Test System-Dependent Clearance of CYP2D6 and CYP3A4/5 Substrates: A Comparison of Human Liver Microsomes and Cryopreserved Human Hepatocytes

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Introduction

The in vitro to in vivo extrapolation (IVIVE) of drug clearance involves the determination of intrinsic clearance in vitro (CL_{int}), based on in vitro measurements of V_{max}/K_m or half-life ($t_{1/2}$) in human liver microsomes or cryopreserved human hepatocytes, which are then scaled to predict hepatic clearance in vivo ($CL_{H,int}$). Although in vitro values of CL_{int} often underpredict in vivo values of $CL_{H,int}$, the values of CL_{int} determined with human liver microsomes would be expected to match those determined in hepatocytes for drugs predominantly cleared by cytochrome P450 (CYP). However, in the case of drugs rapidly cleared by CYP3A4, there are reports showing that CL_{int} values determined in microsomes are much greater than those determined in hepatocytes whereas the opposite has been observed with drugs that are slowly cleared by CYP2D6.[1-2] In the present study we examined the clearance of the CYP3A4/5 substrate midazolam (high intrinsic clearance) and the CYP2D6 substrate dextromethorphan (low intrinsic clearance) in human liver microsomes and cryopreserved human hepatocytes to confirm the findings of previous reports and investigate why hepatocytes cannot support the same high rates of drug clearance supported by human liver microsomes.

Materials and Methods

Chemicals and test system
Dextromethorphan and midazolam were purchased from Sigma-Aldrich (St. Louis, MO); Krebs-Henseleit buffer (KHB) was prepared in house at XenoTech LLC (Lenexa, KS); Cryopreserved human hepatocytes (n=50; mixed gender) were isolated from non-transplantable livers and cryopreserved in house. Pooled human liver microsomes (n=16 or n=200; mixed gender) were prepared from non-transplantable livers and characterized in house as described previously.[3-4] The sources of all other chemicals and reagents used have been described previously.[5-6]

In vitro hepatocyte clearance determinations
The in vitro hepatic clearance of dextromethorphan and midazolam was assessed with cryopreserved human hepatocytes at 1x10^6 cells/mL. Briefly dextromethorphan or midazolam was incubated with cryopreserved human hepatocytes (37˚C with 5% CO_2) at 1 µM or plasma C_{max} (0.014 µM and 0.34 µM, respectively) for 0, 10, 20, 30, 60, 90 and 120 min. At these time points, the reaction was stopped by adding an equal volume of acetonitrile containing internal standard (d_3-dextrorphan or d_4-1'-hydroxymidazolam). Precipitated protein was removed by centrifugation (10 min at 5600 x *g*). The supernatant fraction was analyzed by LC/MS/MS to monitor dextromethorphan and midazolam loss. An API2000 or 4000 (AB Sciex) was used with Shimadzu HPLC pumps and autosampler systems to quantify dextromethorphan and midazolam with calibration curves spanning 0.025 – 1 µM and 0.05 – 1 µM, respectively. MRM transitions for the detection of dextromethorphan and midazolam were 272/147 and 326/291, respectively.

In vitro microsome clearance and kinetics
The in vitro clearance of midazolam and dextromethorphan was also assessed in pooled human liver microsomes (n = 200) at a protein concentration of 0.33 mg/mL, which is equivalent to the concentration of microsomes in hepatocytes at 1x10^6 cells/mL. Briefly, 1 µM midazolam or dextromethorphan was incubated with NADPH-fortified human liver microsomes at 37˚C for 1-8 min or 2-15 min, respectively. Reactions were stopped and processed as described above.

The kinetic parameters, K_m and V_{max}, were determined in human liver microsomes (pooled mix gender; n = 16) for midazolam 1'-hydroxylation and dextromethorphan *O*-demethylation. Briefly, midazolam (0.5, 1, 2.5, 5, 10, 15, 20, and 40 µM) and dextromethorphan (0.5, 1, 2.5, 5, 10, 20, 50, and 100 µM) were incubated for 10 min at 37˚C with 0.1 mg/mL human liver microsomes at pH 7.4 in buffer (50 mM phosphate buffer containing 3 mM $MgCl_2$ and 1 mM EDTA) with an NADPH regenerating system (5 mM glucose 6-phosphate, 1U/mL glucose 6-phosphate dehydrogenase, and 1 mM NADP). Reactions were stopped by adding an equal volume of acetonitrile containing internal standard (d_3-dextrorphan or d_4-1'-hydroxymidazolam). Samples were processed and analyzed by LC/MS/MS dextrorphan and 1'-hydroxymidazolam as described previously.[5]

Microsome isolation from cryopreserved human hepatocytes
Microsomes were prepared from pooled cryopreserved human hepatocytes (n=50) as described previously.[3-4] Briefly, cryopreserved human hepatocytes were thawed and sonicated for 40 sec with homogenization buffer (50 mM Tris-HCl pH 7.4 containing 150 mM KCl and 2 mM EDTA). Homogenate was then centrifuged at ~7000 x *g* for 20 min at 4°C. The supernatant fraction was subjected to further centrifugation at ~100,000 x *g* for 60 min at 4°C. The microsomal pellet was resuspended and washed with buffer (150 mM KCl and 10 mM EDTA at pH 7.4). The washed pellet was then re-isolated by centrifugation at ~100,000 x *g* for 60 min at 4°C. The final pellet was resuspended in 250 mM sucrose. Protein concentration was determined with a Pierce BCA assay (Pierce Chemical, Rockford, IL). CYP3A4 and CYP2D6 activity in microsomes isolated from the pooled hepatocytes was compared with that in pooled human liver microsomes (both at 0.1 mg/mL) with midazolam and dextromethorphan at two substrate concentrations: 1 µM and V_{max} (40 and 75 µM, respectively). After a 5-min incubation at 37°C the reactions were stopped and the samples processed for dextrorphan and 1'-hydroxymidazolam as described above.

In vivo hepatic intrinsic clearance calculations
The scaled in vivo hepatic metabolic clearance (in vivo $CL_{H,int}$) was determined with physiologically based scaling factors (PBSFs) according to the following equation:

$$\textit{in vivo } CL_{H,int} = \textit{in vitro } CL_{int} \bullet \textit{PBSF for microsomes or hepatocytes}$$

Where for human liver microsome scaling:

$$\textit{in vivo } CL_{H,int} = \frac{V_{max}}{K_m} \bullet \frac{40 \text{ mg microsomes}}{g \text{ liver}} \bullet \frac{1650 \text{ g}}{\text{liver}}$$

For human hepatocyte scaling:

$$\textit{in vivo } CL_{H,int} = K_{el} \bullet \frac{\text{Volume of incubation}}{K_m} \bullet \frac{40 \text{ mg microsomes}}{g \text{ liver}} \bullet \frac{1650 \text{ g}}{\text{liver}}$$

Data were processed and all other values were calculated using GraFit 4.0.21 (Erithacus Software Ltd., Horley, Surrey, UK) and Microsoft Excel 2007 (Microsoft, Redmond, VA).

Results

Figure 1 shows the clearance of dextromethorphan and midazolam from pooled cryopreserved human hepatocytes (n = 50; 1x10^6 cells/mL) at 1 µM and at a substrate concentration equal to their in vivo plasma C_{max}. Dextromethorphan $t_{1/2}$ values at 1 µM and 0.014 µM (plasma C_{max}) were 56 and 81 min, respectively. Midazolam $t_{1/2}$ values at 1 µM and 0.34 µM (plasma C_{max}) were 75 and 63 min, respectively. The scaled values of $CL_{H,int}$ are summarized in **Table 1**.

As shown in **Figure 2**, the half-lives of dextromethorphan and midazolam (both at 1 µM) were assessed in pooled human liver microsomes (n = 200) at a protein concentration of 0.33 mg/mL, which is equivalent to the concentration of microsomal protein in hepatocytes at 1 x 10^6 cells/mL. In microsomes, the half-life of midazolam was 7 min (compared with 75 min in hepatocytes) whereas the half-life of dextromethorphan in microsomes was estimated to be 50 min (compared with 56 min in hepatocytes).

Figure 3 shows the kinetics of dextrorphan (CYP2D6) and 1'-hydroxymidazolam (CYP3A4/5) formation by human liver microsomes (n = 16). In vitro clearance was estimated from V_{max}/K_m. When in vitro clearance was scaled to in vivo clearance, $CL_{H,int}$ values for dextromethorphan and midazolam were 115 L/h and 2130 L/h, respectively.

As shown in **Table 1**, the estimate of in vivo clearance of dextromethorphan in microsomes (115 L/h) agreed with well with $CL_{H,int}$ values determined in hepatocytes (102-147 L/h) . However, in the case of midazolam, the estimate of in vivo clearance in microsomes (2130 L/h) was more than order of magnitude greater than $CL_{H,int}$ values determined in hepatocytes (110-129 L/h).

To determine whether this difference in metabolic clearance could be attributed to the functionality of CYP3A4/5 in cryopreserved hepatocytes, microsomes were isolated from pooled hepatocytes (n = 50) and activity was compared with that in pooled human liver microsomes (n= 200). The results are shown in **Table 2**. The microsomes isolated from pooled cryopreserved human hepatocytes had CYP3A4 and CYP2D6 activity comparable (within 20%) to those in pooled human liver microsomes.

These results confirm previous reports[1] that, with rapidly metabolized substrates like midazolam, CYP3A4 activity in hepatocytes is markedly less than CYP3A4 activity in human liver microsomes even when microsomes are isolated from the same hepatocytes used to measure midazolam clearance.

Table 1. Summary of scaled values of hepatic intrinsic clearance ($CL_{H,int}$) from cryopreserved human hepatocytes and human liver microsomes

Substrate	Cryopreserved human hepatocytes						Human liver microsomes		
	[S] = 1 µM			[S] = C_{max}					
	$t_{1/2}$ (min)	k_{el} (min^{-1})	$CL_{H,int}$ [a]	$t_{1/2}$ (min)	k_{el} (min^{-1})	$CL_{H,int}$ [a]	V_{max} (pmol/mg/min)	K_m (µM)	$CL_{H,int}$ [b]
Dextromethorphan	56	0.01234	147 L/h	81	0.00859	102 L/h	276	9.5	115 L/h
Midazolam	75	0.00928	110 L/h	63	0.01092	129 L/h	1290	2.4	2130 L/h

[a] Hepatocyte $CL_{H,int}$ was calculated as follows: k_{el} x volume of incubation/million cells per incubation x 198,000 PBSF. Units were converted to L/h.
[b] Microsomal $CL_{H,int}$ was calculated as follows: V_{max}/K_m x 66,000 PBSF. Units were converted to L/h.
Note: All $CL_{H,int}$ values have not been corrected for binding.

Table 2. Comparison of metabolic rates between microsomes isolated from cryopreserved human hepatocytes and a standard preparation of human liver microsomes

Protein Source	Rate (pmol/mg/min)			
	Midazolam 1'-hydroxylation		Dextromethorphan *O*-demethylation	
	1 µM	40 µM	1 µM	75 µM
HLM (n = 200)	263	1132	45	259
HLM isolated from CHH (n = 50)	206	893	46	220

Figure 1. The in vitro clearance of dextromethorphan and midazolam from pooled cryopreserved human hepatocytes (1x10^6 cells/mL)

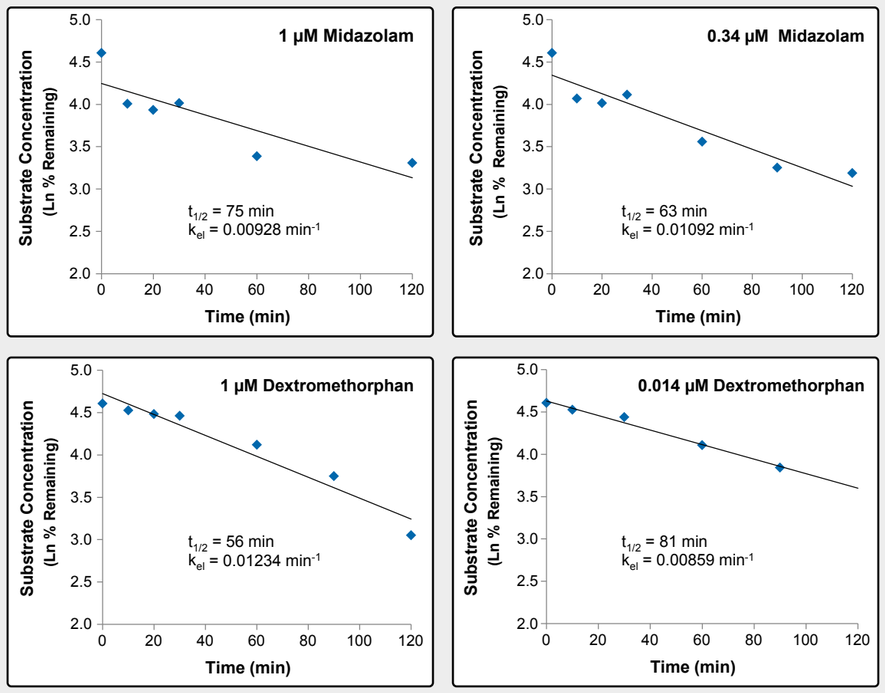


Figure 2. The in vitro clearance of dextromethorphan and midazolam in pooled human liver microsomes at 0.33 mg/mL, a concentration equivalent to the microsomal content of cryopreserved human hepatocytes at 1x10^6 cells/mL

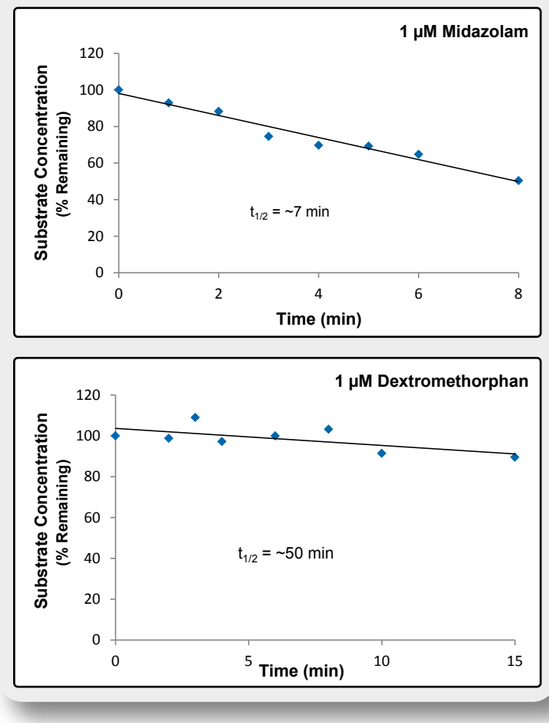
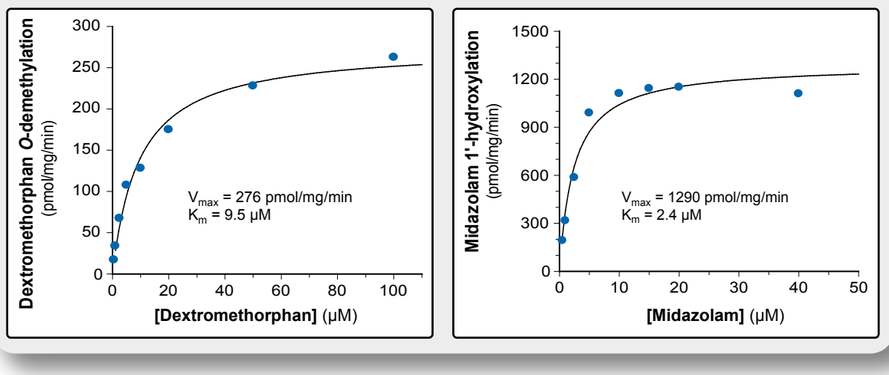


Figure 3. Kinetics for dextrorphan (CYP2D6) and 1'-hydroxymidazolam (CYP3A4/5) formation in pooled human liver microsomes



Conclusions

- The scaled $CL_{H,int}$ for the low intrinsic clearance CYP2D6 substrate dextromethorphan agrees well between human liver microsomes and cryopreserved human hepatocytes.
- The scaled $CL_{H,int}$ for the high intrinsic clearance CYP3A4/5 substrate midazolam is significantly different between human liver microsomes and cryopreserved human hepatocytes, confirming previous reports that clearance of high turnover substrates for CYP3A4/5 in human liver microsomes is substantially greater (by an order of magnitude or more) than that in human hepatocytes.
- Microsomes isolated from cryopreserved human hepatocytes have roughly the same CYP3A4/5 and CYP2D6 activity as that in pooled human liver microsomes,
- These results suggest that some factor, such as membrane permeability, cofactor availability or an unknown factor substantially limits the rate of metabolism of midazolam by CYP3A4/5 in human hepatocytes.

References

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