In our previous work, we reported that neither cofactor availability nor membrane permeability accounted for the much slower in vitro clearance of midazolam in suspended cryopreserved human hepatocytes (CHH) compared with human liver microsomes (HLM). We posited that the difference was possibly due to an impact on the activity of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5 (with multiple substrates) in HLM and CHH. CYP activities were measured at six ionic strength levels (5, 50 and 200 mM phosphate buffer) and in five commonly used cell culture media (KHB, MCM+, DMEM + HEPES, Waymouth’s and William’s E+HEPES) in both HLM and CHH.

**Materials & Methods**

**Chemicals and test system:** William’s E medium and Waymouth’s medium were purchased from Sigma-Aldrich (St. Louis, MO); Dubcco’s modified Eagle medium (DMEM) + HEPES was purchased from Gibco (Grand Island, NY); Krebs-Henseleit buffer (KHB) and modified Chee’s medium with supplementation (MCM+) were prepared in house at XenoTech, LLC (Lenexa, KS). Pooled human liver microsomes (HLM, n = 200, mixed gender) and pooled cryopreserved human hepatocytes (CHH, n = 50, mixed gender) were prepared from non-transplantable livers and characterized at XenoTech, LLC (Lenexa, KS) as described previously (Pearce et al., 1996; Parkinson et al., 2004). The sources of all other chemicals and reagents have been described previously (Parkinson et al., 2011).

**The effect of buffer ionic strength and cell culture media on CYP activity in human liver microsomes:** CYP1A2 (phenacetin O-dealkylation), CYP2A6 (cotrimoxazole 7-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2C8 (amodiaquine N-dealkylation), CYP2C9 (diclofenac 4'-hydroxylation), CYP2C19 (S-mephenytoin 4'-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2E1 (chlorozoxone 6-hydroxylation) and CYP3A4/5 (activity) in HLM and CHH was measured at six ionic strength levels (5, 50 and 200 mM phosphate buffer) as well as in five commonly used cell culture media (KHB, MCM+, DMEM + HEPES, Waymouth’s and William’s E+HEPES) in both HLM and CHH.

**The effect of buffer ionic strength and cell culture media on CYP activity in cryopreserved human hepatocytes:** CYP1A2 (phenacetin O-dealkylation), CYP2A6 (cotrimoxazole 7-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2C8 (amodiaquine N-dealkylation), CYP2C9 (cotrimoxazole hydroxylation), CYP2C19 (S-mephenytoin 4'-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), and CYP3A4/5 activity (midazolam 1'-hydroxylation, midazolam 4'-hydroxylation, nifedipine oxidation, alfentanil N-dealkylation, verapamil N-dealkylation, testosterone 6'-hydroxylation, and atorvastatin ortho-hydroxylation) in CHH was measured at six ionic strength levels (5, 50 and 200 mM phosphate buffer) as well as in five commonly used cell culture media (KHB, MCM+, DMEM + HEPES, Waymouth’s and William’s E). Brieﬂy, phenacetin (40 μM), cotrimoxazole (5 μM), diclofenac (7 μM), S-mephenytoin (40 μM), dextromethorphan (7.5 μM), chlorozoxone (30 μM), midazolam (4 μM), nifedipine (10 μM), alfentanil (40 μM), verapamil (9 μM), testosterone (70 μM) or atorvastatin (40 μM) was incubated at 37°C for 5 min with 0.1 mM potassium liver microsomes (n = 200) or 0.25 mM human liver S9 fraction; n = 200) at three (5, 20 and 200 mM) of concentrations of phosphate buffer (each containing 3 mM MgCl2 and 1 mM EDTA at pH 7.4) or in different cell culture media. Reactions were initiated with an NADPH regenerating system (5 mM glucose 6-phosphate, 1U/mL glucose 6-phosphate dehydrogenase, and 1 mM NADP) and stopped after 5 min with an equal volume of stop reagent (acetonitrile with internal standard). The samples were processed and analyzed by LC/MS/MS as described previously (Parkinson et al., 2011).

**Results**

Figure 1 shows the effect of buffer ionic strength (at 5, 50 and 200 mM phosphate) and various commonly used cell culture media on the CYP activity of pooled human liver microsomes (HLM). Probe substrates for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP2D6 all had the highest activity at 50 mM phosphate (i.e., standard buffering conditions). For CYP2B1 and CYP3A4/5, enzymatic activities were highest at 200 mM phosphate buffer (135% of standard buffering conditions). When incubations in HLM were conducted with various media, MCM+ was found to support the highest activity of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C19 and CYP2D6. For CYP2E1, CYP2C19 and CYP3A4/5, William’s E and KHB media were found to support the highest enzymatic activity levels, though the activity was approximately half the activity in 50 mM phosphate buffer.

When multiple substrates of CYP3A4/5 were assessed in pooled HLM and pooled S9 fraction (as shown in Figure 2), all of them had maximal activity at 200 mM phosphate buffer (137–272% activity of standard buffering conditions). In contrast to midazolam, the five other CYP3A4/5 substrates (nifedipine, alfentanil, verapamil, testosterone and atorvastatin) were all metabolized by HLM to a similar extent in all five media.

Figure 3 shows the effect of five different cell culture media on the time course of CYP activity of pooled cryopreserved human hepatocytes (CHH). For CYP1A2, CYP2B6, CYP2C8 and CYP2D6 there were modest differences in enzymatic activity between the five media. With CYP2C9 and CYP2C19, the highest activity was supported by MCM+ medium; whereas for CYP3A4/5 the highest activity was supported by William’s E medium.

When multiple substrates of CYP3A4/5 were assessed in pooled CHH (as shown in Figure 4), nifedipine was metabolized to the greatest extent in William’s E and KHB media; alfentanil the greatest in Waymouth’s medium; verapamil the greatest in DMEM + HEPES medium; and atorvastatin and testosterone the greatest in MCM+ medium.

**Conclusions**

- The results of this study suggest that the slower clearance of midazolam in CHH relative to HLM is perhaps unique to midazolam because no such marked difference was observed with five other CYP3A4/5 substrates.
- Whereas 50 mM phosphate buffer supported near maximal activity for most CYP enzymes in HLM it did not support maximal activity of CYP3A4/5 or CYP2E1. CYP3A4/5 and CYP2E1 activities were notably lower when HLM were incubated in cell culture medium compared with 50 or 200 mM phosphate buffer. William’s E and KHB media supported the highest CYP3A4/5 activity in both HLM and CHH. These findings have implications for optimizing in vitro conditions for measuring drug clearance and shed further light on the unusual, system-dependent clearance of midazolam. For the clearance of CYP3A4/5 substrates by CHH, William’s E medium and KHB offers advantages over the other media examined. The results do not explain why midazolam clearance in HLM is so much greater than in CHH but they do suggest this characteristic is not shared by five other CYP3A4/5 substrates.

**References**