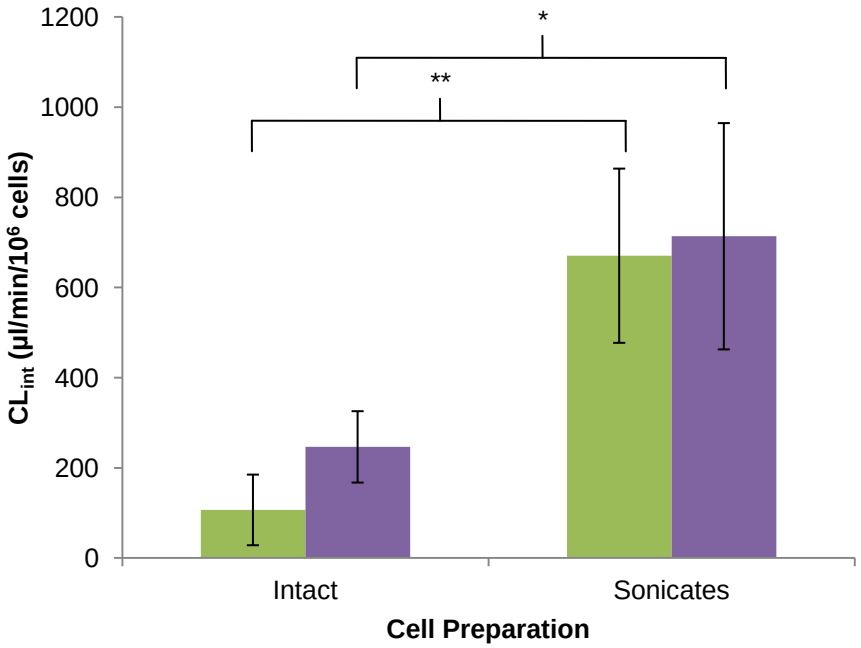


Figure 5-10. (A) individual replicates and (B) mean ± SD of CL_{int} of saquinavir in unsupplemented intact hepatocytes and NADPH-supplemented hepatocyte sonicates in static (■) and shaken (■) incubations. Data are normalised to total cell number, * $p < 0.05$, ** $p < 0.01$ using Student's paired *t*-test.

Whilst the effect of shaking on intact hepatocytes and absolute values of saquinavir CL_{int} were in agreement with those observed previously (Section 5.4.5.3), there was no effect of shaking on saquinavir CL_{int} in hepatocyte sonicates (Figure 5-10). Additional replicates were also performed in this study as one preparation exhibited no effect of shaking on intact hepatocytes (Figure 5-10A). The average CL_{int} of saquinavir in static and shaken hepatocyte sonicates was 670 ± 193 µl/min/10^6 cells and 714 ± 251 µl/min/10^6 cells respectively; this was higher than the average CL_{int} in static incubations of saponin-permeabilised hepatocytes (374 ± 81 µl/min/10^6 cells), but in reasonable agreement with the average CL_{int} in shaken incubations of saponin-permeabilised hepatocytes (753 ± 232 µl/min/10^6 cells).

5.4.7 Effect of shaking on the accuracy of *in vivo* $CL_{int,u}$ predictions

To assess the impact of shaking on the prediction accuracy of *in vivo* $CL_{int,u}$ estimates, scaled $CL_{int,u}$ (ml/min/kg) was compared to *in vivo* $CL_{int,u}$ (ml/min/kg) derived from observed CL_h literature data (Figure 5-11). The AFE and RMSE for predictions of *in vivo* $CL_{int,u}$ in static and shaken assays are given in Table 5-7.

Table 5-7. Accuracy and precision of *in vivo* $CL_{int,u}$ predictions in rat hepatocytes in static and shaken incubations as represented by AFE, RMSE and number of predictions that fall within-, above- and below two-fold of observed *in vivo* $CL_{int,u}$.

	Static	Shaken
AFE	0.67	2.2
Average fold-underprediction	1.5	0.45
RMSE	1163	8043
Number of predictions within two-fold	5	6
Number of predictions above two-fold	2	5
Number of predictions below two-fold	5	1

As a single group, scaled $CL_{int,u}$ determinations from static assays showed on average 1.5-fold underprediction (1/AFE), whilst shaken assays gave an average 2.2-fold overprediction (Table 5-7). The precision of estimates (as represented by RMSE) was greater for static assays than for shaken (Table 5-7). However, for medium to high clearance drugs (all except tolbutamide), predicted *in vivo* $CL_{int,u}$ in shaken assays was either accurately- (within two-fold) or overpredicted. For the same drugs in static assays, *in vivo* $CL_{int,u}$ was accurately-, over- and underpredicted.

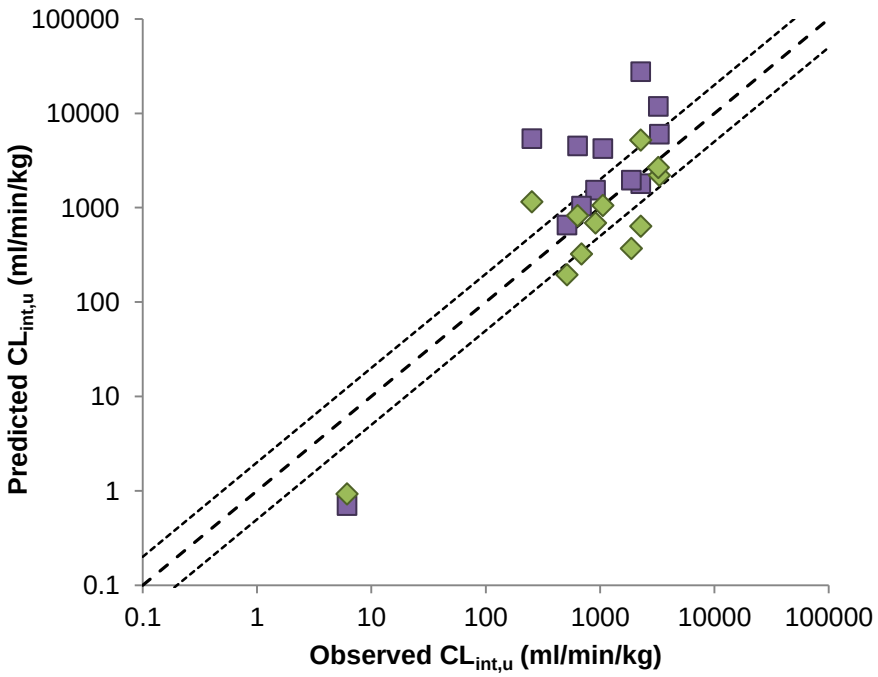


Figure 5-11. Predicted $CL_{int,u}$ determined in static (◆) and shaken (■) *in vitro* assays in comparison to observed $CL_{int,u}$. Dashed line represents exact predictions and dotted lines a two-fold margin of error.

5.5 Discussion

In this chapter, the effect of incubation shaking on CL_{int} determinations in intact hepatocytes has been examined and the underlying mechanism of the observed increases in apparent CL_{int} explored through use of modified preparations including saponin-permeabilised hepatocytes and hepatocyte sonicates.

5.5.1 Effect of shaking on CL_{int} determinations in intact hepatocytes

Initial investigations into the effect of shaking speed on CL_{int} determinations in hepatocyte assays were undertaken using midazolam as a model substrate. The selection of speeds was relatively arbitrary due to the facts that both little information regarding this parameter exists in the literature and that the agitation achieved is unlikely to be equivalent between different apparatus and vessels even at the same speed. 900 rpm was selected based on the use of this speed in earlier work in this project (Chapters 3 and 4) and in published studies (Griffin and Houston, 2004; Parker and Houston, 2008; Jigorel and Houston, 2012). 450 rpm was selected as the mid-point between static and 900 rpm conditions and 1050 rpm was the maximum speed at which there was no risk of incubate overspill between wells. The CL_{int} of midazolam was considerably higher in incubations performed at 900 rpm and 1050 rpm than in incubations performed under static conditions or at 450 rpm. Interestingly, it appeared that it was between 450 rpm and 900 rpm that the centrifugal force exerted became great enough to keep hepatocytes in suspension. This finding has important

implications for the use of shaking in hepatocyte metabolic clearance assays, highlighting a need to optimise shaking conditions rather than regard shaking at any speed as equivalent, as might have been the assumption previously. The variability of CL_{int} determinations also exhibited a similar step change between incubations performed under static conditions and at 450 rpm and incubations performed at 900 rpm and 1050 rpm, suggesting that shaking could be a contributing factor to the observed variability of CL_{int} determinations.

The effect of shaking was confirmed with 10 other small molecule CYP and UGT substrates (Table 5-3, Figure 5-2); increases in apparent CL_{int} in shaken (900 rpm) incubations compared to static incubations ranged between 2.7- and 5.5-fold (Figure 5-3). Although there appeared to be no correlation between this fold increase and *in vitro* CL_{int} (Figure 5-3), there was no effect of shaking observed for tolbutamide, the lowest clearance drug by over 100-fold *in vitro* and approximately 40-fold *in vivo*. It is therefore possible that such effects are only apparent for drugs classified as 'mid-clearance' or above. These findings are supported by the results of a study by Griffin and Houston (2004) who compared *in vitro* CL_{int} in freshly isolated rat hepatocytes incubated in Eppendorf tubes at 900 rpm agitation with that in cryopreserved rat hepatocytes incubated in 24-well plates under static conditions. The CL_{int} of 7-ethoxycoumarin, phenacetin, nordiazepam and dextromethorphan were found to be significantly higher in freshly isolated rat hepatocytes; the formation of 4'-hydroxy-tolbutamide was not significantly different between fresh and cryopreserved hepatocytes. Although this was not a controlled comparison given the additional variables, the authors of this paper suggested that the observed differences between fresh and cryopreserved cells were likely due to the agitation conditions (Griffin and Houston, 2004).

5.5.2 Effect of shaking on the accuracy of *in vivo* $CL_{int,u}$ predictions

The accuracy of predictions of *in vivo* $CL_{int,u}$ from static and shaken *in vitro* assays was evaluated by calculation of AFE and fold-underprediction for the dataset of 12 drugs. CL_{int} determinations in static incubations showed an average 1.5-fold underprediction, whilst those in shaken incubations exhibited a 2.2-fold overprediction of *in vivo* $CL_{int,u}$ (Table 5-7). The apparent increased accuracy in static incubations according to AFE is perhaps misleading however, as over- and underpredictions counteract each other in the calculation of AFE and fewer underpredictions were observed in shaken incubations. A greater number of predictions from shaken assays fall within the generally accepted two-fold of *in vivo* criteria and aside from tolbutamide no other drug is greater than two-fold underpredicted in shaken assays. Although such a large percentage of overpredictions of *in vivo* $CL_{int,u}$ was unexpected, this could be due to number of reasons including the use of a lower drug concentration than in the comparative *in vivo* studies. Additionally, for some drugs, the *in vivo* $CL_{int,u}$ is from a single literature study and therefore the chance of imprecision is greater. A much more extensive comparison is warranted to assess the wider effect of shaking on *in vivo* $CL_{int,u}$ predictions, although this would be a relatively onerous task.

5.5.3 Potential mechanisms of shaking-induced increases in apparent CL_{int}

Postulated mechanisms of the observed shaking-induced increases in apparent CL_{int} included: damage to the plasma membrane of hepatocytes allowing unrestricted access of substrate to the metabolising enzyme(s); reduction in the depth of the UWL surrounding the plasma membrane; and increased distribution of substrate throughout the bulk medium. Investigations were undertaken to explore these hypotheses and determine the true underlying mechanism.

5.5.3.1 Shaking and plasma membrane integrity

To investigate whether the observed increases in apparent CL_{int} with shaking were a result of damage to the plasma membrane, hepatocytes were pre-incubated for a period of 15 minutes (with and without NADPH supplementation) in either static or shaken (900 rpm) conditions, followed by a static incubation with midazolam as the substrate. The CL_{int} of midazolam in the absence of NADPH did not differ between static and shaken pre-incubated hepatocytes (Figure 5-4) and in both conditions was similar to the CL_{int} of midazolam in static incubations following only a 5 minute pre-incubation (Table 5-3). This suggests that the observed increases in CL_{int} with shaking are unlikely to be facilitated by permanent damage to the plasma membrane of hepatocytes. This is further supported by the similar magnitude of the effect on midazolam CL_{int} conferred by NADPH addition in each pre-incubation condition. As the effect of NADPH has been shown to correlate with the proportion of 'non-viable' cells, this suggests similar proportions of 'non-viable' cells in each condition. Furthermore, any notion that shaking could somehow transiently damage or permeabilise the plasma membrane is also detracted from, as in this scenario it would be expected that hepatocytes subject to a shaken pre-incubation with NADPH would have increased intracellular levels of this cofactor and therefore the observed effect of NADPH would be greater in this group.

In addition, there was a statistically significant ($p < 0.05$) increase in the CL_{int} of saquinavir in shaken incubations of permeabilised hepatocytes (supplemented with NADPH) compared to static incubations (Figure 5-8). In permeabilised hepatocytes the membrane barrier is effectively removed, since the pores created in the membrane by saponin treatment are large enough to allow free movement of molecules of up to at least 285 kDa (Bijleveld and Geelen, 1987). The observed increases in CL_{int} with shaking in this system therefore cannot be attributed to altered membrane integrity and thus indicate a change to the rate-limiting process between the static and shaken conditions mediated by an alternative mechanism.

5.5.3.2 Potential alteration of the unstirred water layer

It should be appreciated that the plasma membrane is not the sole barrier to drug permeation into a cell; the UWL surrounding the plasma membrane also provides a potential degree of resistance (Pohl et al., 1998; Naruhashi et al., 2003; Korjamo et al., 2009). In contrast to other techniques such as sonication, the plasma membrane of hepatocytes

permeabilised by saponin (and similar plant glycosides) remains in place and the overall cellular structure largely intact (Zuurendonk and Tager, 1974; Cook et al., 1983; Katz and Wals, 1985; Bijleveld and Geelen, 1987) (for further details see Chapter 4). It is therefore conceivable that an UWL surrounding the permeabilised cell may remain, serving as a residual barrier to drug entry into the cell. As the observed increases in saquinavir CL_{int} in the shaken incubations of NADPH-supplemented permeabilised hepatocytes could not have been mediated by disruption of plasma membrane integrity, it is likely that these effects and therefore those observed in intact hepatocytes are due to a reduction in the depth of the UWL surrounding the (permeabilised) cells. Indeed, it has been documented that the depth of an encountered UWL may be reduced by agitation (Barry and Diamond, 1984; Williams et al., 1990; Pohl et al., 1998; Avdeef et al., 2004; Shibayama et al., 2015).

5.5.3.3 Increased distribution of substrate throughout the bulk medium

Although suggested as an explanation for the observed differences in CL_{int} between shaken freshly isolated and static cryopreserved rat hepatocytes by Griffin and Houston (2004), the likelihood that the effects of shaking are mediated through increased distribution of substrate throughout the bulk medium is low. In the absence of poor solubility at the experimental concentration, all drugs are expected to be distributed evenly throughout the medium by the process of convection (Barry and Diamond, 1984; Korjamo et al., 2009). Nevertheless, this explanation was investigated using hepatocyte sonicates and subsequently rejected based on the findings. In sonicated hepatocytes the metabolic enzymes are likely to be contained within cellular fragments or micelles and therefore any UWL present would be expected to be vastly reduced compared to that surrounding an intact or permeabilised cell. Observed increases in CL_{int} with shaking of hepatocyte sonicates would therefore be due to a mechanism other than reduction in the depth of the UWL. However, the observed CL_{int} of saquinavir was in close agreement between static and shaken incubations of NADPH-supplemented hepatocyte sonicates (670 ± 193 µl/min/10^6 cells and 714 ± 251 µl/min/10^6 cells respectively), the values in both conditions in agreement with the CL_{int} of saquinavir in shaken NADPH-supplemented permeabilised preparations (753 ± 232 µl/min/10^6 cells). This provides evidence that metabolic enzymes remain functional in sonicated hepatocytes supplemented with NADPH; the distribution of substrate is not rate-limiting in static incubations and that the observed increase in CL_{int} with shaking of permeabilised and thus intact hepatocytes is likely to be due to reduction in the depth of the UWL.

5.5.4 Use of membrane permeabilisation and shaking as an approach to determine the rate-limiting process of CL_{int} in hepatocytes

With the conclusion that an UWL is present, the apparent CL_{int} determined in hepatocyte assays can therefore be considered as three sequential clearance processes: clearance through the UWL (CL_{UWL}), clearance through the plasma membrane (CL_{mem}) and intrinsic metabolic clearance ($CL_{int,met}$) (Figure 5-12).

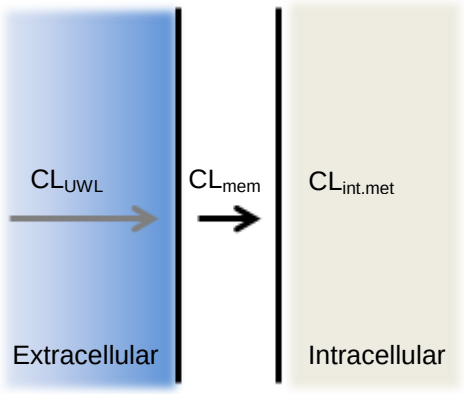


Figure 5-12. *In vitro* apparent CL_{int} can be considered as three sequential clearance processes: CL_{UWL}, CL_{mem} and $CL_{int,met}$.

The $CL_{int,met}$ is not only potentially restricted by CL_{mem}, the principle of which is widely accepted based on the physiological properties of a cell, but also by CL_{UWL}, which has yet to be acknowledged in the context of hepatocyte clearance assays. Identification of each of these potentially rate-determining processes and their influence on a drug's apparent *in vitro* CL_{int} can be accomplished through utilisation of the properties of intact and permeabilised hepatocytes in static and shaken conditions, which are summarised in Figure 5-13.

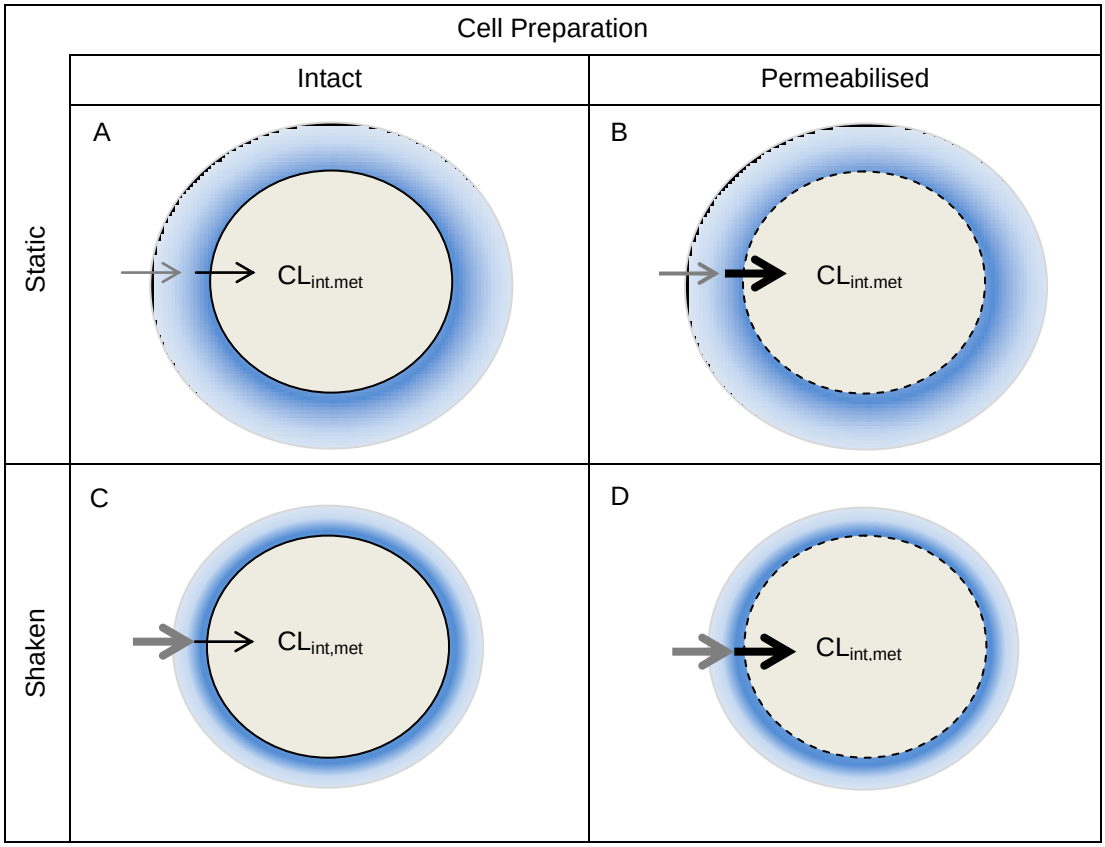


Figure 5-13. The individual and combined effects of membrane permeabilisation and incubation shaking on drug entry into a cell. Blue areas represent the UWL, solid black circles represent an intact plasma membrane and dashed black circles represent a permeabilised plasma membrane. Grey arrows represent drug diffusion through the UWL, black arrows represent drug permeation through the plasma membrane; the thickness of the arrows represents increased or decreased permeation relative to the other conditions.

A rate-limitation presented by UWL permeation can be identified by shaking of intact hepatocytes which reduces the depth of the UWL surrounding the cell (Figure 5-13C). An increase in apparent CL_{int} between static and shaken incubations is indicative of increased drug entry into the cell and therefore an UWL permeation rate-limitation. This was observed for all tested drugs, with the exception of tolbutamide (Figure 5-2), the $CL_{int,met}$ of which does not appear to be restricted by CL_{UWL}. Similarly, a rate-limitation presented by membrane permeation can be identified by permeabilisation of hepatocytes (which effectively removes the membrane barrier) and incubation in shaken conditions (absence of UWL permeation restriction) (Figure 5-13D). An increase in apparent CL_{int} between shaken incubations of intact and permeabilised hepatocytes (supplemented with NADPH) is indicative of increased drug entry into the cell and therefore a membrane permeation rate-limitation. This was observed only for saquinavir (Figure 5-8), indicating that for this drug $CL_{int,met}$ also appears to be restricted by CL_{mem}. The absence of membrane and UWL permeation restrictions for tolbutamide suggests that the overall apparent CL_{int} of this drug is determined by the metabolic rate. In contrast, the $CL_{int,met}$ of saquinavir appears restricted by both UWL

permeation and membrane permeation. The apparent CL_{int} of saquinavir in unshaken intact hepatocytes was increased 2.6-fold in shaken intact hepatocytes and 6.7-fold in shaken permeabilised hepatocytes.

Absolute values for $CL_{int,met}$, CL_{mem} and CL_{UWL} can theoretically be determined from clearance assays using the preparations and conditions given in Figure 5-13. $CL_{int,met}$ can be measured in shaken incubations of (NADPH-supplemented) permeabilised hepatocytes; shaking reduces the depth of the UWL surrounding the porous membrane of the permeabilised cell (Figure 5-13D), thereby facilitating potentially maximal access of substrate into the cell. The restriction of $CL_{int,met}$ by CL_{mem} ($CL_{int,app,mem}$) is represented by the apparent CL_{int} observed in shaken incubations of intact hepatocytes (Figure 5-13C) and can be given by Equation 5.3.

Equation 5.3

$$CL_{int,app,mem} = CL_{int,met} \cdot \frac{CL_{mem}}{CL_{mem} + CL_{int,met}}$$

$CL_{int,met}$ measured in shaken NADPH-supplemented permeabilised hepatocytes.

CL_{mem} may then be obtained by rearrangement of Equation 5.3 to Equation 5.4.

Equation 5.4

$$CL_{mem} = \frac{CL_{int,app,mem} \cdot CL_{int,met}}{CL_{int,met} - CL_{int,app,mem}}$$

The restriction of $CL_{int,met}$ by both CL_{mem} and CL_{UWL} ($CL_{int,app}$) is represented by the apparent CL_{int} observed in static incubations of intact hepatocytes (Figure 5-13A) and can be given by Equation 5.5.

Equation 5.5

$$CL_{int,app} = CL_{int,app,mem} \cdot \frac{CL_{UWL}}{CL_{UWL} + CL_{int,app,mem}}$$

CL_{UWL} may be obtained by rearrangement of Equation 5.5 to Equation 5.6.

Equation 5.6

$$CL_{UWL} = \frac{CL_{int,app} \cdot CL_{int,app,mem}}{CL_{int,app,mem} - CL_{int,app}}$$

Figure 5-14 describes how the $CL_{int,app}$ as a ratio of $CL_{int,met}$ is influenced by changes to CL_{UWL} and CL_{mem} relative to $CL_{int,met}$; CL_{UWL} and CL_{mem} have to be at least 10-fold greater than $CL_{int,met}$ for $CL_{int,app}$ to approach $CL_{int,met}$. A very low CL_{UWL} or CL_{mem} relative to $CL_{int,met}$ severely restricts $CL_{int,app}$.

Knowledge of the rate-determining process of a compound's CL_{int} is advantageous in extrapolation of apparent *in vitro* CL_{int} to *in vivo* $CL_{int,u}$. Determination of CL_{UWL} would be

especially useful for high permeability compounds due to the reported differences in UWL depth between *in vitro* and *in vivo* settings, which are also likely applicable to the liver (Lennernas, 1998; Avdeef et al., 2004).

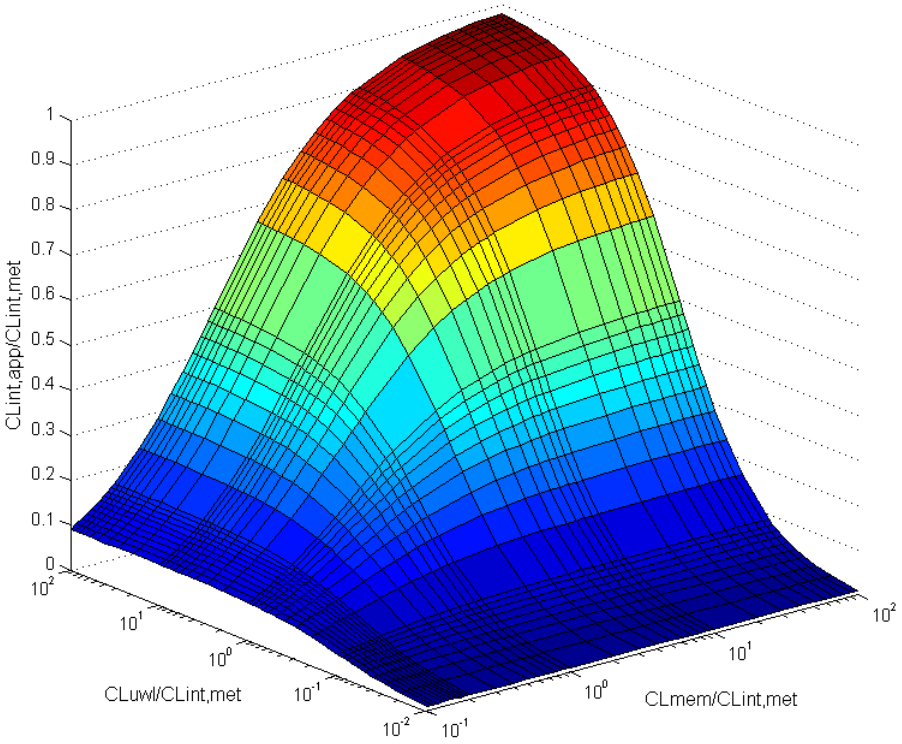


Figure 5-14. Surface plot illustrating the effect of changes in the ratios of $CL_{UWL}/CL_{int,met}$ and $CL_{mem}/CL_{int,met}$ on $CL_{int,app}$ relative to $CL_{int,met}$.

5.6 Conclusion

Despite acceptance of the phenomenon of the UWL for a considerable number of years (Osterthout et al., 1934; Barry, 1969), its presence in *in vitro* assays and the potential implications for CL_{int} determinations in hepatocytes has been largely ignored. A systematic analysis of the mechanisms affecting measurement of clearance in hepatocytes has been lacking; this work has provided not just a demonstration of the critical impact of shaking, but resolved the underlying mechanisms. The applicability of incubation shaking as a physiologically sound method by which to increase *in vitro* CL_{int} determinations has been demonstrated, and therefore qualifies as a potential promising approach to improve the general trend of underprediction of *in vivo* $CL_{int,u}$ from *in vitro* assays. Confirmation of this phenomenon in human hepatocytes and evaluation of the effect on *in vivo* $CL_{int,u}$ predictions is required and will be reported in the next chapter.

As an additional aspect to this work, a framework of experiments and related equations is presented by which the rate-determining process and indeed contribution of individual processes to *in vitro* apparent CL_{int} can be determined. Such information may provide a useful insight into the rate-determining processes in a compound's overall hepatic clearance *in vivo*.

Chapter 6. Investigation into the effect of substrate concentration and shaking on CL_{int} determinations in human hepatocytes

6.1 Introduction

Whilst prediction of metabolic clearance was once largely restricted to the use of animal and human subcellular fractions and animal hepatocytes, the widened availability of cryopreserved human hepatocytes is reflected in their now prevalent use. The vastly increased maximum donor pool size (available in pools of up to 100 donors at the time of writing) reduces the uncertainty posed by interindividual variability of single donor human hepatocytes; large donor pools are expected to be more representative of the average metabolic profile. Despite technical advances in the preparation and preservation of high quality human hepatocytes, underprediction of *in vivo* $CL_{int,u}$ by this system remains (Hallifax et al., 2010; Foster et al., 2011) (see also Section 2.4.1). Reliable predictions of human CL_h are required to inform clinical trial design and reduce avoidable late-stage compound attrition due to poor pharmacokinetic properties, contributing to improved overall success of drug discovery and development programmes.

Over the course of this project, two major variables of metabolic clearance assays, namely substrate concentration and incubation shaking were found to significantly influence CL_{int} determinations of multiple drugs in rat hepatocytes, revealing sub-optimal use in typical situations to date. It is therefore desirable to investigate their impact on CL_{int} determinations in human hepatocytes.

6.2 Aim

The aim of this study was to determine if the factors found to be limiting *in vitro* CL_{int} in rat hepatocytes similarly affect CL_{int} in human hepatocytes. To examine the effect of a lower substrate concentration, the CL_{int} of midazolam and propranolol was determined at 0.1 µM and 1 µM. Midazolam and propranolol are well-characterised CYP3A4 (Patki et al., 2003) and CYP2D6 (McGinnity et al., 2000) substrates respectively which were demonstrated to have significantly higher CL_{int} in rat hepatocytes when determinations were performed at 0.1 µM compared to 1 µM (Section 3.4.4.1). The effect of incubation shaking on CL_{int} in human hepatocytes was assessed using midazolam, propranolol and saquinavir. Saquinavir is a CYP3A4 substrate (Eagling et al., 2002), previously identified as permeability rate-limited in rat hepatocytes (Parker and Houston, 2008). All three drugs were found to have higher CL_{int} in shaken incubations of rat hepatocytes compared to static incubations (Section 5.4.2), indicative of an UWL permeation rate-limitation (see Chapter 5).

6.3 Materials and methods

6.3.1 Materials

Cryopreserved human hepatocytes (pool of 20 donors; see Appendix Table 8-9 for demographic details) and hepatocyte thawing/plating medium (*InVitro*GRO CP medium) were purchased from Bioreclamation IVT (Brussels, Belgium). All other materials and reagents were as detailed previously (Sections 3.3.1 and 4.3.2.1).

6.3.2 Preparation of cryopreserved hepatocytes

Cells were stored in the vapour phase of liquid nitrogen and thawed as per the supplier's instructions (see Appendix 8.8.1). After thawing cells were centrifuged at 50 x g for 5 minutes in 50 ml of *InVitro*GRO CP medium; the resulting supernatant was discarded and cells were re-suspended in a small volume of WME pH 7.4, buffered with HEPES (final concentration 24 mM). Hepatocytes were counted using a haemocytometer and light microscope and viability assessed using the trypan blue exclusion method; the average viability of thawed hepatocytes was 93 ± 2%. Cells were subsequently diluted to the required concentration in HEPES buffered WME.

6.3.3 Substrate depletion assays

Substrate depletion assays were performed in 96-well plates as described in Section 3.3.5.3. Briefly, hepatocytes were pre-incubated for 5 minutes either unshaken on a dry block plate heater or shaken at 900 rpm on a microtiterplate shaker. Following this period, substrate was added to the hepatocytes to initiate the reaction and individual wells were quenched at appropriate time points (n = 6 or 8) to give a substrate depletion profile. Parallel incubations in the absence of cells were also performed as non-specific loss controls. Details of manufacturers and models for the equipment utilised can be found in Appendix 8.4.

Table 6-1. Incubation conditions of midazolam, propranolol and saquinavir in human hepatocyte assays.

Drug	Hepatocyte Concentration (10^6 cells/ml)	Substrate Concentration (µM)	Incubation Time (min)
Midazolam	0.25	0.1 and 1	60
Propranolol	0.75[#] or 1	0.1 and 1	60
Saquinavir	0.4	1	60

[#] Two determinations of CL_{int} of 1 µM propranolol were performed using 0.75 x 10^6 cells/ml.

6.3.4 Sample preparation and liquid chromatography-mass spectrometry analysis

Incubation samples were treated and prepared for LC-MS/MS analysis in the same manner as described in Section 3.3.7. Details of the LC-MS/MS systems and analytical conditions

used for individual compounds and the associated internal standards are given in Appendix 8.5.The procedures for sample quantification were also as given in Section 3.3.7.

6.3.5 Data analysis

Generated *in vitro* data were analysed in the same manner as described in Section 3.3.8.1 and non-specific loss of substrate corrected for as described in Section 4.3.2.7. To allow comparison of hepatocyte CL_{int} data generated in this study with literature *in vivo* data, hepatocyte CL_{int} (µl/min/10^6 cells) was scaled to $CL_{int,u}$ (ml/min/kg) as described in Section 2.3.3. Where possible, fu_{heps} from published experimental data were used and if necessary corrected for cell concentration using Equations 3.2 and 3.3. Where experimental data were unavailable, fu_{heps} was calculated using Equation 2.2.

6.3.6 Statistical analysis

The arithmetic mean and standard deviation were calculated for each condition and substrate concentration for each drug. A two-tailed, paired Student's *t*-test was used to determine the existence of a statistically significant difference between static and shaken conditions for all drugs and between substrate concentrations for midazolam; values were reported as significant when $p < 0.05$. As incubations of 0.1 µM and 1 µM propranolol were performed on separate occasions, a two-tailed, two-sample Student's *t*-test was used to determine the existence of a statistically significant difference between substrate concentrations; values were reported as significant when $p < 0.05$.

6.4 Results

6.4.1 Effect of substrate concentration and shaking on *in vitro* CL_{int} determinations

Midazolam and propranolol were used as model substrates to examine the effect of initial substrate concentration (0.1 µM versus 1 µM) on CL_{int} determinations in human hepatocytes. There was found to be no significant ($p < 0.05$) effect of substrate concentration on the CL_{int} of midazolam in either static or shaken conditions (Table 6-2, Figure 6-1A). For propranolol, there was also no observed effect of substrate concentration in the static condition, however in the shaken condition, CL_{int} determined at 0.1 µM was significantly higher than CL_{int} determined at 1 µM (Table 6-2, Figure 6-1B).

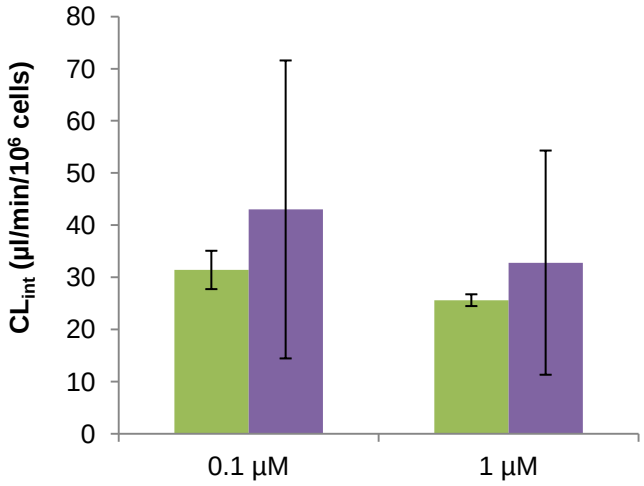
Midazolam, propranolol and saquinavir were used as model substrates to assess the effect of shaking on CL_{int} determinations in human hepatocytes. There was found to be a statistically significant ($p < 0.05$) increase in the CL_{int} of propranolol (at both 0.1 µM and 1 µM) and saquinavir with incubation shaking; an equivalent effect was not observed for midazolam (Table 6-2, Figure 6-1).

Table 6-2. CL_{int} of midazolam, propranolol and saquinavir in cryopreserved human hepatocytes at 0.1 µM and 1 µM in static and shaken incubations. Data represent mean ± SD of three separate experiments.

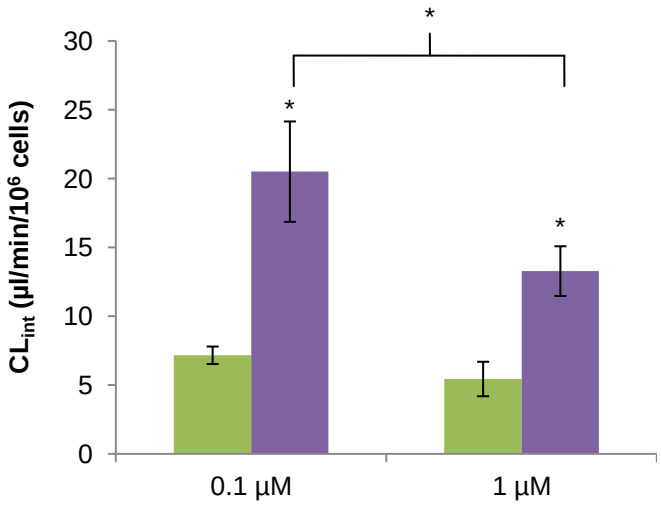
Drug	CL_{int} (µl/min/10^6 cells)			
	0.1 µM		1 µM	
	Static	Shaken	Static	Shaken
Midazolam	28 ± 6	37 ± 23	26 ± 2	27 ± 18
Propranolol	7 ± 1	21 ± 4*	5 ± 1	13 ± 2*[§]
Saquinavir	-	-	29 ± 5	88 ± 22*

* $p < 0.05$ using Student's paired *t*-test compared to static condition. [§] $p < 0.05$ using Student's paired *t*-test compared to 0.1 µM in shaken condition.

A



B



C

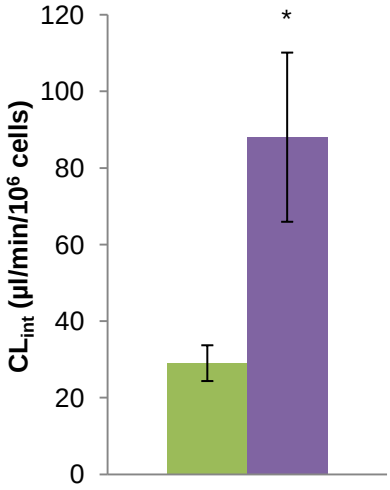


Figure 6-1. CL_{int} of 0.1 µM and 1 µM (A) midazolam (B) propranolol and (C) saquinavir (1 µM only) in human hepatocytes in static (■) and shaken (■) incubations. Data represent mean ± SD of three separate experiments, * $p < 0.05$ using Student's paired *t*-test.

6.4.2 Influence of substrate concentration and shaking on *in vivo* $CL_{int,u}$ predictions

To assess the impact of a lower substrate concentration and incubation shaking on the accuracy of *in vivo* $CL_{int,u}$ predictions in human hepatocytes, predicted $CL_{int,u}$ was compared to observed $CL_{int,u}$ derived from CL_h literature data using the WS model (Table 6-3).

Table 6-3. Predicted $CL_{int,u}$ of midazolam, propranolol and saquinavir in human hepatocytes across different incubation conditions and in the literature in comparison to observed *in vivo* $CL_{int,u.}$ References for observed and predicted (literature) $CL_{int,u}$ can be found in Appendix 8.1.

Drug	Predicted $CL_{int,u}$ (ml/min/kg)					Observed $CL_{int,u}$ (ml/min/kg)
	0.1 µM		1 µM		Literature	
	Static	Shaking	Static	Shaking		
Midazolam	135 ± 30	179 ± 101	127 ± 7	132 ± 85	42 ± 36	390 ± 121
Propranolol	63 ± 6	181 ± 32*	45 ± 12	109 ± 21*[§]	50 ± 23	333 ± 107
Saquinavir	-	-	169 ± 27	514 ± 129*	-	3199

* $p < 0.05$ using Student's paired *t*-test compared to static condition. [§] $p < 0.05$ using Student's paired *t*-test compared to 0.1 µM in shaken condition. References for literature values may be found in Appendix 8.1.

In contrast to rat hepatocytes, scaled *in vitro* $CL_{int,u}$ of the selected drugs in human hepatocytes determined at 0.1 µM and 1 µM in both static and shaken conditions underpredicted *in vivo* $CL_{int,u}$ (Table 6-3). Underpredictions of $CL_{int,u}$ ranged between 2.2- and 3.1-fold for midazolam, 1.8- and 7.4-fold for propranolol and 6.2- and 18.9-fold for saquinavir. For all three drugs, the greatest underpredictions were observed in static incubations with 1 µM substrate; the most accurate predictions of *in vivo* $CL_{int,u}$ were observed in shaken incubations using the lower substrate concentration where investigated (midazolam and propranolol only).

To evaluate the accuracy in the context of the wider range of literature predictions, predicted *in vivo* $CL_{int,u}$ in this study was plotted onto the graph of predicted vs. observed $CL_{int,u}$ of all drugs in human hepatocytes (from Chapter 2) (Figure 6-2). The investigated drugs have clearances in the mid-high range of observed $CL_{int,u}$ and predictions from all *in vitro* conditions (0.1 µM and 1 µM, static and shaking) fall within the overall range of literature predictions (Figure 6-2A). The greatest increases in predicted $CL_{int,u}$ in this study were observed between determinations performed in static and shaken incubations, particularly for saquinavir, where fold-underprediction was largest (Table 6-3, Figure 6-2). The literature prediction of propranolol $CL_{int,u}$ was similar to that in static incubations of both 1 µM and 0.1 µM substrate, but between 2.0- to 3.6-fold lower than determinations in shaken incubations. For midazolam, the literature prediction of $CL_{int,u}$ was at least 3.0-fold lower than predicted $CL_{int,u}$ in this study in static and shaken incubations (Table 6-3). Comparative

literature data was unavailable for saquinavir. In contrast to the study with rat hepatocytes in which an overprediction of $CL_{int,u}$ was observed for propranolol and saquinavir in shaken incubations, no overpredictions of human *in vivo* $CL_{int,u}$ were observed for these drugs.

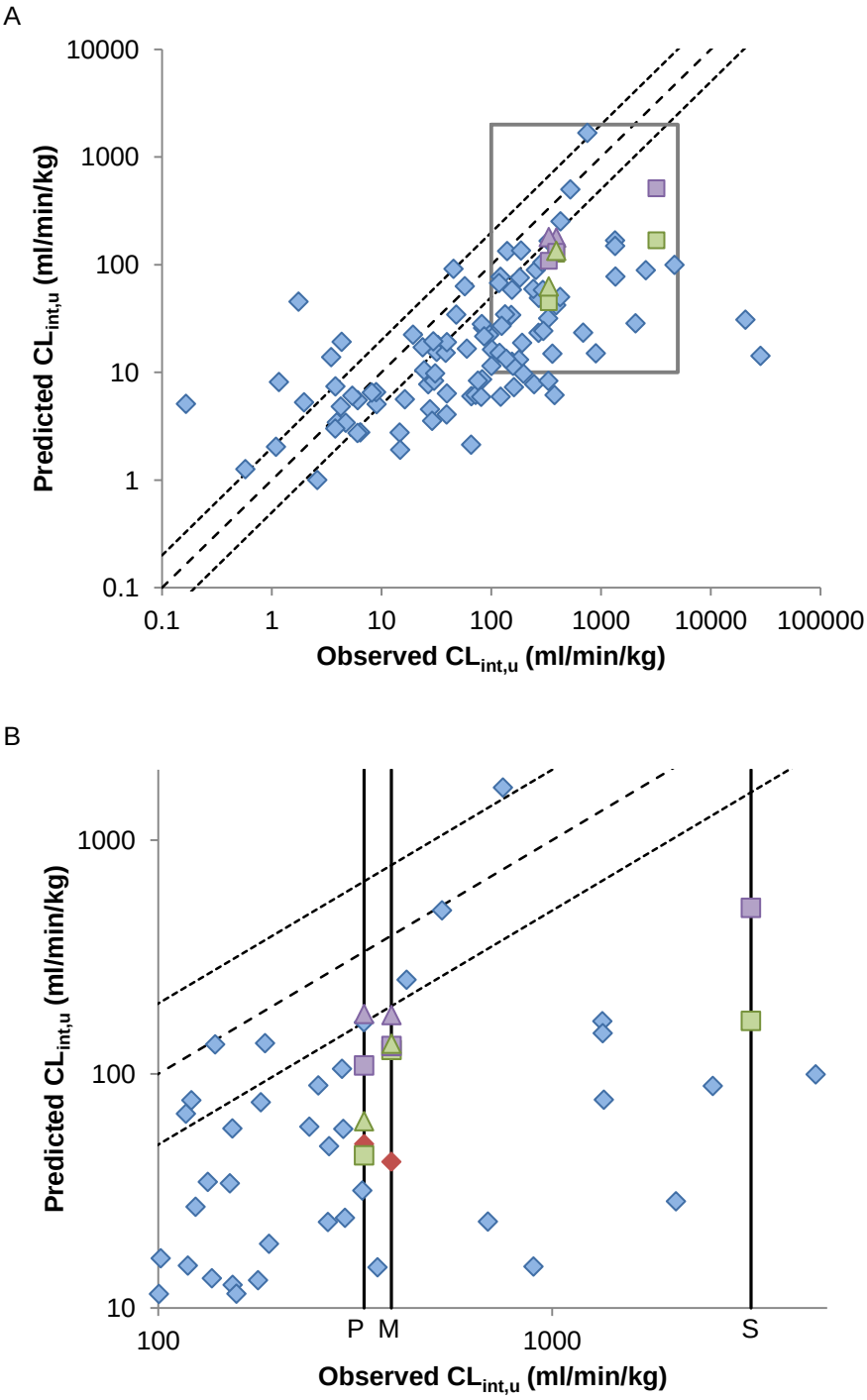


Figure 6-2. (A) Predicted $CL_{int,u}$ of midazolam, propranolol and saquinavir in human hepatocytes in this study superimposed onto literature predictions of *in vivo* $CL_{int,u}$ of all drugs in human hepatocytes. (B) Enlargement of selected section of Plot A. Diamonds (◆) represent literature data (red diamonds (◆) selected drugs); triangles (▲) represent determinations performed at 0.1 µM; squares (■) represent determinations performed at

1 µM; green symbols (■) represent determinations performed in static incubations and purple symbols (■) represent determinations performed in shaken incubations. Dashed lines represent unity and dotted lines a two-fold margin of error, unbroken lines represent observed $CL_{int,u}$ for the selected drugs: P, propranolol; M, midazolam; S, saquinavir.

6.5 Discussion

The current study has investigated, using a limited dataset, the influence on CL_{int} determinations in human hepatocytes of two assay variables shown to significantly affect CL_{int} determinations in rat hepatocytes.

The use of a lower substrate concentration (0.1 µM versus 1 µM) for determinations of CL_{int} in rat hepatocytes resulted in significant ($p < 0.05$) increases in the apparent CL_{int} of seven out of nine investigated drugs, including both midazolam and propranolol (Section 3.4.4). In human hepatocytes however, substrate concentration (0.1 µM versus 1 µM) did not have a significant effect on the observed CL_{int} of midazolam in either static or shaken incubations (Table 6-2, Figure 6-1A). This finding is consistent with the reported K_m for 1'-hydroxylation of midazolam (the major metabolic pathway in human) of 6.0 µM (Hallifax et al., 2005). In contrast, according to the reported K_m of 1.02 µM for 4'-hydroxylation of propranolol in human hepatocytes (Lewis and Ito, 2010), a difference between CL_{int} determined at 0.1 µM and 1 µM would be considered more likely for this drug. A statistically significant ($p < 0.05$) difference between CL_{int} determined at these concentrations was however, only observed in shaken incubations (Table 6-2, Figure 6-1B). The lack of an effect of substrate concentration on the CL_{int} of propranolol in static incubations is most likely due to the presence of a thicker UWL (relative to shaken conditions). Permeation through the UWL is rate-limiting on CL_{int} and therefore the apparent CL_{int} of both 0.1 µM and 1 µM propranolol is similar. In shaken conditions the UWL depth is reduced; when CL_{int} is no longer restricted by UWL permeation, the effects of substrate saturation are observable i.e. higher apparent CL_{int} at a substrate concentration below the K_m. It should be noted that assessment of the effect of substrate concentration on CL_{int} determinations in rat hepatocytes was performed in shaken incubations and therefore UWL permeation was not likely to be rate-limiting for any of the investigated drugs.

As in rat hepatocytes, shaking was also found to significantly ($p <0.05$) affect CL_{int} determinations in human hepatocytes; indeed three-fold increases in the CL_{int} of 0.1 µM propranolol and 1 µM saquinavir were observed in both rat and human hepatocytes. A 2.6-fold increase in the CL_{int} of 1 µM propranolol was also observed in human hepatocytes, however this concentration was not studied in rat. In contrast, no statistically significant ($p < 0.05$) effects of shaking on the CL_{int} of midazolam were observed in human hepatocytes despite the observation in rat. As increased *in vitro* CL_{int} with shaking was established to be indicative of an UWL permeation rate-limitation in rat hepatocytes (Section 5.5.3), it is therefore concluded that such a limitation is also present in static incubations of human

hepatocytes for propranolol and saquinavir, but not midazolam. This would indicate either a membrane permeation or metabolic rate limitation of midazolam CL_{int} in human hepatocytes. As the reported *in vitro* microsomal $CL_{int,u}$ of midazolam (scaled to whole body) is 363 ml/min/kg and 1447 ml/min/kg for human and rat respectively (for references see Appendices 8.1 and 8.3), it is likely that this difference is due to the reduced metabolic clearance of midazolam in humans.

Overall, the use of shaking resulted in improved predictions of *in vivo* $CL_{int,u}$ relative to both static incubations in this study and literature predictions. In contrast to a number of overpredictions determined in shaken incubations of rat hepatocytes with 0.1 µM substrate (across a similar *in vivo* $CL_{int,u}$ range), the same incubation conditions for human hepatocytes resulted in minor underpredictions of *in vivo* $CL_{int,u}$ for both midazolam and propranolol. The average 2.2- and 1.8-fold underpredictions for midazolam and propranolol respectively are close to- and within- the generally accepted 'two-fold of *in vivo*' criteria. The remaining error between *in vitro* and *in vivo* clearance may include interindividual variability. Prediction of the *in vivo* clearance of saquinavir (1 µM) was also considerably improved in shaken incubations, but still 6.2-fold below *in vivo*. This may at least in part however, be due to use of a saturating substrate concentration (the large degree of non-specific binding prevented use of a lower incubation concentration). Assessment of the effect of shaking on additional drugs would be useful in evaluating the wider effect on underprediction of *in vivo* $CL_{int,u}$.

6.6 Conclusion

In conclusion, the experimental variables found to influence determinations of *in vitro* CL_{int} in rat hepatocytes were demonstrated to have a similar effect on such determinations in human hepatocytes. A substrate concentration of 1 µM appeared to be partially saturating in the metabolism of propranolol, highlighting a need for careful consideration of substrate concentration in human metabolic clearance studies also. Increased CL_{int} with shaking was observed for both propranolol and saquinavir in human hepatocytes, therefore indicating presence of the UWL and associated potential permeation rate-limitation in this system which should be accounted for. Species differences were observed in the case of midazolam (no observed effects of substrate concentration or shaking) which likely reflect the differences in metabolism and therefore rate-determining processes of *in vitro* CL_{int} for this drug. Overall, the use of incubation shaking has been demonstrated as an essential component of the quantitative assessment of CL_{int}.

Chapter 7. Final discussion

The overall aim of this thesis was to explore potential contributing factors to the underprediction of *in vivo* clearance, specifically with relation to the *in vitro* methodology of hepatocyte assays. The current chapter will discuss the major findings of this thesis with respect to previously published data and recommendations for future metabolic clearance studies. The limitations of this work are also considered alongside proposed future directions of investigation.

Analysis of the literature regarding *in vitro* predictions of *in vivo* clearance revealed similar fold-underpredictions and trend of clearance-dependent underprediction in human and rat hepatocytes (Table 2-6). This finding gives greater weight to the notion that the source of underprediction lay in the *in vitro* system and is not an artefact of interindividual variability expressed as donor mismatch. Alternative proposed explanations applicable to both species included permeability rate-limitation and cofactor rate-limitation (Hallifax et al., 2010; Foster et al., 2011). These hypotheses were a major focus of investigation of this thesis within the context of *in vitro* methodology. In addition, the effects of assay format and substrate concentration were examined, as well as the usefulness of measurements of viability relative to metabolic activity. Two major findings of this investigation considered of relevance in interpretation of both other findings of this study and previously published studies are discussed primarily, followed by the results of individual sections.

The first (presented in Chapter 4) is that cells typically considered non-viable, due to the inability to exclude trypan blue, may retain enzymatic activity. This was indicated by the higher CL_{int} of midazolam and propranolol in unpurified cryopreserved rat hepatocytes than in the same lot of Percoll-purified hepatocytes of significantly higher viability. This proposition was confirmed by demonstration of metabolic activity of saponin-permeabilised hepatocytes (considered entirely non-viable by trypan blue exclusion) supplemented with cofactor. This signifies that despite damage to the plasma membrane of hepatocytes, metabolic enzymes remain functional, provided adequate concentrations of the required cofactor are available.

The second finding of major importance (presented in Chapter 5) is that shaking increases apparent CL_{int} through reduction in the depth of the encountered UWL. The effects of shaking on apparent CL_{int} were demonstrated across a panel of 12 well-studied small-molecule drugs in rat hepatocytes and subsequently, three of these drugs in human hepatocytes. Three potential mechanisms of shaking-induced increases in CL_{int} were investigated: damage to the plasma membrane of hepatocytes allowing unrestricted access of substrate to the metabolising enzyme(s); reduction in the depth of the UWL surrounding the plasma membrane; increased distribution of substrate throughout the bulk medium. As there was found to be no difference in the CL_{int} of midazolam in hepatocytes subject to a

static and shaken pre-incubation followed by a static incubation with substrate, the theory regarding damage to the plasma membrane was discounted. Similarly, as no differences were observed in the CL_{int} of saquinavir in static and shaken incubations of NADPH-supplemented hepatocyte sonicates, rate-limiting distribution of substrate throughout the bulk medium was also disregarded. The observation of an effect of shaking on CL_{int} in NADPH-supplemented permeabilised hepatocytes indicated an alternative rate-limitation. Consistent with published data regarding reduction in the depth of an encountered UWL with shaking and the effects of the UWL on the apparent permeability of drugs (Pohl et al., 1998; Avdeef et al., 2004; Korjamo et al., 2009), increases in apparent CL_{int} with shaking were concluded to be due to increased permeation through the UWL.

7.1 Influence of assay format selection on *in vitro* intrinsic clearance

Comparison of assay formats in this study revealed differences in apparent CL_{int} both between suspended and short-term monolayer cultured hepatocytes and suspended hepatocytes in different formats. These observations are likely to be largely due to the differing effects of shaking on the UWL surrounding cells across vessels of different dimensions. In addition, short-term monolayer cultured hepatocytes were subject to a reduced shaking speed relative to suspensions, which may (partially) account for the lower CL_{int} observed. Interestingly, previous studies which have reported differences in CL_{int} between suspended and short-term monolayer cultured hepatocytes have compared static cultured hepatocytes with shaken suspended hepatocytes and such differences were only observed for medium and high clearance drugs (Utesch et al., 1991; Blanchard et al., 2004; Hewitt and Utesch, 2004; Blanchard et al., 2005; Griffin and Houston, 2005; Jouin et al., 2006; Lundquist et al., 2014). Although Lundquist et al. (2014) observed comparable CL_{int} in cultured and suspended rat hepatocytes, both formats were unshaken. This provides further support for the suggestion that observed differences in CL_{int} between assay formats are related to the effect of incubation shaking on the UWL surrounding cells.

In this study, there were also found to be large differences in the variability of *in vitro* CL_{int} determinations between formats. Whilst some variability in metabolic activity between different preparations may be inherent, it is not expected to account for the large discrepancies between formats. As ‘non-viable’ cells may retain enzymatic activity, the high variability observed in these suspension assays could be due to the varying proportion of (discounted) ‘non-viable’ cells within each incubation. The lower variability in monolayer assays would be explained by fact that the percentage viability (plasma membrane integrity) of cells in monolayer culture is likely to be very high, since cells with a damaged membrane are unlikely to adhere to a surface. Consequently, the proportion of ‘non-viable’ but enzymatically active cells (which are unaccounted for) contributing to metabolic clearance will consistently be very small.

7.2 Influence of substrate concentration on *in vitro* intrinsic clearance

The use of a generic incubation concentration (typically 1 µM) is common practice in the high throughput drug discovery setting (Klopf and Worboys, 2010). However, the findings of this study clearly demonstrate, through comparison of 0.1 µM and 1 µM substrate concentrations, that the use of 1 µM substrate is not consistent with accurate determinations of CL_{int} in rat hepatocytes (Figure 3-4). The lesser effect of a lower substrate concentration in human hepatocytes, investigated using two of the same drugs, is almost certainly due to species differences in hepatic metabolism. Xenobiotic metabolism is typically much faster in rats than humans, resulting in higher CL_{int} in the former species (Yang et al., 2010; Nishimuta et al., 2013). Whilst this can reflect metabolism of a substrate via a greater number of metabolic pathways, for these drugs, it most likely reflects lower K_m values of metabolism by rat isozymes and therefore lower saturating substrate concentrations. This may also explain the observation of overpredictions of *in vivo* $CL_{int,u}$ in rat hepatocytes with use of 0.1 µM substrate in this study, but a residual underprediction of *in vivo* $CL_{int,u}$ in human hepatocytes using this concentration. Overpredictions observed in rat may be a reflection of (partially) saturating drug concentrations used for *in vivo* studies, a scenario which may be less likely to occur for clinical dosages due to the postulated lower affinity of human metabolic enzymes for these substrates. Although inappropriate *in vitro* substrate concentration is likely to be a contributing factor to the overall underprediction of (rat) hepatic clearance, it is unlikely to explain the entirety of such underprediction, especially in human.

7.3 Consideration of the cofactor depletion hypothesis of underprediction of hepatic clearance

There has been speculation that the underlying cause of underprediction of *in vivo* clearance in hepatocytes is endogenous cofactor depletion (Swales and Utesch, 1998; Steinberg et al., 1999; Hengstler et al., 2000; Wang et al., 2005; Hallifax et al., 2010; Foster et al., 2011). Much of the supporting evidence of apparent cofactor depletion is in the form of increased CL_{int} in cofactor-supplemented hepatocyte sonicates or permeabilised cells (Swales et al., 1996; Swales and Utesch, 1998; Hewitt et al., 2000; Hewitt and Utesch, 2004). However, as clearly demonstrated in this work, the act of sonication or permeabilisation of hepatocytes and supplementation with cofactor, not only facilitates an increased concentration of cofactor available to the metabolising enzyme(s), but also a potentially increased concentration of substrate. Higher CL_{int} in such preparations is therefore not necessarily due to increased cofactor availability, but may also or instead signify removal of a permeation restriction (membrane and/or UWL) of the substrate.

Moderate increases in CL_{int} were observed with the addition of NADPH to intact hepatocytes in both this (Section 3.4.3.1) and previous (Wang et al., 2005; Kuester and Sipes, 2007) studies. However, following further investigation it became apparent that such increases were representative of the effects of this cofactor on the enzymatically-functional, but membrane-damaged cells considered ‘non-viable’ within the incubation. The large variability

in effect of cofactor supplementation on midazolam CL_{int} and differences observed between assay formats in this study are therefore likely due to the varying proportions of 'non-viable' cells. Although some degree of cofactor depletion is plausible, it seems unlikely to be the major cause underlying underprediction of hepatic clearance.

7.4 Measures of viability

The use of a hepatocyte preparation for metabolic or other pharmacokinetic studies is often dependent on the measured viability in relation to a pre-defined limit of experimental acceptability. Reported viability of hepatocytes is most commonly a measure of plasma membrane integrity as determined by trypan blue exclusion. However, as discussed previously, it was concluded that cells determined non-viable by trypan blue exclusion may in fact retain a degree of enzymatic activity. Such findings have important implications regarding the use of low viability preparations in both past and future studies. Apparent high CL_{int} in preparations of low viability (for example in the study by Jouin et al. (2006)) may be due to the presence of a large number of metabolically active hepatocytes which are unaccounted for. In addition, the use of low viability hepatocytes could result in an overestimation of CL_{int} for passive permeability or active uptake rate-limited compounds. Although the overall viability of a preparation does not appear to affect the metabolic activity of hepatocytes within it, should little be known about the rate-determining processes of a compound's CL_{int}, it may be advisable to Percoll-purify very low viability preparations to circumvent such issues.

Neither trypan blue exclusion nor ATP content was found to correlate with the metabolic activity of hepatocyte preparations as assessed by the CL_{int} of a panel of nine drugs encompassing a range of *in vivo* clearances and metabolic pathways. Interestingly, significant correlations were found between the CL_{int} of individual drugs with common metabolic enzymes, for example midazolam and triazolam, naloxone and chlorpromazine. This either suggests that the activity of different metabolic enzymes could be differentially affected by hepatocyte isolation, reflects a degree of interindividual variation within these animals, or a combination of the two. Metabolism of a metabolically relevant probe substrate or positive control is therefore proposed as the most accurate indication of the metabolic activity of a hepatocyte preparation, potentially reflecting both interindividual variation and extrinsic factors which may have altered metabolic activity post-isolation. Such a measure may only be feasible in certain situations, for example determination of the CL_{int} of multiple compounds within a chemical series expected to be metabolised via the same pathway(s). Initially, a considerable number of CL_{int} determinations of such a probe substrate (or substrates) would need to be performed to determine an average value and acceptance range. Following this, the reliability of an CL_{int} determination of an untested compound would be assessed based on the CL_{int} of the probe substrate relative to previous determinations. Such an approach may not be applicable in the case of analysis of a chemically diverse group of drugs should the metabolic pathways be unknown. However, it may be possible to

use *in silico* prediction of metabolic liabilities to guide selection of appropriate probe substrates. Alternatively, a panel of probe substrates could be employed, such as the U.S. Food and Drug Administration's preferred chemical substrates for *in vitro* experiments (FDA, 2006).

7.5 Influence of shaking on *in vitro* intrinsic clearance

The use of shaking in hepatocyte clearance assays appears typically infrequent and variable; shaking conditions reported in published studies considered in this thesis range from a complete absence of shaking to shaking at 900 rpm. Although the general consensus appeared to be that shaking may be damaging to cells (Berry et al., 1991; Seglen, 2013), the true effects of shaking on hepatocyte metabolic clearance assays appear to have never been extensively examined.

As discussed previously, shaking has been demonstrated to reduce the depth of an UWL adjacent to a biological membrane (Pohl et al., 1998; Avdeef et al., 2004; Korjamo et al., 2009). However, whilst the potential and even observed effects of the UWL diffusion barrier on apparent permeability and uptake transport parameters have been reported across many years (Winne, 1973; Williams et al., 1990; Naruhashi et al., 2003; Avdeef et al., 2004; Korjamo et al., 2009; Shibayama et al., 2015), consideration of the implication of such research on metabolic clearance studies has been limited, if at all. A large disconnection between research relating to intestinal membrane permeability and hepatic clearance studies is apparent, but whilst there are considerable differences between the *in vivo* situations, there are important similarities *in vitro*, including the presence of the UWL.

7.5.1 Optimisation of shaking speed

The depth of an encountered UWL is reported to be related to the shaking speed, as well as aqueous diffusivity of the solute and viscosity of the solvent (Avdeef et al., 2004; Korjamo et al., 2009). In initial investigations regarding shaking speed, the CL_{int} of midazolam was found to be considerably higher in incubations performed at 900 rpm and 1050 rpm than in incubations performed under static conditions or at 450 rpm. The large step change in CL_{int} between 450 rpm and 900 rpm is likely to represent reduction in the depth of the UWL to a degree, between these two shaking speeds, where UWL permeation becomes no longer the rate-determining process of *in vitro* CL_{int}. Instead, either membrane permeation or metabolism becomes rate-determining and therefore a further reduction in the depth of the UWL at a shaking speed of 1050 rpm has no impact on the observed *in vitro* CL_{int} of midazolam. Under these circumstances however, an increased CL_{int} at 450 rpm relative to that in static incubations may also be expected, as some reduction in the depth of the UWL with shaking and therefore increase in UWL permeation, would be anticipated. The lack of such an increase may be due to the fact that at this speed cells are not suspended within the incubation media, but aggregated at the bottom of the incubation vessel. The action of shaking cannot reduce the UWL depth around individual cells which are aggregated and

therefore a considerable effect on CL_{int} is not observed until (somewhere between 450 rpm and) 900 rpm when cells become suspended. Further characterisation of the effect on the CL_{int} of multiple drugs across an increased number of shaking speeds would have been useful in determining the influence of both hepatocyte suspension and further reduction in UWL depth on apparent CL_{int}.

Nevertheless, these findings highlight the importance of optimisation of shaking speed for individual incubation vessels and apparatus, and a definitive need to ensure constant suspension of incubated hepatocytes. The point at which UWL permeation becomes no longer rate-determining, and therefore shaking speed at which apparent CL_{int} is reflective of the true *in vitro* CL_{int}, may differ between compounds. Although selection of a high shaking speed such as 900 rpm will increase the likelihood that this point is surpassed, it is important to be aware of the limitations of this method; for very high clearance compounds, there is the possibility that UWL permeation may still be rate-limiting at this speed. Though there is evidence to suggest the presence of an UWL in the liver (Ichikawa et al., 1992), this is expected to be minimal due to the narrow diameter of sinusoids and constant blood flow.

Noticeably, the variability of CL_{int} determinations exhibited a similar step change between shaking speeds and may therefore also be linked to UWL permeation. In the example of midazolam, at 0 rpm and 450 rpm the apparent CL_{int} is assumed to be determined by the rate of permeation through the UWL surrounding the aggregated cells. This is unlikely to differ greatly between hepatocyte preparations. At 900 rpm and 1050 rpm however, the apparent CL_{int} is most likely determined by the rate of metabolism; this would be expected to exhibit greater variability between preparations as a result of either intrinsic differences in enzymatic functionality or extrinsic factors which may for example, have altered the energetic status of the isolated cells. In addition, occasionally there may be instances in which cells fall out of suspension and this may therefore also increase variability of CL_{int} determinations in shaken incubations.

7.5.2 Substrate specificity in effect of shaking

During further investigation of the effects of shaking with additional drugs, increases in apparent CL_{int} were observable for other medium and high clearance drugs, but not the low clearance drug tolbutamide (Figure 5-2). These findings are supported by those of Griffin and Houston (2004), who found a similar pattern of increased CL_{int} in shaken suspensions of freshly isolated hepatocytes compared to static suspensions of cryopreserved hepatocytes, for medium and high, but not low, clearance drugs. This suggests that for a likely considerable proportion of medium and high clearance drugs, UWL permeation may be the rate-determining process of *in vitro* CL_{int} and therefore significantly contribute to the observed underprediction of *in vivo* clearance. Low clearance drugs such as tolbutamide, are unlikely to be UWL permeation rate-limited, but instead either membrane permeation or metabolism rate-limited. This explanation would account for the differences in prediction

accuracy across *in vivo* clearance i.e. typically accurate predictions of low clearance drugs and considerable underpredictions of high clearance drugs.

The use of shaking resulted in 50% of predictions of *in vivo* $CL_{int,u}$ in rat hepatocytes within two-fold of observed, although a slight average overprediction (2.2-fold) of the 12 investigated drugs as a single group. However, the effect of shaking on CL_{int} determinations in rat hepatocytes was investigated using 0.1 µM substrate and as alluded to previously, it is possible that higher drug concentrations may have been used in the cited *in vivo* studies. Therefore a comparison of CL_{int} determinations in static and shaken incubations using 1 µM substrate would be useful in further assessing the effect of shaking on *in vivo* $CL_{int,u}$ prediction accuracy in rat. The use of shaking in human hepatocyte studies improved predictions of *in vivo* $CL_{int,u}$ for all three tested drugs, with considerable improvements observed for propranolol and saquinavir.

7.6 Implications regarding the presence of the unstirred water layer in *in vitro* assays

The apparent CL_{int} determined in hepatocyte assays can be considered as three sequential clearance processes: clearance through the UWL, clearance through the plasma membrane and intrinsic metabolic clearance (Figure 5-12). In Chapter 5, a series of experiments was proposed in which the properties of intact and permeabilised hepatocytes in static and shaken conditions could be utilised to determine each of these clearance processes, and identify which is rate-limiting (Section 5.5.4). The limitation of this approach is that it is only applicable to compounds which do not show accumulation of unbound drug within the cell. In the present assay, active transport or passive permeation into and accumulation within hepatocytes will not be identified as determination of parameters relies on drug metabolism. The presence of the UWL however, may be of equal importance in determination of CL_{int} for drugs with such disposition. The movement of drug into a cell, whether passively through the membrane or by an active uptake transporter, is governed by the concentration at the cell surface; the presence of the UWL reduces this concentration relative to the bulk medium and therefore the amount of drug taken into the cell. The implication regarding determination of uptake clearance is the same as for metabolism - underprediction of *in vivo* clearance.

Although not considered as part of this thesis, in addition, the presence of the UWL may also affect determination of the kinetic parameter K_m in both uptake and metabolism studies. As far back as 1973, Winne described how the presence of an UWL may increase the apparent K_m of a substrate of a 'carrier-mediated transport system'. Very recently this was experimentally demonstrated in the context of hepatic clearance by Shibayama et al. (2015) who reported that vigorous stirring reduced the apparent Michaelis constant, but did not alter the maximal rate of transport of tetraethylammonium and 1-methyl-4-phenylpyridinium by human Organic Cation Transporter 2 (OCT-2). In theory, the same principles apply to the determination of K_m for enzymatic reactions within hepatocytes. Notably, the reported literature K_m values for metabolism of the substrates used to investigate the effect of

substrate concentration in rat hepatocytes (Table 3-7) were higher than would be expected based on comparisons of CL_{int} determined at 0.1 µM and 1 µM substrate in shaken incubations (Section 3.4.4). This was attributed to potential inaccuracies in substrate concentration range used for determinations, or the obscuration of alternative metabolic pathways involving higher affinity enzyme components. However, it could also be the case that kinetic parameters were determined in static incubations of hepatocytes without consideration of the effects of the UWL, therefore leading to overestimation of K_m.

7.6.1 The unstirred water layer and the serum incubation effect

In the investigation of *in vitro* methodology relating to the prediction of metabolic clearance, some researchers have advocated the incubation of hepatocytes in serum (Shibata et al., 2000; Bachmann et al., 2003; Blanchard et al., 2004; Blanchard et al., 2005; Blanchard et al., 2006). This method was initially proposed as a means by which to equate *in vitro* and *in vivo* binding and negate the need for such measurements and corrections (Shibata et al., 2000). However, one particular research group reported improved predictions of *in vivo* $CL_{int,u}$ and CL_h in hepatocytes incubated in serum compared to serum-free media (Blanchard et al., 2004; Blanchard et al., 2005; Blanchard et al., 2006). A suggested explanation for this observation was that certain facilitated or active transport processes may be activated in the presence of serum (Blanchard et al., 2005; Blanchard et al., 2006). Binding of molecules to albumin had previously been shown to decrease the overall uptake into hepatocytes, but increase the apparent uptake of the free fraction, however the mechanism of such a phenomenon was undefined (Barnhart et al., 1983; Tsao et al., 1988). Indeed, the presence of the UWL may also explain observations attributed to and the underlying mechanism of this so-called protein-facilitated drug uptake.

Diffusion of a molecule through the UWL is partially dependent on its size; larger molecules with a smaller diffusion coefficient encounter an apparently narrower UWL than smaller molecules with a greater diffusion coefficient (Pohl et al., 1998). The binding of a drug to serum proteins would therefore effectively narrow the UWL encountered and potentially increase the observed $CL_{int,u}$. Such effects on CL_{int} (corrected for protein binding) would therefore be expected to be more apparent for highly protein bound drugs. In support of this hypothesis, Blanchard et al. (2004; 2005; 2006) found large increases in predictions of *in vivo* $CL_{int,u}$ and CL_h in serum incubations for highly protein bound drugs (e.g. midazolam), but a lack of an effect for low protein bound drugs (e.g. naloxone). In addition, Blanchard et al. (2004) reported a considerably decreased $K_{m,u}$ (K_m corrected for protein binding) for both midazolam (17-fold) and mibefradil (145-fold) in rat hepatocytes incubated in serum. This is consistent with the proposed effects of reduction of the UWL on apparent K_m. The idea that protein-facilitated drug uptake may be explained by increased diffusion of protein-bound drug through the UWL has in fact been proposed previously in the context of the perfused rat liver (Ichikawa et al., 1992). It is a hypothesis that warrants deeper investigation outside of the scope of this thesis.

7.7 Conclusions and recommendations

Although the underprediction of hepatic clearance from *in vitro* systems is widely accepted, there is a definite need to elucidate and resolve the causes underlying this phenomenon. The current study sought to investigate potential contributing factors to the underprediction of *in vivo* clearance, specifically with relation to the *in vitro* methodology of hepatocyte metabolic clearance assays. Soars et al. (2007b) suggested that "investigators who claim that *in vitro* CL_{int} data are not predictive of *in vivo* clearance may simply not be considering the data (including its origin and quality) with due attention". To a degree, the findings of this study reveal that to be true; the results of this work have highlighted potential oversights in the *in vitro* methodology as well as a need to reassess current practices, as opposed to flaws in the basic premise. The following are recommendations of best practice based on the principal findings of this thesis.

It is recommended that metabolic clearance assays using the substrate depletion approach (human and animal) are performed at both 0.1 µM and 1 µM. This would facilitate more accurate determinations of CL_{int} and highlight any potential for enzymatic saturation in future *in vitro* and *in vivo* studies. Trypan blue exclusion should be viewed as a measure of plasma membrane integrity only and not an indicator of the metabolic capacity of a hepatocyte preparation. Instead, the use of a metabolically relevant probe substrate may be more appropriate in identifying anomalous results in metabolic clearance assays. ATP content is not recommended as a measure of viability for such studies. Trypan blue exclusion may be used to judge the necessity for Percoll purification of preparations. In the case of compounds which are metabolism rate-limited, Percoll purification of preparations below the typically employed 85% viability cut-off is likely to be unnecessary. However, for compounds which are uptake/permeability rate-limited (or for which the rate-limiting clearance process is unknown, including drugs considered permeable or highly metabolically cleared), Percoll purification of preparations below 85% viability as determined by trypan blue exclusion is recommended to avoid overpredictions of CL_{int}. Both metabolic and uptake clearance studies should be performed using suspended hepatocytes under shaken conditions (speed optimised relative to apparatus), as the presence of the UWL *in vitro* may reduce the concentration of compound at the cellular surface resulting in an overestimation of K_m for enzymes and transporters and an underestimation of clearance. Lastly, studies following the afore-described protocol using both intact and permeabilised hepatocytes in static and shaken conditions (Chapter 5) may be used to delineate clearance processes (UWL permeation, membrane permeation/transport and metabolic clearance) *in vitro.* This approach would also be appropriate for substrates of uptake transporters, although further investigation would be required in relation to this application.

Taken together, these technically moderate but influential changes to the *in vitro* methodology prove promising in achieving accurate predictions of *in vivo* clearance from hepatocyte studies.

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Chapter 8. Appendix

8.1 Database of *in vivo* and *in vitro* clearance of drugs in human.

Table 8-1. Database of *in vivo* and *in vitro* clearance of drugs in human. References are given in numbered format in square brackets.

Drug	*In Vivo*				*In Vitro*					
					Hepatocytes			Microsomes		
	n	CL_h (ml/min/kg)	fu_b	$CL_{int,u}$ (ml/min/kg)	n	fu_{hep}*	$CL_{int,u}$ (ml/min/kg)	n	fu_{mic}*	$CL_{int,u}$ (ml/min/kg)
Acebutolol	3	5.4 [1-3]	0.81 [1, 3]	9.0	1	0.91	5.1 [4]			
Acetaminophen	5	4.4 [5-8]	0.88 [5, 9]	6.4	3	0.933 [6]	2.8 [6, 10, 11]	1	0.92	7.2 [10]
Alfentanil	6	6.9 [12-16]	0.16 [12-15]	64				1	a	116 [13]
Alprazolam	4	0.78 [5, 13, 14, 17]	0.31 [5, 13, 14]	2.6	2	0.93	1.0 [18, 19]			
Alprenolol	2	14 [5, 20]	0.27 [5]	154	1	0.85	59 [5]	1	0.79	45 [5]
Amitryptyline	5	9.6 [5, 14, 21, 22]	0.054 [5, 14, 21, 23]	330	1	0.15	32 [10]	1	0.10	79 [10]
Antipyrine	2	0.55 [5, 24]	0.99 [5, 24]	0.57	1	0.95	1.3[25]	1	0.93	0.13 [26]
Atenolol	3	0.13 [5, 14, 27]	0.79 [5, 14]	0.16	1	0.95	5.1[10]	1	0.93	12 [10]
Atorvastatin	1	16 [13]	0.036 [13]	2071	2			1	a	51 [13]
Betaxolol	2	2.9 [5, 28]	0.56 [5]	6.1	2	0.79	5.5 [4, 29]	1	0.70	2.7 [10]
Bosentan	2	3.5 [30, 31]	0.064 [30]	66	4	0.81	6.0 [32]	1	0.73	14 [10]
Bupivacaine	3	7.1 [5, 14, 33]	0.071 [5, 14, 33]	152	1	0.55	34 [5]	1	0.43	87 [5]
Buprenorphine	3	15.0 [5, 14, 34]	0.040 [5, 14, 34]	1354	1	0.35	78 [5]	2	0.10 [35]	661 [5, 35]
Caffeine	2	1.2 [25, 36]	0.65 [36, 37]	2.0	3	0.96	5.3 [4, 11, 25]	1	0.93	0.40 [38]
Carbamazepine	1	1.6 [37]	0.31 [37]	5.4	1	0.84	6.1 [4]			
Carvedilol	3	10 [14, 39, 40]	0.050 [14, 39]	427	4	0.38	253 [4, 10, 11, 39]	1	0.28	330 [10]
Chlorpheniramine	2	1.6 [5, 41]	0.44 [5, 41]	3.8	2	0.66	7.4 [4, 10]			
Chlorpromazine	3	11 [5, 36, 42]	0.043 [5, 11, 36, 37]	525	4	0.07	501 [5, 10, 11, 25]	2	0.04	448 [5, 10]
Cimetidine	4	2.6 [14, 39, 43, 44]	0.87 [14, 39]	3.5	3	0.95	14 [4, 10, 39]	1	0.92	8.9 [10]
Clozapine	2	4.1 [14, 45]	0.054 [14, 45]	96	4	0.70	22 [4, 10, 11, 29]	1	0.59	18 [10]

Codeine	1	13.8 [46]	0.73 [46]	57	1	0.93	63 [4]			
Cyclosporine A	3	7.6 [13, 39, 47]	0.047 [13, 39, 47]	154	2	0.77	13 [4, 39]	1	a	66 [13]
Desipramine	1	10 [48]	0.21 [48]	121	5	0.16	77 [4, 5, 10, 11, 25]	1	0.10	104 [10]
Dexamethasone	2	5.7 [49, 50]	0.29 [50]	28	1	0.90	4.6 [10]	1	0.85	9.3 [10]
Diazepam	6	0.57 [25, 51-54]	0.036 [25, 51, 52, 54]	16	8	0.544 [6]	5.6 [4, 6, 10, 11, 18, 19, 25, 55]	1	0.66	2.4 [10]
Diclofenac	1	7.7 [56]	0.0090 [56]	1342	10	0.94	169 [4, 5, 10, 11, 36, 55, 57, 58]	3	0.84 [35]	136 [5, 10, 35]
Diflusinal	2	0.14 [39, 59]	0.0053 [39]	27	2	0.95	7.8 [11, 39]			
Diltiazem	4	12 [14, 25, 60, 61]	0.22 [14, 51]	134	9	0.360 [6]	35 [4-6, 10, 11, 25, 29]	2	0.72	31 [5, 10]
Diphenhydramine	3	18 [62-64]	0.34 [62]	360	2	0.69	15 [4, 10]	1	0.58	11 [10]
Domperidone	2	12 [1, 12]	0.097 [1, 12]	271	1	0.48	49 [5]	1	0.37	306 [5]
Felodipine	2	16 [13, 65]	0.0057 [13]	14012				1	0.06 [66]	1703 [13]
Fenoprofen	1	7. 3 [39]	0.0055 [39]	2063	4	0.95	29 [5, 10, 11, 39]	1	0.92	13 [5]
Flumazenil	4	15 [14, 67-69]	0.60 [14, 67-69]	101	1	0.94	216 [5]			
Flunitrazepam	2	3.1 [1, 36]	0.25 [1, 36]	15	2	0.89	2.8 [18, 19]			
Fluphenazine	1	0.58 [14]	0.14 [14]	4.3	1	0.63	19 [5]	1	0.51	92 [5]
Furosemide	4	1.2 [14, 36, 70]	0.020 [10, 14, 36]	62					0.92	27 [10]
Gemfibrozil	2	3.1 [36, 39]	0.026 [36, 39]	139	10	0.93	134 [4, 5, 10, 11, 39, 57, 58]	2	0.91 [35]	31 [5, 10, 35]
Glimepiride	2	1.0 [1, 14]	0.0055 [1, 14]	196	1	0.90	9.8 [5]	1	0.84	37 [5]
Glipizide	2	0.75 [1, 39]	0.020 [1, 39]	39	3	0.96	4.1 [10, 11, 39]			
Glyburide	3	2.0 [14, 71]	0.038 [72]	60	3	0.93	17 [5]	1	0.89	57 [5]
Hydrocortisone	2	3.3 [73, 74]	0.20 [73]	19	1	0.93	18 [10]	1	0.89	45 [10]
Ibuprofen	2	1.4 [25, 75]	0.018 [75]	84	4	0.94	26 [5, 10, 11, 25]	1	0.91	31 [10]
Imipramine	5	13 [4, 36, 37, 48, 76]	0.13 [23, 36, 48]	255	8	0.18	90 [4, 5, 10, 11, 25, 29]	1	0.12	125 [10]

Indomethacin	3	2.1 [14, 39, 77]	0.019 [14, 39, 77]	124	1	0.95	27 [39]			
Irbesartan	3	3.8 [14, 39, 78]	0.10 [14, 39]	48	4	0.94	35 [10, 11, 29, 39]			
Ketanserin	4	9.7 [79-82]	0.097 [79, 80, 82]	187	4	0.87	136 [57, 58]			
Ketoprofen	3	2.1 [5, 36, 39, 83]	0.015 [5, 11, 36, 39, 84]	158	6	0.96	12 [4, 5, 10, 11, 39]	1	0.93	7.2 [10]
Labetalol	3	14 [85-87]	0.38 [86, 87]	119	1	0.93	15 [5]	1	0.89	17 [5]
Levoprotiline	1	14 [88]	0.19 [5]	256	1	0.66	7.8 [5]			
Lidocaine	7	14 [14, 36, 89-91]	0.34 [10, 14, 36, 92]	137	2	0.90	13 [5, 10]	1	0.85	4.3 [93]
Lorazepam	4	1.1 [25, 36, 94-96]	0.080 [10, 14, 36, 96]	15	2	0.85	1.9 [4, 25]	1	0.78	22 [10]
Lorcainide	3	20 [10, 97, 98]	0.17 [10, 98]	2559	1	0.17	89 [10]	1	0.11	806 [10]
Lovastatin	2	15 [1, 37]	0.082 [1, 37]	740				1	a	4096 [13]
Methadone	4	1.7 [13, 14, 99, 100]	0.21 [13, 14]	8.7				1	a	19 [13]
Methylprednisolone	4	5.9 [10, 36, 101, 102]	0.18 [10, 36]	45	3	0.87	13.5 [10, 11, 25]	1	0.81	27 [10]
Metoclopramide	1	1.6 [103]	0.60 [103]	8.9	1	0.82	6.6 [5]			
Metoprolol	4	12 [14, 36, 104, 105]	0.83 [11, 14, 36, 104]	32	7	0.90	16 [4, 5, 10, 11, 29]	1	0.85	4.0 [10]
Mianserin	1	18 [106]	0.14 [107]	897	1	0.83	15 [5]	1	0.76	25 [5]
Midazolam	5	9.2 [13, 36, 37, 108, 109]	0.043 [11, 13, 14, 36, 37, 108]	390	10	0.92	42 [4, 5, 10, 11, 18, 19, 25, 32, 55]	3	0.54 [66]	363 [5, 10, 13]
Montelukast	6	1.1 [14, 39, 110, 111]	0.0062 [112]	182	2	0.41	76 [10, 39]			
Nadolol	33	0.92 [4, 14, 113]	0.83 [14, 114]	1.2	1	0.94	8.2 [10]	1	0.91	18 [10]
Naloxone	2	18 [25, 115]	0.51 [14, 116]	333	10	0.93	167 [4, 5, 10, 11, 25, 32, 57, 58]	2	0.87 [35]	13 [10, 35]
Naltrexone	1	1.4 [117]	0.83 [117]	1.8	4	0.94	46[57, 58]			
Naproxen	2	0.12 [14, 36]	0.0010 [10]	117	5	0.91	68 [4, 10, 57]	1	0.86	19 [10]

Nifedipine	6	5.4 [4, 25, 36, 39, 118-121]	0.030 [14, 36, 118, 119]	242	4	0.79	60 [4, 10, 25, 36]	2	0.70 [66]	178 [10, 13]
Nisoldipine	3	12 [13, 122, 123]	0.0030 [13]	9837				1	a	5993 [13]
Nitrendipine	4	20 [4]	0.029 [14]	20700	1	0.61	31 [4]			
Omeprazole	2	11 [4, 124, 125]	0.067 [125]	331	3	0.87	8.4 [4, 10, 11]	1	0.81	17 [10]
Ondansetron	3	6.5 [4, 14, 126]	0.33 [14]	29	5	0.88	3.6 [4, 10, 11, 29, 39]			
Oxaprozin	1	0.07 [39]	0.0007 [39]	100	3	0.94	12 [10, 11, 39]			
Oxazepam	2	1.2 [36, 127]	0.043 [36, 127]	30	3	0.87	8.4 [10, 11, 25]			
Oxprenolol	1	5.4 [128]	0.30 [5]	24	2	0.88	11 [5, 29]			
Phenacetin	2	20 [14, 129]	0.57 [10, 14, 130]	687	4	0.91	24 [5, 10, 29, 55]	2	0.87	34 [5, 10]
Phenytoin	2	5.3 [14, 37]	0.11 [14]	65	1	0.84	2.1 [29]			
Pindolol	3	3.7 [1, 14, 37]	0.56 [1, 14, 37]	8.1	3	0.91	6.5 [4, 10, 11]			
Prazosin	3	4.4 [4, 14, 131]	0.067 [10, 14, 132]	83	6	0.94	8.6 [4, 5, 10, 11, 29, 39]	1	0.91	7.3 6[10]
Prochlorperazine	3	16 [133]	0.0027 [94]	28402	1	0.76	14 [5]	1	0.66	70 [5]
Promazine	1	12 [1]	0.092 [134]	295	1	0.26	58 [5]			
Promethazine	1	16 [135]	0.22 [135]	293	1	0.18	106 [5]	1	0.12	79 [5]
Propafenone	2	19 [51, 136]	0.057 [51]	4672	2	0.50	100 [5, 10]	2	0.39	212 [5, 10]
Propranolol	15	15 [4, 14, 36, 87, 137-143]	0.15 [14, 36, 141]	333	8	0.63	50 [4, 5, 10, 11, 25, 29]	2	0.51	18 [5, 10]
(-)-Propranolol	4	13 [141-143]	0.17 [141]	191	1	0.63	19 [5]	1	0.51	14 [5]
(+)-Propranolol	4	15 [141-143]	0.19 [141]	270	1	0.63	23 [5]	1	0.51	32 [5]
Quinidine	5	4.1 [13, 14, 36, 39, 144]	0.21 [13, 14, 36, 39, 144]	24	3	0.64	17 [10, 11, 36]	2	0.53	19 [10, 145]
Ranitidine	4	2.7 [14, 39, 146, 147]	0.80 [14, 39]	3.9	4	0.95	3.4 [4, 10, 11, 39]			
Repaglinide	2	13 [1, 13]	0.025 [13]	1388				1	0.73 [66]	110 [13]
Rifabutin	2	4.1 [13, 148]	0.48 [13]	11				1	a	68 [13]
Risperidone	3	7.9 [12, 14, 149]	0.15 [12, 14]	82	2	0.74	28 [5, 10]	2	0.64	46 [5, 10]
Ritonavir	1	1.2 [39]	0.015 [39]	86	3	0.39	22 [4, 11, 39]			

Saquinavir	2	18 [13, 150]	0.038 [13]	3199				1	0.10 [66]	6386 [13]
Scopolamine	3	13 [4, 14, 151]	0.90 [14]	39	1	0.94	19 [4]			
Sildenafil	2	7.6 [152, 153]	0.040 [152]	298	4	0.92	24 [5, 10, 11, 25]	3	0.88,a	103 [5, 10, 13]
Tacrolimus	3	0.71 [154]	0.0040 [13, 155, 156]	181				1	0.18 [66]	889 [13]
Temazepam	2	1.9 [4, 157]	0.017 [157]	122	2	0.91	6.0 [4, 5]			
Tenoxicam	1	0.054 [158]	0.013 [158]	4.2	2	0.96	4.8 [10, 25]			
Theophylline	3	0.47 [25, 159, 160]	0.45 [14, 84]	1.1	2	0.96	2.0 [11, 25]	1	0.93	2.8 [10]
Timolol	3	9.7 [14, 37, 161]	0.59 [14, 37, 161]	31	2	0.90	9.8 [10, 11]			
Tolbutamide	2	0.35 [25, 162]	0.076 [162]	4.7	5	0.83	3.4 [5, 10, 11, 25, 36]	1	0.76	2.1 [10]
Trazodone	3	2.3 [13, 14, 163]	0.086 [13, 14]	30	1	0.82	20 [5]	2	0.74, a	63 [5, 13]
Triazolam	4	4.3 [1, 4, 36, 164]	0.14 [1, 36]	39	4	0.91	6.4 [4, 5, 18, 19]	1	0.87	40 [5]
Trimipramine	1	16 [165]	0.051	1344	1	0.10	150 [5]	1	0.06	222 [5]
Verapamil	6	16 [4, 13, 37, 166, 167]	0.096 [13, 37, 51, 166, 167]	750	8	0.52	106 [4, 5, 10, 11, 25, 29]	3	0.47 [66]	224 [5, 10, 13]
Warfarin	2	0.086 [25, 168]	0.023 [14, 168]	3.8	1	0.94	3.0 [25]	1	0.90	2.0 [10]
Wafarin (S-)	1	0.11 [36]	0.018 [36]	6.0	1	0.94	2.7 [36]			
Zaleplon	1	16 [169]	0.40 [14]	161	5	0.92	7.3 [5, 57, 58]			
Zidovudine	2	19 [170]	0.82 [171]	377	3	1.00 [6]	6.2 [6, 57]			
Zileuton	1	6 [4]	0.10 [172]	81	1	0.91	5.9 [4]			
Zolpidem	4	5.7 [1, 13, 14, 173]	0.10 [1, 13, 14, 173]	76	3	0.86	8.4 [5, 10, 11]	3	0.79, a	16 [5, 10, 13]

* fu_{hep} values are normalised to 1 x 10^6 cells/ml and fu_{mic} values are normalised to 1 mg microsomal protein/ml. Experimentally determined values are followed by the accompanying reference; all other values were determined using lipophilicity relationship algorithms (Equations 2.2 and 2.1, fu_{hep} and fu_{mic} respectively). a, only $CL_{int,u}$ or equivalent reported, no value for fu_{mic} given. Freshly isolated hepatocytes were used by [19, 39, 55] and both freshly isolated and cryopreserved hepatocytes were used by [4, 32, 58]. All other studies used cryopreserved hepatocytes.

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8.2 Drugs subject to glucuronidation in human

Table 8-2. Drugs subject to glucuronidation and reported fm_{UGT}

Drug	fm_{UGT}	Reference
Imipramine	0.1	Soars et al. (2002)
Amitriptyline	0.25	Miners and Mackenzie (1991)
Oxprenolol	0.25-0.29	Laethem et al. (1995)
Alprenolol	0.36	Miners and Mackenzie (1991)
Oxaprozin	0.39	Miners and Mackenzie (1991)
Gemfibrozil	0.40	Soars et al. (2002)
Fenoprofen	0.49	Miners and Mackenzie (1991)
Labetolol	0.55	Miners and Mackenzie (1991)
Naproxen	0.57/0.65	Miners and Mackenzie (1991); Soars et al. (2002)
Naloxone	0.60/0.65	Miners and Mackenzie (1991); Soars et al. (2002)
Acetaminophen	0.63	Miners and Mackenzie (1991)
Codeine	0.70	Soars et al. (2002)
Temazepam	0.73	Miners and Mackenzie (1991)
Zidovudine	0.75	Miners and Mackenzie (1991)
Diflunisal	0.76	Miners and Mackenzie (1991)
Oxazepam	0.86	Miners and Mackenzie (1991)
Ketoprofen	0.90	Soars et al. (2002)
Lorazepam	0.92	Miners and Mackenzie (1991)
Levoprotiline	0.97-0.99	Kaiser et al. (1992)

8.3 Database of *in vivo* and *in vitro* clearance of drugs in rat.

Table 8-3. Database of *in vivo* and *in vitro* clearance of drugs in rat. References are given in numbered format in square brackets.

Compound	*In Vivo*				*In Vitro*					
					Hepatocytes			Microsomes		
	n	CL_h (ml/min/kg)	fu_b	$CL_{int,u}$ (ml/min/kg)	n	fu_{hep*}	$CL_{int,u}$ (ml/min/kg)	n	fu_{mic*}	$CL_{int,u}$ (ml/min/kg)
Acetaminophen	1	24 [1]	0.82 [1]	39	1	0.93 [1]	76 [1]			
Alfentanil	1	45 [2]	0.24 [2]					1	0.97 [2]	572 [2]
Alprazolam	1	19 [3]	0.35 [3]	67	1	0.93	149 [4]	1	1.00 [4]	101 [4]
Antipyrine	5	5.1 [5-7]	1.00 [5]	5.4	2	1.0 [8]	413 [8]	1	0.93	8.1 [9]
Atorvastatin	3	35 [10-12]	0.036 [10, 12]	1470	3	0.92	45 [12-14]			
Bosentan	5	30 [15-17]	0.015 [15, 17]	2792	2	0.81	7.0 [15]			
Caffeine	1	0.02 [18]	1.00 [18]	0.02	1	0.96	12 [18]	1	0.93	11 [18]
Cerivastatin	1	39 [12]	0.041 [12]	1516	1	0.94	12 [12]			
Chlordiazepoxide	1	10 [3]	0.15 [3]	74	1	0.87	49 [4]	1	1.00 [4]	11 [4]
Chlorpromazine	1	61 [5]	0.068 [5]	2264						
Clobazam	1	32 [3]	0.21 [3]	220	1	0.90	137 [4]	1	1.00 [4]	264 [4]
Clonazepam	1	20 [3]	0.21 [3]	116	1	0.85	89 [4]	1	0.76 [4]	233 [4]
Dextromethorphan	1	62 [19]	0.26 [20]	635	5	0.52	696 [15, 20-22]	2	0.92 [20]	596 [20, 23]
Diazepam	5	51 [5, 24-27]	0.10 [5, 24-27]	1000	7	0.54 [1]	432 [1, 4, 21, 22, 28, 29]	2	0.93 [4]	438 [4, 29]
Diclofenac	5	22 [30-33]	0.041 [30, 31]	687	3	0.94	369 [15, 28, 31]			
Diltiazem	2	71 [34, 35]	0.18 [34-36]	1342	2	0.36 [1]	3277 [1, 21]			
Domperidone	1	67 [2]	0.07 [2]					1	0.34 [2]	698 [2]
Erythromycin	2	32 [34, 37]	0.60 [34, 37]	76	1	0.74	169 [14]			
Ethoxycoumarin	2	54 [3, 38]	0.22 [3]	515	5	0.86	282 [15, 22, 39]	3	0.79	248 [23, 39, 40]
Felodipine	1	3.7 [41]	0.070 [41]					1	0.09	13904 [41]
Fexofenadine	8	38 [42-45]	0.34 [42]	168	1	0.81	101 [14]			

FK079	1	2.6 [1]	0.10 [1]	28	1	0.91 [1]	6.7 [1]			
Galantamine	1	32 [2]	0.76 [2]					1	0.86 [2]	33 [2]
Granisetron	1	41 [31]	0.61 [31]	114	1	0.87	302 [31]			
Ibuprofen	55	4.9 [46, 47]	0.038 [46]					1	0.91	83 [47]
Indinavir	2	51 [48, 49]	0.65 [48, 49]					2	0.77	326 [48, 50]
Indomethacin	1	0.60 [46]	0.005 [46]	121	1	0.95	5.1 [12]			
Ketanserin	1	5.9 [2]	0.018 [2]					1	0.32 [2]	42 [2]
Lorcainide	1	86 [2]	0.22 [2]					1	0.45 [2]	1849 [2]
Lubeluzole	1	33 [2]	0.010 [2]					1	0.05 [2]	1387 [2]
Mazapertine	1	62 [2]	0.047 [2]					1	0.13 [2]	6390 [2]
Metoprolol	2	73 [6, 51]	0.53 [6]	513	1	0.90	101 [21]	1	0.85	124 [52]
Midazolam	7	54 [3, 31, 53-55]	0.062 [31, 54, 56]	1925	7	0.98 [8]	460 [4, 8, 28, 31, 39]	2	0.68 [4, 39]	1447 [4, 39]
Naloxone	1	59 [57]	0.57 [57]	253	2	1.00 [8]	1273[8]			
Nebivolol	1	41 [2]	0.013 [2]					1	0.10 [2]	990 [2]
Nelfinavir	1	37 [49]	0.041 [49]	1426	1	0.26	41070 [58]	1	0.02 [58]	342857 [58]
Norcisapride	1	27 [2]	0.43 [2]					1	0.79 [2]	7.4 [2]
Oxodipine	1	18 [3]	1.00 [3]					1	0.58	31 [59]
Phenytoin	6	18 [5, 60-63]	0.23 [5, 61, 62]	97	4	0.84	82 [21, 22, 60]			
Pindolol	1	59 [31]	0.64 [31]	225	1	0.91	251 [31]			
Prazosin	2	49 [31, 64]	0.33 [31]	296	1	0.94	41 [31]			
Propafenone	3	42 [65]	0.023 [65]	3227	1	0.50	10977 [65]	1	0.39	11753 [65]
Propranolol	8	74 [3, 5, 6, 15, 17, 51]	0.088 [5, 6, 15, 51]	3295	7	0.75 [66]	4527 [15, 21, 22, 39, 67, 68]	3	0.57 [68]	10705 [39, 68, 69]
Quinidine	3	28 [5, 70, 71]	0.20 [5, 70, 71]					1	0.53	19 [23]
Quintoloast	2	54 [72]	0.051 [72]	2341	1	0.88 [1]	58 [1]			
Risperidone	1	76 [2]	0.14 [2]					1	0.34 [2]	980 [2]
Ritonavir	2	30 [31, 49]	0.048 [31, 49]	894	2	0.39	7225 [58]	1	0.23 [58]	38764 [58]
Rosuvastatin	5	51 [15, 43, 73-75]	0.084 [15, 75, 76]	1228	3	0.96	60 [14, 15]			
Salbeluzole	1	43 [2]	0.019 [2]					1	0.06 [2]	956 [2]
Saquinavir	1	36 [49]	0.062 [49]	911	2	0.66	8428 [14, 58]	2	0.09 [58]	37818 [58, 77]

Tolbutamide	4	0.81 [5, 7, 78, 79]	0.13 [5, 78, 79]	6.1	4	0.83	9.8 [15, 22, 60]	3	0.76	11 [60, 80, 81]
Triazolam	1	84 [3]	0.28 [3]	1875	1	0.91	854 [4]	1	0.84 [4]	552 [4]
Troglitazone	2	37 [82]	0.16 [82]	386	1	0.12 [1]	4277 [1]			
Verapamil	4	43 [34, 35, 83, 84]	0.071 [34, 35]	1056	2	0.52	664 [15, 21]			
S-Warfarin	4	0.24 [85-88]	0.021 [85-87]	12	2	0.94	0.90 [22]			
Zidovudine	1	41 [1]	0.79 [1]	87	1	1.00 [1]	14 [1]			
AZ1	1	11 [31]	0.025 [31]	499	1	0.82 [31]	68 [31]			
AZ2	1	16 [31]	0.071 [31]	265	1	0.85 [31]	113 [31]			
AZ3	1	12 [31]	0.035 [31]	388	1	0.89 [31]	36 [31]			
AZ4	1	36 [31]	0.032 [31]	1781	1	0.71 [31]	138 [31]			
AZ5	1	57 [31]	0.16 [31]	805	1	0.70 [31]	167 [31]			
AZ6	1	9.3 [31]	0.016 [31]	641	1	0.64 [31]	220 [31]			
AZ7	1	13 [31]	0.038 [31]	404	1	0.61 [31]	32 [31]			
AZ8	1	57 [31]	0.14 [31]	987	1	0.90 [31]	160 [31]			
AZ9	1	46 [31]	0.050 [31]	1697	1	0.75 [31]	182 [31]			
AZ10	1	23 [31]	0.029 [31]	1042	1	0.05 [31]	1126 [31]			
AZ11	1	40 [31]	0.086 [31]	775	1	0.82 [31]	29 [31]			
AZ12	1	50 [31]	0.086 [31]	1167	1	0.72 [31]	93 [31]			
AZ13	1	33 [31]	0.076 [31]	648	1	0.76 [31]	23 [31]			
AZ14	1	21 [31]	0.036 [31]	756	1	0.71 [31]	47 [31]			
AZ15	1	32 [31]	0.089 [31]	529	1	0.85 [31]	80 [31]			
AZ16	1	17 [31]	0.030 [31]	697	1	0.73 [31]	49 [31]			
AZ17	1	13 [31]	0.19 [31]	81	1	0.95 [31]	17 [31]			
AZ18	1	47 [31]	0.085 [31]	1060	1	0.77 [31]	105 [31]			
AZ19	1	53 [31]	0.090 [31]	1258	1	0.91 [31]	194 [31]			
AZ20	1	39 [31]	0.013 [31]	4856	1	0.44 [31]	250 [31]			
AZ21	1	41 [31]	0.10 [31]	682	1	0.86 [31]	228 [31]			
AZ22	1	18 [31]	0.011 [31]	1955	1	0.36 [31]	497 [31]			
AZ23	1	8.7 [31]	0.0036 [31]	2629	1	0.60 [31]	135 [31]			
AZ24	1	7.9 [31]	0.062 [31]	138	1	0.60 [31]	40 [31]			
AZ25	1	17 [31]	0.13 [31]	162	1	0.90 [31]	25 [31]			
AZ26	1	7.3 [31]	0.036 [31]	219	1	0.85 [31]	33 [31]			
AZ27	1	19 [31]	0.017 [31]	1407	1	0.51 [31]	385 [31]			
AZ28	1	17 [31]	0.084 [31]	244	1	0.79 [31]	59 [31]			

AZ29	1	22 [31]	0.045 [31]	630	1	0.55 [31]	205 [31]			
AZ30	1	50 [31]	0.056 [31]	1800	1	0.50 [31]	486 [31]			
AZ31	1	5.7 [31]	0.062 [31]	2028	1	0.78 [31]	159 [31]			
AZ32	1	25 [31]	0.064 [31]	515	1	0.79 [31]	63 [31]			
AZ33	1	51 [31]	0.13 [31]	792	1	0.77 [31]	130 [31]			
AZ34	1	32 [31]	0.076 [31]	631	1	0.61 [31]	92 [31]			
AZ35	1	1.5 [31]	0.0018 [31]	812	1	0.46 [31]	29 [31]			
AZ36	1	16 [31]	0.083 [31]	233	1	0.59 [31]	71 [31]			
AZ37	1	2.0 [31]	0.0018 [31]	1122	1	0.44 [31]	135 [31]			
AZ38	1	40 [31]	0.19 [31]	349	1	0.74 [31]	207 [31]			
AZ39	1	42 [31]	0.047 [31]	1516	1	0.63 [31]	228 [31]			
AZ40	1	13 [31]	0.14 [31]	110	1	0.88 [31]	48 [31]			
AZ41	1	8.7 [31]	0.14 [31]	68	1	0.90 [31]	39 [31]			
AZ42	1	40 [31]	0.16 [31]	415	1	0.88 [31]	69 [31]			
AZ43	1	14 [31]	0.022 [31]	722	1	0.84 [31]	80 [31]			
AZ44	1	19 [31]	0.11 [31]	212	1	0.72 [31]	112 [31]			
AZ45	1	25 [31]	0.28 [31]	117	1	0.59 [31]	114 [31]			
AZ46	1	2.2 [31]	0.0018 [31]	1227	1	0.43 [31]	152 [31]			
AZ47	1	3.6 [31]	0.0090 [31]	415	1	0.61 [31]	82 [31]			
AZ48	1	33 [31]	0.29 [31]	171	1	0.77 [31]	145 [31]			
AZ49	1	4.4 [31]	0.0080 [31]	575	1	0.82 [31]	157 [31]			
AZ50	1	15 [31]	0.011[31]	1642	1	0.83 [31]	278 [31]			
H1	1	44 [89]	0.035 [89]	2275	1	0.60 [89]	338 [89]	1	0.46 [89]	542 [89]
H2	1	4.9 [89]	0.042 [89]	121				1	0.74 [89]	42 [89]
H3	1	0.05 [89]	0.0010 [89]	50	1	0.73 [89]	124 [89]	1	0.48 [89]	240 [89]
H4	1	0.17 [89]	0.0010 [89]	167	1	0.85 [89]	21 [89]	1	0.67 [89]	70 [89]
H5	1	1.4 [89]	0.011 [89]	132	1	0.81 [89]	105 [89]	1	0.89 [89]	178 [89]
H6	1	0.55 [89]	0.003 [89]	184	1	0.71 [89]	66 [89]	1	0.59 [89]	189 [89]
H7	1	0.58 [89]	0.003 [89]	196	1	0.69 [89]	209 [89]	1	0.53 [89]	158 [89]
H8	1	3.8 [89]	0.010 [89]	393	1	0.78 [89]	130 [89]	1	0.69 [89]	128 [89]
H9	1	4.2 [89]	0.01 [89]	487	1	0.57 [89]	212 [89]	1	0.48 [89]	213 [89]
H10	1	0.23 [89]	0.0090 [89]	234	1	0.75 [89]	98 [89]	1	0.63 [89]	157 [89]
H11	1	15 [89]	0.041 [89]	416	1	0.29 [89]	180 [89]	1	0.23 [89]	231 [89]
H12	1	5.4 [89]	0.020 [89]	284	1	0.63 [89]	85 [89]	1	0.46 [89]	402 [89]
H13	1	100 [89]	0.051 [89]	390196	1	0.56 [89]	201 [89]	1	0.47 [89]	237 [89]
H14	1	5.0 [89]	0.0020 [89]	2632	1	0.38 [89]	333 [89]	1	0.25 [89]	1966 [89]
H15	1	2.0 [89]	0.0060 [89]	343	1	0.20 [89]	429 [89]	1	0.23 [89]	507 [89]

H16	1	0.72 [89]	0.0020 [89]	361				1	0.32 [89]	199 [89]
H17	1	3.1 [89]	0.0060 [89]	530	1	0.79 [89]	106 [89]	1	0.59 [89]	186 [89]
H18	1	4.9 [89]	0.0080 [89]	639	1	0.70 [89]	67 [89]	1	0.46 [89]	53 [89]
H19	1	6.5 [89]	0.0060 [89]	1165	1	0.89 [89]	114 [89]	1	0.89 [89]	77 [89]
H20	1	31 [89]	0.0050 [89]	8777	1	0.63 [89]	828 [89]	1	0.69 [89]	880 [89]
H22	1	9.0 [89]	0.018 [89]	549	1	0.45 [89]	143 [89]	1	0.24 [89]	249 [89]
H23	1	0.25 [89]	0.0010 [89]	251				1	0.74 [89]	86 [89]
H24	1	44 [89]	0.0030 [89]	25873	1	0.69 [89]	191 [89]	1	0.48 [89]	343 [89]
H25	1	14 [89]	0.0040 [89]	4042	1	0.57 [89]	78 [89]	1	0.63 [89]	240 [89]
H26	1	2.2 [89]	0.008 [89]	279	1	0.82 [89]	71 [89]	1	0.77 [89]	62 [89]
H27	1	7.4 [89]	0.014 [89]	567	1	0.63 [89]	158 [89]	1	0.69 [89]	275 [89]
H28	1	4.1 [89]	0.0040 [89]	1064	1	0.88 [89]	110 [89]	1	0.27 [89]	44 [89]
H29	1	13 [89]	0.025 [89]	575	1	0.79 [89]	33 [89]	1	0.74 [89]	115 [89]
H30	1	6.3 [89]	0.097 [89]	70	1	0.94 [89]	65 [89]	1	1.00 [89]	19 [89]
H31	1	9.4 [89]	0.013 [89]	801	1	0.81 [89]	55 [89]	1	0.92 [89]	76 [89]
H32	1	1.8 [89]	0.0030 [89]	605	1	0.89 [89]	52 [89]	1	0.63 [89]	25 [89]
H33	1	88 [89]	0.019 [89]	39214				1	0.07 [89]	1287 [89]
H34	1	29 [89]	0.0060 [89]	6891				1	0.65 [89]	183 [89]
H35	1	47 [89]	0.017 [89]	5251				1	0.30 [89]	598 [89]
H37	1	60 [89]	0.0020 [89]	75576	1	0.47 [89]	368 [89]	1	0.19 [89]	3071 [89]
H39	1	79 [89]	0.015 [89]	25436	1	0.75 [89]	72 [89]	1	0.46 [89]	16 [89]
H40	1	54 [89]	0.0080 [89]	14952	1	0.63 [89]	499 [89]	1	0.61 [89]	17 [89]
H2a	1	79 [15]	0.036 [15]	10383	1	0.63 [15]	602 [15]			
H12a	1	77 [15]	0.0069 [15]	49471	1	0.23 [15]	330 [15]			
H13a	1	8.0 [15]	0.0018 [15]	4870	1	0.62 [15]	125 [15]			
H15a	1	64 [15]	0.0035 [15]	49940	1	0.64 [15]	296 [15]			

* fu_{hep} values are normalised to 1 x 10^6 cells/ml and fu_{mic} values are normalised to 1 mg microsomal protein/ml. Experimentally determined values are followed by the accompanying reference; all other values were determined using lipophilicity relationship algorithms (Equations 2.2 and 2.1, fu_{hep} and fu_{mic} respectively). Cryopreserved hepatocytes were used by [39] and both cryopreserved and freshly isolated hepatocytes were used by [22]. All other studies used freshly isolated hepatocytes.

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8.4 Laboratory equipment used in this study

Table 8-4. Laboratory equipment used in this study with details on manufacturer and model.

Description	Manufacturer	Model
96-well plates	Fisher Scientific UK Ltd. (Loughborough, UK)	Nunc round-bottomed 0.5 ml polypropylene
96 deep-well plates	VWR International Ltd (Lutterworth, UK)	96-square well, 1.2 ml polypropylene microplate
96-well black clear-bottomed plates	Fisher Scientific UK Ltd. (Loughborough, UK)	96-well black/clear bottom, polystyrene flat bottom
24-well uncoated plates	Greiner Bio-One (Stonehouse, UK)	Cellstar 24 well cell culture multiwell polystyrene plates
24-well collagen-coated plates	Scientific Lab Supplies Ltd. (Hessle, UK)	BioCoat
Microcentrifuge tubes	STARLAB UK (Milton Keynes, UK)	1.5 ml TubeOne microcentrifuge tubes
Falcon tubes	Greiner Bio One (Stonehouse, UK)	Centrifuge tube, conical base, 50 ml
Round-bottomed tubes	Sigma-Aldrich Company Ltd. (Dorset, UK)	Plastibrand centrifuge tubes, round bottomed, 48 ml
Round-bottomed tube caps	Sigma-Aldrich Company Ltd. (Dorset, UK)	Plastibrand centrifuge tube caps
Bijou vials	Sigma-Aldrich Company Ltd. (Dorset, UK)	Bijou sample container, polystyrene, 7 ml
MS vials (Ultima I)	VWR International Ltd (Lutterworth, UK)	Webseal insert, 700 µl, clear, conical
MS plate sealing mat (Ultima I)	VWR International Ltd (Lutterworth, UK)	Webseal mat for 700 µl inserts
MS vials (Ultima II)	Agilent Technologies UK Ltd. (Cheshire, UK)	Glass inserts, 350 µl
MS vial caps (Ultima II)	Agilent Technologies UK Ltd. (Cheshire, UK)	Cap for 350 µl glass inserts
Reservoirs	STARLAB UK (Milton Keynes, UK)	Three-way polypropylene reservoir: single (50 ml), 8-channel (8 x 5 ml), 12-channel (12 x 5 ml)
Scintillation vials	Fisher Scientific (Loughborough, UK)	Screw neck vial, 20 ml
Screw vial	Kinesis (St. Neots, UK)	Screw vial, 8 mm, 2 ml
Vial cap	Kinesis (St. Neots, UK)	Screw cap, 8 mm, black
Pipettes (single-channel)	Gilson Inc., Anachem Ltd (Luton, UK)	Pipetman (air displacement)

Pipettes (single-channel)	Gilson Inc., Anachem Ltd (Luton, UK)	Microman (positive displacement)
Pipettes (multi-channel)	Mettler-Toledo Ltd (Beaumont Leys, UK)	Rainin, Pipet Lite
Gloves	VWR International Ltd (Lutterworth, UK)	Powder free nitrile
Liver perfusion kit	Custom made	Custom made
Eppendorf Thermomixer	Eppendorf AG (Hamburg, Germany)	Eppendorf Thermomixer Compact
Microtiterplate shaker	Heidolph instruments (Scwabach Germany)	Titramax 1000
Dual block dry bath heating system	STARLAB UK (Milton Keynes, UK)	Dry bath heating system, dual block, digital
Ultrasonic probe	CamLab Ltd. (Cambridge, UK)	Omni Ruptor 400 ultrasonic probe homogeniser
Fluorescence plate reader	Tecan Group Ltd (Männedorf, Switzerland)	Safire
Microcentrifuge	Eppendorf (Hamburg, Germany)	MiniSpin
Centrifuge	Eppendorf (Hamburg, Germany)	5804
Vortex Mixer	Nickel Electro Ltd (Weston-S-Mare, Somerset, UK)	Clifton cyclone
pH meter	Mettler-Toledo (Beaumont Leys, UK)	340
Water bath	Stuart, BibbySterilin Ltd (Stone, UK)	Shaker bath SBS30
Microscope	Olympus (Southend-on-Sea, UK)	Olympus CK2
Haemocytometer	Immune Systems Ltd. (Devon, UK)	FastRead counting slides
Balance (high precision)	Mettler-Toledo Ltd. (Beaumont Leys, UK)	NewClassic MS
Balance (low precision)	Precisa (Tongwell, Milton Keynes, UK)	AG XB620M
CO_2 Incubator	Leec Ltd.(Nottingham, UK)	Precision 190D
Mass Spectrometer	Waters (Elstree, UK)	Quattro Ultima and Quattro Micro with Mass Lynx 4.1 software
HPLC	Waters (Elstree, UK)	Alliance HT2790 Separations module
HPLC column	Phenomenex Inc (Macclesfield, UK)	Luna C18 (3 µm 50 x 4.6 mm) Phenyl hexyl (3 µm 50 x 4.6 mm)
Safety cabinet	Walker Safety Cabinets Ltd (Glossop, UK)	Class II Microbiological safety cabinet

8.5 LC-MS/MS methods used for quantification of samples

Samples were analysed using either a Waters Alliance 2795 (Waters, Watford, UK) or Agilent 1100 (Agilent Technologies, Stockport, UK) high-performance liquid chromatography (HPLC) system coupled to either a Micromass Quattro Ultima (Waters, Watford, UK) or Micromass Quattro Micro (Waters, Watford, UK) mass spectrometer. All drugs and associated internal standards were separated on a Luna C18 column (3 µm, 50 x 4.6 mm) (Phenomenex, Macclesfield, UK), with the exception of propranolol, propafenone and metoprolol, which were separated on a Luna Phenyl-Hexyl column (3 µm, 50 x 4.6 mm) (Phenomenex, Macclesflield, UK). The following mobile phases were used: Solvent A, 90% water, 10% methanol and 0.05% formic acid; Solvent B, 10% water, 90% methanol and 0.05% formic acid; Solvent C, 90% water, 10% methanol and 1 mM ammonium acetate; and Solvent D, 10% water, 90% methanol and 1 mM ammonium acetate. HPLC flow rate was set to 1 ml/min; the gradient of mobile phases varied for each drug. HPLC eluent was split to 0.25 ml/min before analysis by electrospray atmospheric pressure ionization combined with multiple reaction monitoring (MRM) of manually optimized product ions using tandem mass spectrometry (MS/MS). The capillary voltage was 3.5 kV; desolvation and source temperatures were 350°C and 125°C respectively; desolvation gas flow rate was 600 l/hr. MS/MS conditions for each individual analytes are detailed in Tables 8-4, 8-5 and 8-6.

Table 8-5. **MS/MS conditions used on Micromass Quattro Ultima (I).**

Drug	Internal Standard	Mode	Parent (m/z)	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)	Cone gas (l/h)
Chlorpromazine	Dextromethorphan	+	319.10	86.15	30	18	150
Dextromethorphan	Chlorpromazine	+	272.10	171.05	95	35	150
Metoprolol	Propranolol	+	268.10	115.95	60	20	70
-	Propranolol	+	260.05	116.20	70	16	70
Naloxone	Buspirone	+	328.15	310.05	80	20	150
-	Buspirone	+	386.25	122.20	60	30	150
Propafenone	Propranolol	+	342.1	116.2	70	20	150
Propranolol	Propafenone	+	260.05	183.15	70	18	150
Triazolam	Diazepam	+	343.00	308.15	80	25	150
-	Diazepam	+	285.05	257.05	60	21	150
Verapamil	Midazolam	+	455.25	165.10	60	30	150
	Midazolam	+	326.00	291.20	70	25	150

Table 8-6. **MS/MS conditions used on Micromass Quattro Ultima (II).**

Drug/Metabolite	Internal Standard	Mode	Parent (m/z)	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)	Cone gas (L/h)
Tolbutamide	Diclofenac	-	269.55	170.90	45	17	70
4'-hydroxy-tolbutamide	Diclofenac	-	285.60	186.85	50	17	70
-	Diclofenac	-	294.50	250.75	40	11	70

Table 8-7. **MS/MS conditions used on Micromass Quattro Micro.**

Drug	Internal Standard	Mode	Parent (m/z)	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)	Cone gas (L/h)
Midazolam	Diazepam	+	326.15	291.45	40	30	150
-	Diazepam	+	285.15	153.65	34	30	150
Saquinavir	Terfenadine	+	671.65	570.40	48	35	150
-	Terfenadine	+	472.25	436.60	36	31	150

8.6 Thawing of cryopreserved rat hepatocytes

Supplier: Tebu-Bio Ltd. (Cambridgeshire, UK) (supplied by Sekisui XenoTech LLC (Kansas, USA)).

Thawing Kit Contents

Tube A: Supplemental DMEM (without penicillin/streptomycin) and isotonic Percoll

Tube B: Supplemental DMEM (without penicillin/streptomycin)

1. Warm tubes A and B to 37 ± 1°C in water bath before use (typically takes ~15-20 minutes).
2. Remove two cryotubes from the liquid nitrogen storage unit and immediately place in a 37 ± 1°C waterbath for ~80 seconds, shaking gently. The frozen cell pellet should move freely when the cryotube is inverted. *Do not over-thaw.*
3. Dump the frozen pellet of one of the cryotubes into Tube A. Rinse the cryotube with 1.5 ml of media contained in Tube B. Pour this rinse into Tube A. Gently invert Tube A until all ice is melted.
4. Centrifuge Tube A at 100 x g for 5 minutes at room temperature or 2-8°C.
5. Aspirate and discard the supernatant fluid without disturbing the cell pellet. Resuspend in 1 ml WME.
6. Withdraw 1.5 ml media from Tube B. Dump the frozen pellet of the second vial into Tube B. Rinse the vial with the 1.5 ml media, pour this rinse back into Tube B. Gently invert Tube B until all ice is melted.
7. Centrifuge Tube B at 60 x g for 3 minutes at room temperature or 2-8°C.
8. Aspirate and discard the supernatant fluid without disturbing the cell pellet. Resuspend in 1 ml WME.

8.7 AbCam ATP assay protocol

8.7.1 Pre-assay preparation

- **ATP Standard:** Reconstitute ATP Standard (1 µmol) with 100 µl of double-distilled water to generate a 10 mM ATP standard stock solution. Keep on ice while in use. Aliquot ATP standard so that you have enough to perform the desired number of assays. Store at -20°C.
- **ATP Probe:** Ready to use as supplied. Warm by placing in a 37°C bath for 1-5 minutes to thaw the DMSO solution before use. Store at -20°C, protect from light. Once the probe is thawed, use within two months.
- **ATP Converter:** Dissolve in 220 µl ATP assay buffer. Aliquot ATP converter needed to perform the desired number of assays. Store at -20°C.
- **Developer Mix:** Dissolve in 220 µl ATP assay buffer. Aliquot developer needed to perform the desired number of assays. Store at -20°C.

8.7.2 Assay protocol for cell (adherent or suspension) samples

8.7.2.1. Sample preparation

1. Harvest 10^6 cells.
2. Wash cells with cold PBS.
3. Resuspend cells in 100 µl of ATP assay buffer
4. Homogenize cells quickly by pipetting up and down a few times.
5. Centrifuge 2 min at 4°C (top speed using a microcentrifuge, ~13,000 rpm) to remove any insoluble material.
6. Collect supernatant and transfer to a clean tube. Keep on ice.
7. Make sample volume up to 550 µl with ATP assay buffer.
8. Mix with 100 µl of ice cold PCA (4 M) in 1.5 ml microcentrifuge tubes.
9. Vortex briefly and incubate on ice for 5 minutes.
10. Centrifuge at 13,000 rpm for 2 minutes at 4°C and transfer supernatant to a fresh tube. Measure volume of supernatant.
11. Precipitate excess PCA by adding an equal volume of ice cold 2 M KOH (to supernatant obtained in previous step) and vortexing briefly.
12. Centrifuge at 13,000 rpm for 15 minutes at 4°C and collect supernatant.

8.7.2.2 ATP measurement

1. Prepare a 1 mM ATP standard from 10 mM standard stock.
2. Dilute 10 µl of 1 mM ATP standard into 90 µl of double-distilled water to create 100 µl of 0.1 mM standard.
3. Prepare standard curve dilution ranging from 0-20 µM in a 96-well microplate (Table 8-8).

Table 8-8. ATP standard curve preparation.

End [ATP] in well (µM)	ATP 0.1 mM standard (µl)	Assay buffer (µl)	Total volume (µl)
0	0	150	150
4	6	144	150
8	12	138	150
12	18	132	150
16	24	126	150
20	30	120	150

4. Prepare ATP reaction master mix. Add (number of samples + standards + 1) of each of the following components:

ATP Assay Buffer	45.8 µl
ATP Probe	0.2 µl
ATP Converter	2 µl
Developer Mix	2 µl
Total	50 µl

5. Assay standard curve and samples in duplicate.

   a) Add 50 µl of ATP reaction mix to each well.

   b) Add 50 µl of standard or sample to each well.

   c) Incubate at room temperature for 30 minutes protected from light.

   d) Measure output on a microplate reader, excitation/emission = 535/587 nm

   The reaction is stable for at least 2 hours.

8.8 Cryopreserved human hepatocytes

Table 8-9. Demographic information for pooled cryopreserved human hepatocytes supplied by Bioreclamation IVT.

Product No.	Lot No.	Gender	Age range	Race
X008000	FHQ	Females (10), Males (10)	4 - 77 years	Caucasian (18), African American (1), Hispanic (1).

8.8.1 Thawing protocol

Supplier: Bioreclamation IVT (Brussels, Belgium).

For thawing of 1-3 vials:

1. Pre-warm *InVitro*GRO CP Medium to 37° C.
2. Transfer (50 – 2 x no. of vials) ml of warm *InVitro*GRO CP Medium to a sterile 50 ml conical tube.
3. Carefully remove the vial from the the liquid nitrogen storage unit. If the vial was stored in the liquid phase, carefully remove the cap and pour off any liquid nitrogen. Close the cap firmly before placing the vial into the water bath.
4. Immediately immerse the vial(s) into a 37° C water bath. Shake gently until the ice is entirely melted, but no longer than it takes to completely thaw the vial. It may be helpful to remove the label from the vial so it is easier to view the vial contents.
5. Empty the contents of the vial into the pre-warmed *InVitro*GRO CP Medium.
6. Add 1 ml of pre-warmed *InVitro*GRO CP Medium to each vial to resuspend any remaining cells. Decant or pipette the contents into the hepatocyte suspension.
7. Resuspend the hepatocytes by gently inverting the tube several times (3 times is sufficient).
8. Centrifuge the cell suspension at 50 x g in a room temperature centrifuge for 5 minutes.
9. Discard the supernatant by either pouring in one motion (do not pour partially and re-invert centrifuge tube), or aspirating using a vacuum pump.
10. Loosen the cell pellet by gently swirling the centrifuge tube.
11. Add 2-3 ml of WME buffer. Invert the tube gently to resuspend the hepatocytes.
12. Determine the total cell count and the number of viable cells using the trypan blue exclusion method.