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COMPARISON OF INTRINSIC CLEARANCE IN LIVER MICROSOMES AND HEPATOCYTES FROM RATS AND HUMANS - EVALUATION OF FREE FRACTION AND UPTAKE IN HEPATOCYTES

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Running title

INTRINSIC CLEARANCE IN MICROSOMES AND HEPATOCYTES

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LIST OF ABBREVIATIONS

CYP, cytochrome P450; KHB, Krebs-henseleit buffer; LC/MS/MS, liquid chromatography coupled to tandem mass spectrometry; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form

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ABSTRACT

Apparent intrinsic clearance ($CL_{int,app}$) of the 7-ethoxycoumarin, phenacetin, propranolol, and midazolam was measured using rat and human liver microsomes, freshly isolated and cryopreserved hepatocytes to determine factors responsible for differences in rates of metabolism in these systems. The cryopreserved and freshly isolated hepatocytes generally provided similar results, albeit there was greater variability using the latter system. The $CL_{int,app}$ values in hepatocytes are observed to be lower than that in microsomes and this difference becomes greater for compounds with high $CL_{int,app}$. This could partly be attributed to the differences in the free fraction (f_u). The f_u in hepatocyte incubations ($f_{u,hep\text{-}inc}$) was influenced not only by the free fraction of compounds in the incubation buffer ($f_{u,buffer}$) but also by the rate constants of uptake (k_{up}) and metabolism (k_{met}). This report provides a new derivation for $f_{u,hep}$ which can be expressed as: $f_{u,hep\text{-}inc} = [k_{up} / (k_{met} + k_{up})] / [1 + (C_{hep} / C_{buffer}) \times (V_{hep} / V_{buffer})]$, where the C_{hep}, C_{buffer}, V_{hep}, and V_{buffer} represent the concentrations of a compound in hepatocytes and buffer, and volumes of hepatocytes and buffer, respectively. For midazolam, the $f_{u,hep\text{-}inc}$ was calculated and the maximum metabolism rate in hepatocytes was shown to be limited by the uptake rate.

INTRODUCTION

The determination of in vitro intrinsic clearance (CL_{int}) for drug candidates in the early discovery stage is a common practice in the pharmaceutical industry (Lave et al. 1997, Houston 1994, Obach et al.1997). The CL_{int} values of drug candidates can help to confirm if metabolism is the main clearance pathway when it is compared to the total body clearance in vivo. It is also helpful in rank ordering drug candidates based on their metabolic stabilities, assessing species and gender differences in metabolic clearance, and projecting the metabolic clearance of drug candidates in humans. The in vitro CL_{int} may be derived from enzyme kinetic data such as V_{max}/K_m (Griffin and Houston 2004, Tan and Pang 2001, Lin et al. 1996) or from the in vitro $t_{1/2}$ values where sub-K_m substrate concentrations are used (Lave et al. 1997, Obach 1999, Lau et al. 2002, Jones and Houston 2004). The CL_{int} can be calculated from the experimental apparent intrinsic clearance, $CL_{int,app}$, by correcting for free fraction of test compounds in the incubations. To further predict the in vivo hepatic clearance from the in vitro intrinsic clearance, a well-stirred model is often used (Naritomi et al. 2001, Ito and Houston 2004). A survey of literature revealed that in hepatocyte incubations, the free fraction of test compound has not been well defined. Simply assuming a steady state where the intracellular free concentration equals the extracellular free concentration may allow one to roughly estimate CL_{int} for some compounds. However clearance, after a dose in vitro or in vivo, is actually a dynamic system such that at any given time typically the amount of compound getting into a cell equals the amount of compound leaving the cell by diffusion and by metabolism (Figure 1). Thus the intracellular free concentration is always somewhat lower than the extracellular free concentration because metabolism

constantly removes compound from hepatocytes and an extracellular - intracellular free concentration gradient is needed to replenish the metabolized and outfluxed compound. For some rapidly metabolized compounds, the intracellular free concentration may be much lower than the extracellular free concentration, because the removal of compounds by metabolism could be faster than the uptake. In the literature, a few attempts have been reported on measurement of free fraction using metabolically inactivated or dead hepatocytes by equilibrium dialysis (Austin et al. 2005), or using the ratio of free concentration in the buffer over the total concentration in hepatocytes – free and bound (Witherow and Houston, 1999). Both approaches assumed the buffer concentration equals the intracellular free concentrations of compound without considering metabolism. The current report provides a derivation of free fraction in the hepatocytes using the more dynamic system with intact diffusional and metabolic processes.

Among the routinely used in vitro systems, such as microsomes and hepatocytes (Houston and Carlile 1997), microsomes are usually used to determine CYP mediated metabolism (phase I). Hepatocytes, having intact cell membranes and physiological concentrations of enzymes and cofactors, are believed to be a model close to whole liver for drug clearance measurements (Bachmann et al. 2003, Ito and Houston 2004, McGinnity et al. 2004). The aims of this work were 1) to compare intrinsic clearance determinations in hepatic microsomes and hepatocytes for a set of compounds including marketed drugs that are primarily metabolized by phase I enzymes, 2) to explore the relationship of the free fraction with uptake and metabolism of the test compound in

hepatocytes, and 3) to provide an explanation for the low apparent intrinsic clearances observed in hepatocyte incubations compared to that in microsomal incubations.

Materials and Methods

Reagents. Pooled human and rat liver microsomes and pooled cryopreserved rat hepatocytes were purchased from XenoTech LLC (Kansas City, KS). Cryopreserved human hepatocytes (pool of 4 in the experiment) were purchased from In Vitro Technologies, Inc. (Baltimore, MD). Fresh human hepatocytes were purchased from BD Gentest (Woburn, MA) and In Vitro Technologies, Inc. Fresh rat hepatocytes were prepared in house. All hepatocytes used in this study had viability of > 80%. Midazolam, 7-ethoxycoumarin, phenacetin, and propranolol were purchased from Sigma-Aldrich (St. Louis, MO).

$CL_{int,app}$ determination in microsomes and hepatocytes. Microsomes (0.5 mg/mL) were pre-incubated with 2 μM test compound for 5 min at 37°C in 0.1 M phosphate buffer, pH 7.4. The reactions were initiated by adding pre-warmed cofactors (2 mM NADPH and 3 mM $MgCl_2$). After 0, 3, 7, 12, 20, and 30 min incubations at 37°C, the reactions were stopped by adding an equal volume of acetonitrile containing 1 μM of carbutamide (internal standard). The samples were kept in a refrigerator for 30 min and then centrifuged at 3,000g for 10 min. The supernatants were analyzed with LC/MS/MS for the amount of parent compound remaining.

Calculation of Apparent Intrinsic Clearance:

$CL_{int,app}$ = (0.693 / in vitro $t_{½}$) (incubation volume/ mg of microsomal protein) (45 mg microsomal protein / gram of liver) (20^a gm of liver / kg body weight)

a: Lin et al. (1996), 20 and 45 gm of liver / kg body weight were used for human and rat, respectively.

Similarly, $CL_{int,app}$ determinations in hepatocytes were performed in KHB buffer, pH 7.4, with 1.0 x 10^6 hepatocytes/mL (viability > 80%) and 2 μM test compound. The incubations were carried out in a 37°C CO_2 (5%) incubator as previously described (Li et al., 1999). The reactions were stopped at 0, 30, 60, 120, 180, and 240 min with addition of an equal volume of acetonitrile containing 1 μM of carbutamide. A value of 135 x 10^6 hepatocyte / gm of liver (Houston 1994) was employed in the $CL_{int,app}$ calculation (equation above).

Hepatocyte uptake. Cryopreserved hepatocytes were thawed out and suspended in KHB buffer at 2.0 x 10^6 hepatocytes/mL as previously described (Shitara et al. 2003). Centrifuge tubes were prepared by loading 150 μL silicone/mineral oil on top of 50 μL of 5 N NaCl/0.2% Saponin. In a 37°C water bath, 0.5 mL hepatocytes suspensions were pre-incubated in the presence or absence of 10 μM ketoconazole for 10 min. Uptake was initiated by adding 0.5 mL 4 μM midazolam into the hepatocytes. At 0.5, 2, 4, 10, 20, and 30 min, 100 μL of the cell suspensions were transferred into the centrifuge tubes containing the silicone/mineral oil and the NaCl/0.2% Saponin, and the uptake was terminated by separating the hepatocytes from the midazolam solution with a 10 sec centrifugation at 10,000g. Midazolam concentrations in the hepatocytes as well as the uptake solution were analyzed using LC/MS/MS with appropriate standard curves.

Microsomal protein binding. Microsomal protein binding assay was adapted from a published procedure (Obach, 1997): microsomes (0.5 mg/mL) were mixed with 2 μM test compound in 0.1 M phosphate buffer, pH 7.4 containing 3 mM $MgCl_2$ (the dialysis buffer). The mixture was subjected to an overnight dialysis at 37^{o}C against the dialysis buffer using the Spectrum apparatus (Spectrum, Los Angeles, CA). The retrieved microsomes were then diluted in two volumes of the dialysis buffer and dialysate from the receiving side was diluted in half volume of the control microsomes. After the protein in the samples from the donor and the receiver was precipitated in an equal volume of acetonitrile containing 1 μM of carbutamide, the supernatants were analyzed using LC/MS/MS for the amount of parent compound remaining. The free fractions were calculated as:

$f_{u,mic}$ = (concentration at the receiving side x 1.5) / (concentration at the donor side x 3)

LC/MS/MS analyses. Peak area ratios of test compounds and carbutamide (internal standard) were determined by a LC/MS/MS system which consisted of an Agilent 1100 HPLC (Agilent, Palo Alto, CA), a Leap CTC PAL autosampler (LEAP Technologies Inc., Carrboro, NC), and a SCIEX API 4000 detector (Applied Biosystems, Concord, Ontario, Canada). Separation was performed on a Waters YMC Basic, 3 μM, 50 mm x 2.0 mm column (Waters, Milford, MA), eluted at a flow rate of 0.5 mL/min. Mobile phase A was 0.1 % (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The gradient consisted of 30% of mobile phase B for 0.5 minutes after injection and increased linearly to 90% B from 0.5 to 1.5 minutes. Mobile phase B was held at 90% from 1.5 to 2.3 minutes and the column was re-equilibrated to 30% B from 2.3 to 3.5 minutes. All compounds were detected by positive ion spray in the

multiple-reaction monitoring (MRM) mode using pre-determined parent/product mass transition ion pairs.

RESULTS

The apparent intrinsic clearance results are presented in Table 1. Phenacetin, 7-ethoxycoumarin (7EC), propranolol, and midazolam are primarily metabolized by phase I enzymes. The $CL_{int,app}$ values for all compounds in hepatocytes were much lower compared to those in microsomes, with midazolam showing the highest differential, and hence has been studied in detail. All four compounds showed comparable $CL_{int,app}$ in freshly isolated (3 experiments) and cryopreserved (5 experiments) rat hepatocytes with a similar inter-experimental variation (Table 2). Furthermore, the mean $CL_{int,app}$ values in freshly isolated and cryopreserved rat hepatocytes were comparable suggesting that the enzyme activity in the cryopreserved rat hepatocytes was at levels comparable to those in the freshly isolated rat hepatocytes. In the cryopreserved human hepatocytes study, these four compounds also showed a similar inter-day variation in enzyme activity as that in cryopreserved rat hepatocytes. However, in freshly isolated human hepatocytes, the inter-experimental variation was notably higher (Table 2). Unlike the cryopreserved hepatocyte study, which uses the same characterized and pooled hepatocytes, the fresh human hepatocyte studies were subject to inter-individual variation in enzyme activities, limited characterization at the time of the experiment, and a possibility of damaged hepatocytes during shipment. On the other hand, cryopreservation is known to preserve most of the CYP activities of the original freshly isolated hepatocytes (Li et al. 1999a, Madan et al. 1999). Since the mean values of $CL_{int,app}$ in cryopreserved and freshly isolated human hepatocytes in our study appeared to be similar, cryopreserved human hepatocytes could be a valid model for $CL_{int,app}$ determinations.

In the hepatocyte uptake study, after separating hepatocytes from the incubation buffer at different time points, the concentrations of midazolam in both hepatocytes and buffer were measured. In the human hepatocyte incubations, midazolam was depleted from the incubation buffer at the same rate as in the rat hepatocyte incubations. In contrast to hepatocytes, the clearance of midazolam in the liver microsomal incubation from rats was faster than that from humans (Table 1). When the selective CYP3A4 inhibitor ketoconazole was co-incubated with midazolam (a selective CYP3A4 substrate), midazolam was no longer cleared from the incubation buffer (Figure 2). These data suggested that 1) the removal of midazolam from the incubation buffer is also due to metabolism in hepatocytes and 2) the uptake is the rate limiting step. Taken together, these observations suggest that the midazolam uptake rates in rats and humans are similar to each other, and that both rates are lower compared to the corresponding metabolism rates, i.e. as soon as midazolam gets into the hepatocytes, it is cleared rapidly by metabolism. It is reasonable to believe that midazolam uptake rates are similar in human and rat hepatocytes because they reflect simple diffusion across the cell membranes. Midazolam is not reported to be a substrate of any active uptake or efflux transporters, therefore its uptake is not affected by ketoconazole. Ketoconazole is an inhibitor of several uptake and efflux transporters and CYP3A (Salphati and Benet 1998, Azer et al. 1995).

Table 3 summarizes the free fractions (f_u) of midazolam in human and rat liver microsomal incubations. From the protein binding studies, midazolam had f_u values of 0.83 and 0.84 in human and rat microsomes, respectively, showing that the binding was

similar in rat and human systems. The f_u values in hepatocyte incubations were calculated from the parameters obtained from the hepatocyte uptake study using equation (12) (derivation discussion below). The uptake rate constants in rats and humans were determined to be 0.0419 and 0.0371 /min/(million hepatocytes/mL), respectively; the volume ratios (V_{hep} / V_{buffer}) was 1/100 for both rats and humans; and the concentration ratios (C_{hep} / C_{buffer}) were 35 and 41 for rats and humans, respectively. The CL_{int} values of midazolam in microsomal and hepatocyte incubations were calculated from the $CL_{int,app}$ and the f_u and are presented in Table 3 for comparison.

DISCUSSION

In our study, the $CL_{int,app}$ values of 7EC, phenacetin, propranolol, and midazolam, all cleared by phase I metabolism as the first step, were significantly lower in hepatocyte incubations compared to microsomal incubations. Similar observation was recently reported by Hallifax and colleagues (2005a) where they compared CYP1A2, 2C9, 2D6, 2E1 and 3A4 activities in cryopreserved hepatocytes from about 200 donors with that in microsomes from about 100 donors. The CYP activities in hepatocytes were found to be 2.5- to 20-fold lower than in microsomes. For example, using testosterone as the substrate, CYP3A4 activity was 11-fold lower in hepatocytes. Many factors could attribute to this difference, such as nonspecific binding or quality of cryopreserved hepatocytes. The present work provides an explanation for this difference from the perspective of free fraction in hepatocytes for compounds that involve diffusional movement across cell membrane. Since hepatocytes are intact cells, compounds have to get into the cells before they can reach the metabolizing enzymes. The rate of compound clearance is, therefore, dependent on the uptake rate as well as the metabolism rate. Two important scenarios arise. If the uptake rate is much faster than the metabolism rate, then the overall clearance is metabolic rate limited. Conversely, if the metabolism rate is much faster than the uptake rate, the overall clearance is uptake rate limited.

Transcellular (diffusional) uptake is the primary route of entry into hepatocytes for most of compounds (Brayden, 1997). It is usually fast since it does not require an energy source or a salt gradient as most active uptake processes do. This discussion focuses on the transcellular uptake to quantitatively explore the relationship between hepatocyte

clearance and hepatocyte uptake/metabolism. In hepatocytes, when the intracellular free concentration ($C_{hep,u}$) is << the Michaelis-Menten constant (K_m),

the apparent metabolic rate is: $v_{met,app} = k_{met} \times C_{hep,u}$ (1)

the uptake rate in the hepatocytes is: $v_{up} = k_{up} \times C_{buffer,u}$ (2)

where k_{up} is the uptake rate constant, k_{met} is the metabolism rate constant, and $C_{buffer,u}$ is the free concentration in buffer which is same as the extracellular free concentration (Figure 1). Assuming that diffusion across membrane is identical in both directions,

the removal rate from the hepatocytes is: $(k_{met} \times C_{hep,u}) + (k_{up} \times C_{hep,u})$ (3)

where the term $k_{met} \times C_{hep,u}$ represents metabolic rate, and $k_{up} \times C_{hep,u}$ represents the diffusion of compound from inside the hepatocytes to the outside buffer. After the compound is added to the incubation mixture, a dynamic equilibrium is established where the rate of compound being taken up by the hepatocytes equals the rate of compound being removed from the hepatocytes (by diffusional and metabolism) at any given time (Figure 1), thus the uptake rate can be expressed as:

$v_{up} = (k_{up} \times C_{buffer,u}) = (k_{met} \times C_{hep,u}) + (k_{up} \times C_{hep,u})$ (4)

Dividing (1) by (4):

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$$v_{met,app}/v_{up} = (k_{met} \times C_{hep,u})/ [(k_{met} \times C_{hep,u}) + (k_{up} \times C_{hep,u})] \quad (5)$$

Multiply of both numerator and denominator of the right side of equation 5 by $C_{buffer,u}$ and cancel out the $C_{hep,u}$, the equation (5) becomes

$$v_{met,app}/v_{up} = (k_{met} \times C_{buffer,u})/ [(k_{met} \times C_{buffer,u}) + (k_{up} \times C_{buffer,u})] \quad (6)$$

equation (6) can then be re-written as:

$$v_{met,app} = \frac{v_{met,max} \times v_{up}}{v_{met,max} + v_{up}} \quad (7)$$

where $v_{met,max} = k_{met} \times C_{buffer}$ is the maximum metabolic rate if $C_{buffer,u} = C_{hep,u}$. In other words, this would be the highest possible metabolic rate of the compound in hepatocytes as if the enzymes were exposed to the extracellular concentration. Equation (7) demonstrates the hyperbolic relationship between apparent metabolic rate and the maximum metabolic rate where the uptake rate is the upper limit. For compounds that have a lower rate of metabolism, the effect of v_{up} on the clearance rate is minimal. However for a rapidly metabolized compound, the apparent metabolic rate could plateau at v_{up}.

Intrinsic clearance can be calculated from the apparent intrinsic clearance by correcting with the free fraction of the compound to which the enzymes are exposed (Obach 1999). The f_u in hepatocyte incubation ($f_{u,hep-inc}$) could be expressed as the ratio of intracellular free concentration ($C_{hep,u}$) to total incubation concentration (C_{total}) which is the amount of

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compound in hepatocytes ($C_{hep,}$ x V_{hep}) and buffer (C_{buffer} x V_{buffer}) divided by the incubation volume (V_{buffer} + V_{hep}):

$$f_{u,hep\text{-}inc} = C_{hep,u} / C_{total} = C_{hep,u} / \{[(C_{buffer} \text{ x } V_{buffer}) + (C_{hep,} \text{ x } V_{hep})] / (V_{buffer} + V_{hep})\} \quad (8)$$

where C_{hep} is the total concentration of compound in hepatocytes, i.e. free + bound to intracellular and membrane proteins and C_{buffer} equals the $C_{buffer,u}$ since no protein is present in the incubation buffer. The $f_{u,hep\text{-}inc}$ could be simplified assuming that the volume of the hepatocytes compared to the volume of the buffer is minimal (V_{hep} << V_{buffer}):

$$f_{u,hep\text{-}inc} = C_{hep,u} / C_{total} = C_{hep,u} / \{[(C_{buffer} \text{ x } V_{buffer}) + (C_{hep,} \text{ x } V_{hep})] / V_{buffer}\} \quad (9)$$

Equation (9) can be rearranged to:

$$f_{u,hep\text{-}inc} = \frac{\frac{C_{hep,u}}{C_{buffer}}}{1 + \frac{C_{hep} \text{ x } V_{hep}}{C_{buffer} \text{ x } V_{buffer}}} \quad (10)$$

Equation (4) can be rearranged as:

$$\frac{C_{hep,u}}{C_{buffer}} = \frac{k_{up}}{k_{up} + k_{met}} \quad (11)$$

By placing equation (11) into (10), we get:

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$$f_{u,hep\text{-}inc} = \frac{\frac{k_{up}}{k_{up} + k_{met}}}{1 + \frac{C_{hep} \text{ x } V_{hep}}{C_{buffer} \text{ x } V_{buffer}}} \qquad (12)$$

At a true steady state where there is no metabolism to remove the compound (k_{met} = 0 or << k_{up}), the intracellular free drug concentration equilibriums with the extracellular free drug concentration. Equation (12) can be simplified to:

$$f_{u,hep\text{-}inc} = \frac{1}{1 + \frac{C_{hep} \text{ x } V_{hep}}{C_{buffer} \text{ x } V_{buffer}}} = f_{u,buffer} \qquad (13)$$

Thus the intracellular free drug concentration approaches the extracellular free drug concentration. However, in equation (12) when metabolism is very high compared to the uptake, the numerator becomes a small fraction, and the $f_{u,hep\text{-}inc}$ becomes less than $f_{u,buffer}$. Therefore, the intracellular free concentration becomes much lower than the extracellular free concentration. This explains why the rapidly metabolized compound midazolam has a much lower $CL_{int,app}$ in hepatocytes compared to that in microsomal incubations. As illustrated in equation (12) the f_u in hepatocyte incubations can be calculated from the experimental data using C_{hep}, V_{hep}, C_{buffer}, V_{buffer}, k_{met}, and k_{up}.

The uptake rate in a hepatocyte incubation can be calculated from the amount of a compound appearing in hepatocytes at any given period of time. But this method cannot distinguish the fraction of compound non-specifically bound to the outer membrane of the hepatocytes from the fraction uptaken into the hepatocytes. In an incubation where a

compound's metabolism rate is greater than its uptake rate, monitoring the disappearance of the compound from the incubation buffer provides an easier way to determine the uptake rate. In our study, the metabolism rate constants of midazolam in rats and humans, converted from the microsomal data with correction of f_u, were 0.370 and 0.167 /min/(million hepatocytes/mL), respectively. Putting values of rate constants of metabolism and uptake, as well the concentrations and volumes of the hepatocytes and incubation buffer in equation (12), the f_u values of midazolam in rat and human hepatocyte incubations were calculated to be 0.075 and 0.129, respectively (Table 3). Noticeably, before being corrected for f_u, the apparent intrinsic clearance of midazolam in rat microsomal incubation was about 40-fold higher than that in hepatocytes. However, after a correction for f_u in both systems the difference in CL_{int} is reduced to about 3.8-fold. Considering that the microsomal and hepatocyte incubations are two widely different experimental systems involving two different scale up factors to calculate the f_u, this 3.8-fold difference may be considered acceptable. Similarly, a smaller difference (3.5-fold) was observed in intrinsic clearances between human microsomal and hepatocyte incubations after a correction of both values with f_u. The intrinsic clearance of midazolam using human hepatocytes was significantly lower than that in rat hepatocytes. This was consistent with the reported lower plasma clearance of 0.40 L/h/kg in humans (Goodman & Gilman 2001) compared to 4.75 L/h/kg in rats (Kotegawa et al. 2002). A recent study suggested that the metabolism of midazolam in human hepatocytes would not reach saturation until the total incubation concentration reaches 100 μM (Zhao et al. 2005). This is consistent with our study. The $f_{u,hep\text{-}inc}$ of midazolam in human hepatocytes calculated from equation (12) is 0.129 (Table 3). Therefore, a 100

μM concentration in hepatocyte incubation would have an intracellular free concentration around 12.9 μM. At such a concentration midazolam, which has K_m around 4 μM determined in human liver microsomes (Pelkonen et al. 1998), would have its metabolism rate at saturation.

The $CL_{int,app}$ values for midazolam in hepatocytes, measured at sub-K_m concentrations using the method of disappearance of parent compound, are consistent with those reported by Lave et al. (1997) and Lau et al. (2002). It is interesting to note that several reports (Hallifax et al., 2005; Obach, 1999; Walsky and Obach, 2004; Kotegawa et al. 2002) show different $CL_{int,app}$ values measured using the alternate method of V_{max}/K_m. This discrepancy may be attributable to the V_{max}/K_m approach for estimating $CL_{int,app}$ where saturating concentrations of midazolam were used and enzymes in hepatocytes metabolized midazolam at full capacity. One has to be cautious when using the data from the V_{max}/K_m approach since midazolam, at high, nonphysiological concentrations, is known to be a 'substrate inhibitor' of CYP3A (Galetin et al. 2003; Hallifax et al. 2005), thereby also showing reduced clearance in microsomes.

In conclusion, hepatocytes, being closer to the in vivo liver system, are ideally suited for the prediction of in vivo hepatic clearance. Microsomes are still valuable for clearance determination for compounds metabolized primarily by phase I enzymes. The determination of intrinsic clearance using hepatocytes is dependent on $CL_{int,app}$ which in turn is dependent on the metabolism and cellular uptake rates. A derivation of an equation to calculate $f_{u,hep\text{-}inc}$ is provided, and its use to account for the lower in vitro

clearance in human and rat hepatocytes compared to microsomes for midazolam, a compound which shows high clearance using microsomal system, is demonstrated.

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Legends for Figures

Figure 1 Hepatocyte suspension model, a dynamic system where the rate of uptake equals the rate of metabolism plus diffusion from inside to outside at any given time. The rate constant of diffusion across either sides are assumed to be the same. The active uptake and efflux transporters were ignored for simplicity.

Figure 2 Midazolam uptake in human and rat hepatocytes

Footnote: Midazolam disappearance in the incubation buffer. Hepatocytes were removed at each incubation time point

Table 1. Apparent Intrinsic Hepatic Clearances from Different In Vitro Systems

In vitro system	$CL_{int,app}$ (L/hr/kg)					
	Human			Rat		
Compound	Hepatocytes		Microsomes	Hepatocytes		Microsomes
	Fresh (N=4)	Cryopreserved (N=7)	(N=3)	Fresh (N=3)	Cryopreserved (N=5)	(N=3)
7- EC	1.80	1.57	7.34	2.50	2.69	16.8
Phenacetin	0.74	0.33	1.15	0.49	0.58	6.38
Propranolol	0.37	0.63	1.72	2.73	3.20	79.0
Midazolam	0.69	0.98	22.4	2.46	2.74	113

ND: not done

Table 2. Apparent Clearance (L/hr/kg) in Freshly Isolated and Cryopreserved Rat and Human Hepatocytes

Cryopreserved	Rat Hepatocytes[1]			Human Hepatocytes[3]		
	Mean	SD	%CV	Mean	SD	%CV
7- EC	2.69	0.85	31.7	1.57	0.68	43.5
Phenacetin	0.58	0.09	15.8	0.33	0.14	42.2
Propranolol	3.20	0.26	8.2	0.63	0.32	51.5
Midazolam	2.74	0.44	16.0	0.98	0.40	40.9

Freshly Isolated	Rat Hepatocytes[2]			Human Hepatocytes[4]		
	Mean	SD	%CV	Mean	SD	%CV
7- EC	2.50	0.33	13.2	1.80	1.39	77.2
Phenacetin	0.49	0.18	36.0	0.74	0.77	103
Propranolol	2.73	0.41	15.0	0.37	0.41	109
Midazolam	2.46	0.17	7.0	0.69	0.90	131

ND: not done; [1] N=5; [2] N=3; [3] N=7; [4] N=4

Table 3 Midazolam clearances (L/hr/kg) and unbound fraction in microsomes and hepatocytes

	Human			Rat		
	$CL_{int, app}$	CL_{int}	f_u	$CL_{int, app}$	CL_{int}	f_u
Microsomes	22.4	27.0	0.83	113	135	0.84
Hepatocytes	0.98	7.6	0.129 [a]	2.75	36.7	0.075[a]

[a] Calculated from equation (12)

Figure 1

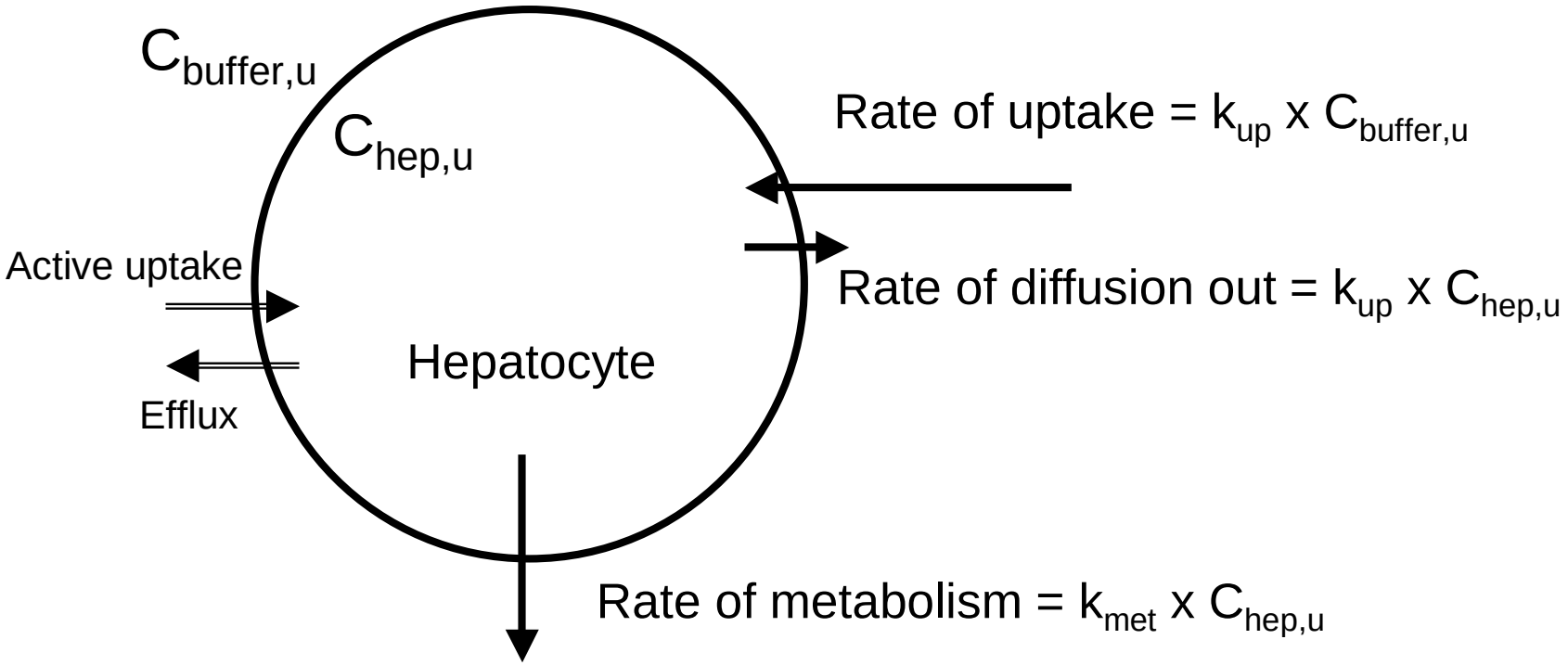

Figure 2