**HLM-beads: Rapid assessment of non-specific binding to human liver microsomes using magnetizable beads**

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Abstract

In early drug development, drug-drug interaction (DDI) risk is routinely assessed using human liver microsomes (HLM). Non-specific binding of drugs to HLM can affect the determination of accurate enzyme parameters ($K_m$, $K_i$, $K_I$). Previously, we described a novel in vitro model consisting of HLM bound to magnetizable beads (HLM-beads). The HLM-beads enable rapid separation of HLM from incubation media by applying a magnetic field. Here, HLM-beads were further characterized and evaluated as a tool to assess HLM non-specific binding of small molecules. The free fractions ($f_{u,mic}$) of 13 compounds (chosen based on their pKa values) were determined using HLM-beads under three HLM concentrations (0.025, 0.50 and 1.0 mg/mL) and compared to those determined by equilibrium dialysis. Most $f_{u,mic}$ values obtained using HLM-beads were within 0.5 to 2-fold of the values determined using equilibrium dialysis. The highest fold difference were observed for high binders itraconazole and BIRT2584 (1.9- to 2.9-fold), as the pronounced adsorption of these compounds to the equilibrium dialysis apparatus interfered with their $f_{u,mic}$ determination. Correlation and linear regression analysis of the $f_{u,mic}$ values generated using HLM-beads and equilibrium dialysis was conducted. Overall, a good correlation of $f_{u,mic}$ values obtained by the two methods were observed as the $r$ and $R^2$ values from correlational analysis and linear regression analysis were >0.9 and >0.89, respectively. These studies demonstrate that HLM-beads can produce comparable $f_{u,mic}$ values as determined by equilibrium dialysis, while reducing the time required for this type of study from hours to only ten minutes and compound apparatus adsorption.
Significance Statement

This work introduces a new method of rapidly assessing non-specific microsomal binding using human liver microsomes bound to magnetizable beads.
Introduction

The potential for drug-drug interactions (DDI) is routinely evaluated during the development of small molecule drugs (Veehof et al., 1999). Typically, in drug development, preclinical assessments of DDI risk or predictions of drug clearance are conducted using hepatic tissue fractions. Human liver microsomes (HLM) are the most widely used in vitro tool for this purpose, due to their inclusion of the most relevant drug metabolizing enzymes at physiological levels and low cost to purchase. Furthermore, an abundance of historical data regarding HLM performance and limitations make HLM a well-defined system for studying glucuronidation or cytochrome P450 oxidation, two major pathways of drug metabolism (Asha and Vidyavathi, 2010). For clearance predictions, HLM are used when sufficient turnover of parent compound can be observed and where clearance pathways are known to involve enzymes that are abundant in the hepatocyte endoplasmic reticulum (Obach, 2001; Argikar et al., 2016). It is well-known that the accuracy of DDI or clearance predictions depends on understanding the free concentration of drug in plasma as it is generally accepted tenet that only free drug is available interact with drug metabolizing enzymes. More recently, it has also been recognized that a good understanding of the free concentration of drug in in vitro HLM incubations is necessary to derive accurate enzyme kinetic parameters ($K_m$, $K_i$, and $K_I$) since drugs can bind to membrane or protein components in HLM (Obach, 1997; Austin et al., 2002; Hallifax and Houston, 2006). Accounting for non-specific binding of drugs to HLM has been shown to improve the prediction of in vivo clearance (Obach, 1999).

In silico algorithms that rely on physicochemical parameters such as pKa, LogP, or LogD have shown success in predicting $f_{u,mic}$ values for compounds where $f_{u,mic}$ values exceed 0.5 and LogP values are less than 3 (Winiwarter et al., 2019). The accuracy of prediction decreases for more
lipophilic compounds, highlighting the need to experimentally assess microsomal binding. The most commonly used method to determine non-specific binding of drugs to HLM is equilibrium dialysis, where compounds diffuse across a semi-permeable membrane separating a suspension of HLM from buffer (McLure et al., 2000). In a typical equilibrium dialysis experiment, the time required for the system to reach equilibrium can be as long as 8 hours (Burns et al., 2015).

Previously, we have demonstrated that HLM are able to bind to silica-coated magnetizable beads (Horspool et al., 2020). The HLM could not be released from the beads by multiple washing steps, indicating strong binding. Furthermore, the HLM-beads retained functional drug metabolizing enzyme activities and could be rapidly removed from the incubation media using a strong magnet. In this work, we have developed a method to assess non-specific binding of compounds to HLM using HLM-beads and evaluated its accuracy by comparing the results to parallel studies using equilibrium dialysis. An illustration of the steps involved in conducting a microsomal binding study using HLM-beads is shown in Fig. 1.
**Materials and Methods**

**Materials**

Clozapine, diclofenac, diltiazem, imipramine, itraconazole, nevirapine, tolbutamide, warfarin and verapamil were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Alprazolam, chlorpromazine, diazepam and midazolam were purchased from Cerilliant Corp. (Round Rock, TX, USA). BIRT2584 was synthesized in-house (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA). Diclofenac-d$_4$, (+/-)-verapamil-d$_3$ and warfarin-d$_5$ were purchased from CDN Isotopes (Point-Claire, Quebec, Canada). HLM (Lot 38291, mixed gender 150 donors) were acquired from Corning Inc. (Glendale, AZ, USA). Silica-coated magnetizable beads (catalog # 501036426) were obtained from G-Biosciences (St. Louis, MO, USA). Coomassie Plus – The Better Bradford Assay Reagent and Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) set were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rapid equilibrium dialysis (RED) device was obtained from Thermo Fisher Scientific Pierce Labs (Waltham, MA, USA).

**Methods**

**Selection of compounds.** Alprazolam, chlorpromazine, clozapine, diazepam, diclofenac, diltiazem, imipramine, itraconazole, midazolam, tolbutamide, warfarin, verapamil and BIRT2584 were selected for the HLM binding studies. The structure and physicochemical properties of the compounds are listed in Fig. 2 and Table S1, respectively. Diclofenac-d$_4$, nevirapine, (+/-)-verapamil-d$_3$ and warfarin-d$_5$ were used as internal standards (IS) for LC-MS/MS analysis.

Microsomal binding of all tested compounds was evaluated at 1.0 µM and at HLM concentrations of 0.025, 0.50 or 1.0 mg/mL.
**Volume determination of the aqueous phase of the beads stock solution.** Samples of 100, 250, 500 and 1000 µL of well-suspended magnetizable beads (beads) from G-Biosciences were aliquoted into four 1.5 mL microcentrifuge tubes. The supernatants were carefully removed using a magnetic tube rack (Dynamag-2, Life Technologies Inc.) and the volume of the extracted liquid was measured.

**Time course of HLM binding to beads.** The stock HLM suspension (20 mg/mL, 30 µL) was diluted into phosphate buffer (50 mM, pH 7.4, 570 µL) (PB) to produce a suspension of HLM (1.0 mg/mL, 600 µL). Well-suspended beads from the manufacturer (100 µL) were aliquoted into a 1.5 mL microcentrifuge tube. The supernatant was removed using a magnetic tube rack and the beads were washed three times using 600 µL of PB each time. After the final wash, the supernatant was removed from the beads. The HLM suspension (1.0 mg/mL, 600 µL) was then mixed with the beads. The protein content of the supernatant of the mixture (5.0 µL) was measured at 0.25, 0.50, 1.0, 5.0 and 10 minutes (min) after the initiation of the incubation. The HLM suspension prepared (1.0 mg/mL) prior to the exposure to beads was used as a control. The total supernatant removed at the end of the experiment was <5% of the initial incubation volume. The protein content in each sample was measured using Bradford Coomassie Blue Assay. Briefly, Coomassie reagent (300 µL) was added into individual wells on a 96-well clear-bottom plate followed by addition of a sample (5.0 µL). Protein content was quantified using a bovine serum albumin standard curve. The free fraction of protein remaining in the supernatant at each incubation time point was compared to the protein content of the HLM suspension without beads. Protein content at each time point was determined in triplicate.

**Determination of the HLM binding capacity of beads.** Well-suspended beads (500 µL) were aliquoted into a 1.5 mL microcentrifuge tube and washed as described above. To maintain the
concentration of the stock beads during the experiments, washed beads were reconstituted in an equivalent volume of incubation buffer as the initial volume removed from the stock beads. The supernatant volume was determined to be 78 ± 3 % of the volume of the stock bead suspension. After washing, the beads were resuspended into 390 µL PB to bring the suspension volume back up to 500 µL. Sixteen aliquots (30 µL of each) of the suspension were dispensed into individual microcentrifuge tubes and supernatants were removed as described above. HLM suspension (1.0 mg/mL, 1.0 mL) was then added to the first tube containing beads, gently mixed by inverting the tube 5-6 times and incubated for 5 min at 4°C. After the incubation, the suspension was subjected to a magnetic field and an aliquot of the supernatant (3.0 µL) was taken for protein quantification. The remaining suspension was then transferred to a second tube containing free beads, mixed gently and incubated for 5 min at 4°C. After the incubation, the suspension was subjected to a magnetic field and the supernatant (3.0 µL) was taken for protein quantification. The procedure was repeated until protein content in the supernatant no longer decreased with the addition of beads. The total volume of supernatant removed from the suspension was <5% of the total initial volume. The protein content in each sample was quantified as described above. A sample of the HLM suspension (1.0 mg/mL, 3.0 µL) taken prior to the exposure to the beads was used as a control. Adsorption of HLM onto beads was determined by comparing the protein content in the supernatant before and after the addition of HLM to the beads. The determination was performed in triplicate.

**Preparation of HLM-beads for microsomal binding study.** Well-suspended beads (200 µL) were aliquoted into a 1.5 mL microcentrifuge tube and washed as described above. All the solvent was removed after the last wash and replaced with HLM (20 mg/mL, 50 µL) and PB
(906 µL). The suspension was gently mixed and incubated at 4 °C for 5 min. The resulting HLM-bead suspension was previously determined to be equivalent to a 1.0 mg/mL HLM suspension in PB (1.0 mL).

**Time course of compound binding to HLM-beads.** The progress of compound binding to HLM-beads was studied using diazepam, clozapine and imipramine. HLM-beads (1.0 mg/mL, 1.5 mL) were aliquoted into 2 mL microcentrifuge tubes and equilibrated at 37 °C for 5 min, and then the supernatant was removed. Pre-warmed (37 °C) test compound (1.0 µM, 1.5 mL) was mixed with the HLM-beads. The suspension was incubated at 37°C on an orbital shaker with 750 rotations per minute (rpm). At 1.0, 2.5, 5.0, 10, 15 and 30 min after initiation of incubation, an aliquot of supernatant (10 µL) was mixed with an equal volume of acetonitrile (ACN) containing formic acid (FA, 0.1%) and IS. The preparation was then centrifuged at 3500 revolutions per minute (RPM), 4°C for 10 min. The compound solution in the absence of HLM-beads was taken as t₀. The total volume of sample taken at the end of experiment was <5% of the initial incubation volume. The samples were then analyzed using LC-MS/MS. The compound free fraction at each incubation time point was calculated according to Eq. 1.

\[
\text{Compound free fraction} = \frac{[\text{Compound}]_t}{[\text{Compound}]_{t0}} \quad \text{Eq. 1}
\]

Where \([\text{Compound}]_t\) represents the compound concentration in the supernatant at any given incubation time point and \([\text{Compound}]_{t0}\) is the time point before the addition of HLM-beads. The incubations were performed in duplicate.
**Microsomal binding study using HLM-beads.** HLM-beads (equivalent to 1.0 mg/mL HLM) were prepared as described above and diluted in PB to achieve HLM concentrations equivalent to 0.025 and 0.50 mg/mL. Aliquots of HLM-beads (150 µL) were dispensed into microcentrifuge tubes. The supernatant in each of the tubes was removed using a 96-well magnetic plate. The test compounds (1.0 µM, 150 µL) were added into the microcentrifuge tubes containing the HLM-beads. The suspension was gently mixed, then incubated for 5 min, at 750 rpm on an orbital shaker set to 37 °C. The supernatant (75 µL) was then removed and mixed with an equal volume of ACN (0.1% FA, IS) followed by centrifugation at 3500 RPM, 4°C for 10 min. The compound solution in the absence of HLM-beads was used as a control. The concentration of the free test compound in the supernatants was analyzed by LC-MS/MS. The f_{u,mic} value was calculated according to Eq. 2. The incubations were conducted in triplicate.

\[
f_{u,mic} = \frac{[\text{Compound}]_{+HLM\text{-beads}}}{[\text{Compound}]_{PB}}
\]

Eq. 2

[Compound]_{+HLM\text{-beads}} and [Compound]_{PB} represent the compound concentrations in the supernatants with HLM-beads and with PB only, respectively.

**Microsomal binding study using rapid equilibrium dialysis (RED)** The RED device consists of inserts in a 48-position plate. Each insert consists of a compound donor and receiver compartment, separated by a cellulose dialysis membrane (M.W. cutoff, 8 kDa). Test compound (200 µL) and HLM (0.025, 0.50 or 1.0 mg/mL) were added to the donor compartment and PB (400 µL) was added to the receiver compartment. The samples were then incubated on an orbital shaker set to 65 rpm for six hours at 37°C. At the end of incubation, 25 µL of sample from each chamber was removed and equalized by adding an equal volume of buffer to the donor samples.
and an equal volume of HLM (free of test compound) to the receiver samples. An aliquot of each sample was diluted 4-fold with ACN (0.1% FA, IS) followed by centrifugation at 3500 RPM, 4°C for 10 min. the test compounds remaining in the supernatant were then analyzed by LC-MS/MS. The $f_{u,mic}$ was calculated according to Eq. 3.

$$f_{u,mic} = \frac{[\text{Compound}]_{\text{receiver}}}{[\text{Compound}]_{\text{donor}}}$$  

Eq. 3

Where $[\text{Compound}]_{\text{donor}}$ and $[\text{Compound}]_{\text{receiver}}$ represent the concentrations of compound in the donor and receiver compartments, respectively. The incubations were performed in triplicate.

For compounds such as chlorpromazine, itraconazole and BIRT 2584, that extensively adsorb onto materials in the RED device, a second experiment was conducted whereby the RED inserts were pre-soaked with test compound (1.0 µM) prior to the binding assay. The experimental procedures were identical as described above except that each compartment of the device was pre-incubated with the test compound in PB for 30 min. All the test compound solutions were removed and the binding assays were performed as described previously. The incubations were performed in triplicate.

**LC-MS/MS analysis.** Compounds were eluted by reverse phase HPLC using a Waters Acquity or an Agilent 1290 series ultrahigh performance pump. A Waters Acquity UPLC BEH, C18, 1.7 µm, 2.1 x 50 mm, part#188002350). An Applied Biosystems 5000 or 6500 Qtrap mass spectrometer equipped with electron spray ionization source in multiple reaction monitoring mode was used for detection.

The mobile phases A and B for all analytes were water (0.1% FA) and ACN (0.1% FA), respectively. The MRM transitions of the analytes are compiled in the Supplemental Section.
Table S2. Quantitation of test compounds was achieved by comparing the peak area ratios of analyte over IS to test compounds with known concentration, i.e. standard curve.

**Calculation of LogD.** Where experimentally determined LogD values could not be found, calculated values were produced under pH 7.4 using Marvin View (ChemAxon Ltd.).

**Statistics.** Comparisons between \( f_{u,mic} \) values determined using HLM-beads and RED were conducted using a two-tailed Pearson Correlation analysis and linear regression. Both analyses were conducted using GraphPad Prism (version 8.3.0).

**Results**

**Characterization of the HLM binding to magnetizable beads.** Both the HLM-binding capacity of the beads and the time required for HLM binding to the beads was assessed.

Previously, a 30 min incubation time was used to ensure that the binding of HLM to the beads reached equilibrium (Horspool *et al*., 2020). To optimize the time required for the binding, the time course of HLM binding to the beads was investigated. As shown in Fig. 3, HLM binding to the beads reached equilibrium approximately 1 min after the incubation was initiated. Thus, to ensure consistency in incubation time and minimize the time required to complete binding, the incubation time for HLM binding to beads was set to 5 min. Incorporation of more automated techniques could reduce the incubation time even further.

To determine the HLM-binding capacity of the beads, washed beads were titrated into HLM (1.0 mg/mL, 1.0 mL). The protein remaining in the supernatant after each addition of beads was quantified and normalized to the protein content of a 1.0 mg/mL suspension of HLM (without...
beads), Fig. 4. It was determined that 185 ± 13 µL of commercially supplied bead suspension is capable of binding 1.0 mg of HLM.

In subsequent studies, complete binding of HLM was accomplished by incubating 200 µL of the manufacturer-supplied suspension of beads with each mg of HLM used in an incubation.

**Time course of test compound binding to HLM-beads.** The time required for compound to reach binding equilibrium to HLM (when fraction of unbound compound does not change with time) was investigated using three compounds: diazepam, clozapine and imipramine. As shown in Fig. 5, binding reached completion between only 1 and 2.5 min after the initiation of the incubation. A five min incubation time was then used in subsequent experiments to ensure that the of binding equilibrium achieved.

**Microsomal binding study using HLM-beads & RED.** The $f_{u,mic}$ values of a set of 13 test compounds obtained using HLM-beads and RED at three HLM concentrations were listed in Tables 1. Regression and correlational analysis of $f_{u,mic}$ values obtained using the two methods was shown in Fig. 6. The $r$ values are ≥ 0.9 indicating a high correlation of $f_{u,mic}$ values obtained using HLM-beads and RED. Chlorpromazine, itraconazole and BIRT 2584 and itraconazole are exceptions in that their $f_{u,mic}$ values are up to 2.9-fold higher using HLM-beads vs. RED due to their extensive adsorption onto the RED device.

For these three compounds, the microsomal binding studies using RED were conducted with and without pre-soaking the apparatus with 1.0 µM of compound. A comparison of the $f_{u,mic}$ values obtained with and without pre-soaking are listed in Table S3. The ratio of $f_{u,mic}$ obtained by HLM-beads and RED changed from 5.1 to 1.3 for chlorpromazine, 26 to 2.9 for itraconazole and
7.6 to 1.9 for BIRT 2584 when the dialysis membrane of RED device not pre-soak and pre-soak with the test compound.
Discussion

HLM are vesicular subcellular fractions composed of endoplasmic reticulum and are commonly used to study hepatic metabolism in support of small-molecule drug development. Previously, we demonstrated that HLM can bind strongly to silica-coated magnetizable beads and that these HLM-beads retain functional drug metabolizing enzymes (Horspool et al., 2020).

Detailed characterization of the HLM-beads system demonstrated that the binding of HLM to beads is a fast event, reaching completion within one minute after combining HLM with the beads (Fig. 3). The binding capacity of the beads is determined to be 185 µL of the manufacturer-supplied suspension of beads /mg HLM (Fig. 4). The capacity of the beads to capture HLM is dependent on the total surface area of each bead, which is inversely related to the diameter of each bead (Ahmed and Wunder, 2009). As such, it is likely that HLM-loading capacity varies with the size of bead being used. If the average surface area of the beads differs across batches (e.g. due to changes in manufacturing equipment or process), HLM-binding capacity should be determined for each batch. The ease with which the HLM-beads could be separated from their incubation media prompted the current study to explore the application of the HLM-beads as a tool to assess the binding of small molecules to HLM.

Non-specific binding of small molecules to microsomes is a well-known in vitro artifact that can contribute to inaccurate projection of clearance from in vitro data or the inaccurate determination of concentration-dependent enzyme kinetic constants such as the Michaelis constant (Km), reversible inhibition constant (Ki) or inactivation constant (Ki) (Obach, 1997; Tran et al., 2002; Venkatakrishnan et al., 2003). Several different methods to measure the non-specific binding of compounds to HLM have been described which use principles of centrifugation or
filtration (Zhou et al., 2002; Niwa et al., 2005; Brown et al., 2006; Ballard and Rowland, 2011). However, equilibrium dialysis, remains the most commonly used method to determine the $f_{u,\text{mic}}$ values (Kurz et al., 1977; Vuignier et al., 2010). Although greater efficiency of determining $f_{u,\text{mic}}$ values using equilibrium dialysis have been realized with high throughput, 48 or 96-position devices, the long incubation times (up to 8 hours) often required to allow test compounds to permeate across the dialysis membrane continues to be a challenge, particularly for unstable compounds (Zentz et al., 1978; Horowitz and Barnes, 1983; Eriksson et al., 2005; Waters et al., 2008).

Based on their moderate to high binding to HLM, diazepam, clozapine and imipramine were chosen to evaluate the time required for compounds to bind to HLM on HLM-beads. As shown in Fig. 5, binding of these compounds to HLM reached completion within 5 min regardless of the compound tested. Thus, compared to established methods such as equilibrium dialysis or ultracentrifugation that require lengthy permeation or centrifugation steps, significant time-savings can be achieved using HLM-beads. Indeed, conducting a complete incubation using HLM-beads requires as little as ten minutes as opposed to at least several hours for the previously mentioned methods.

To determine if HLM-beads can be used to assess microsomal binding of small molecules, the $f_{u,\text{mic}}$ values of 13 compounds were determined using HLM-beads at three HLM concentrations. These compounds were selected based on their pKa and the extent of ionization at pH 7.4. Among the tested compounds, three are acidic, four are neutral and six are basic at physiological pH. The structure and the pKa, LogP, LogD$_{7.4}$ values of the compounds are listed in Fig. 2 and Table S1, respectively. Previous studies have highlighted the relative propensity of basic compounds to bind non-specifically to HLM as opposed to neutral and acidic compounds that
generally exhibit less binding possibly due to electrostatic interactions between the protonated base and negatively charged head of the phospholipids (Kramer et al., 1998; Obach, 1999; Li et al., 2009).

The pattern of binding across acidic, neutral and basic compounds is in good agreement with previous studies demonstrating a propensity of basic compounds to exhibit lower $f_{u,mic}$ values (Obach, 1999; McLure et al., 2000; Li et al., 2009) (Fig. 6). Previous work comparing the $f_{u,mic}$ values determined by equilibrium dialysis and by analyzing the intrinsic clearance of substrates at varying concentrations of HLM, demonstrated a high concordance of $f_{u,mic}$ values between methods for compounds with $\log D_{7,4}$ values less than 3.5 (Chen et al., 2017). Because the latter method involves the direct assessment of enzyme activity, it is considered a more accurate assessment of non-specific binding in microsomes. For compounds possessing $\log D_{7,4}$ values greater than 3.5, $f_{u,mic}$ values determined by equilibrium dialysis were consistently greater than 2-fold lower than those values determined using the intrinsic clearance method. This suggests that the 2- to 3-fold higher $f_{u,mic}$ values determined for itraconazole ($c\log D_{7,4} = 7.31$) (Treyer et al., 2019) and BIRT2584 ($c\log D_{7,4} = 4.4$) using HLM-beads may be a more accurate assessments of the free concentration of compound in each HLM incubation. Although not assessed in this work, it should be relatively easy to simultaneously assess $f_{u,mic}$ values using the intrinsic clearance method and the HLM-bead method as long as the test compounds exhibit sufficient turnover at various concentrations of HLM.

Adsorption of test compounds to apparatus material is always a concern for obtaining accurate $f_{u,mic}$ values (Ballard and Rowland, 2011). Three high binders, itraconazole, chlorpromazine and BIRT2584 were selected to test the effect of non-specific adsorption of compound to the RED apparatus on $f_{u,mic}$ values (see Supplemental information for detailed discussion). Pre-soaking the
RED device with test compound reduce the ratios of $f_{u,\text{mic}}$ obtained by HLM-beads and RED of chlorpromazine, itraconazole and BIRT 2584 down to 1.3, 2.9 and 1.9 folds compare to non-soak, 5.1, 26 and 7.6 folds. As the data shown that $f_{u,\text{mic}}$ values obtained for these three high binders have a better agreement between the HLM-beads and RED device with reduced apparatus non-specific binding, i.e. pre-soak. The results indicates that the HLM-beads has much less extent of apparatus adsorption, thus could provide more accurate $f_{u,\text{mic}}$ for compounds with high apparatus adsorption. Moreover, our studies show that the extent of compound adsorption to beads in the absence of HLM is negligible (data not shown). Furthermore, we previously showed that phospholipids can block HLM from binding to the beads, which indicates that it is possible to block the binding of material to the beads (Horspool et al., 2020). Thus, we hypothesized that the HLM would have the same effect of blocking other compounds from binding to the beads. Further investigations on the non-specific binding of compounds to the beads is ongoing. Overall, HLM-beads can be used as a fast and reliable way to determine the binding of small molecules to HLM with accuracy and precision that is comparable to values obtained using equilibrium dialysis. Because the method does not rely on centrifugation or dialysis, the time required to complete an experiment is reduced considerably and can benefit the evaluation of test compounds that are chemically unstable. Furthermore, unlike equilibrium dialysis that relies on a dialysis membrane, the apparatus used for the HLM-beads can be chosen to avoid any effects of material adsorption on the determination of $f_{u,\text{mic}}$ (e.g. silanization of glass tubes). Since HLM-beads enables the quick separation of HLM from solvent, the sample analysis process has been simplified compared to RED. Procedures such as developing separated bioanalytical methods required for RED to assess donor or recipient compartments or preparing cross-matrix normalization of samples, and having to account for differences in bioanalytical matrix-
dependent sensitivity due to different concentrations of HLM are no longer required, which reduce the effort associated with downstream LC/MS analysis markedly.

When $f_{u,mic}$ values are determined using only a few concentrations to cover wide ranges of compound and HLM concentrations, inaccuracies can be introduced to DDI or clearance predictions (McLure et al., 2000). Using HLM-beads, it becomes feasible to routinely determine the $f_{u,mic}$ value of each compound concentration used in the assays to make a more accurate prediction. Indeed, as the HLM-beads retain functional enzymes, it is also possible to determine $f_{u,mic}$ values in the same incubations used in the in vitro metabolism studies.

As stated previously, accurate predictions of drug clearance or DDI also depends on a good understanding of unbound plasma concentration of drug. Unfortunately, the mechanism by which HLMs bind to the magnetizable beads (via phospholipid interactions with silica coating) is not amenable to non-membrane proteins. Our previous work showed that binding of human serum albumin to silica coated beads is not as complete as the binding of phospholipids to the beads (Horspool et al., 2020). It is however worth noting that at least one marketed product exists which utilizes binding of plasma proteins to non-magnetizable beads (Schuhmacher et al., 2004). We do not think that it would be difficult to adapt such a product into a magnetizable bead format.

In summary, we have demonstrated that HLM-beads can be used to assess the non-specific binding of drugs to human liver microsomes. The method is much faster, with fewer sample processing steps, but can produce comparable results as the gold-standard method equilibrium dialysis. As the mechanism of HLM binding to silica-coated beads is driven by the interaction between phospholipids and silica coating, it is likely that this technology can be expanded to
include assessment of non-specific binding to other subcellular fractions from other sources such as animals, plants and cellular systems.
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**Authorship contributions:**

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Wrote or contributed to the writing of the manuscript: Ting Wang, Tom S Chan, and Andrea Whitcher-Johnstone
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**Figure Legends**

**Figure 1.** General procedure for HLM-beads microsomal binding study.

**Figure 2.** Structures of the compounds tested.

**Figure 3.** Time course of HLM binding to beads. The progress of HLM binding to beads was monitored by through determining the amount of protein remaining in the supernatant. The free protein content in the supernatant at each given incubation time point was normalized to the protein content prior to the exposure of HLM to the beads. The protein content in each sample was quantified by a Bradford assay at 595 nm. Each data point represents a mean of three determinations ± SD.

**Figure 4.** Capacity of beads to capture HLM. The stoichiometry of HLM binding to beads was monitored by determining the amount of protein remaining in the supernatant at each addition of beads. The free protein content in the supernatant at each addition of beads was normalized to the protein content prior to the exposure of HLM to beads. Interception of the straight line to X-axis represents the stoichiometry of the beads to bind 1.0 mg of HLM. Beads volume on the X-axis refers to the well-mixed beads-aqueous mixture from commercial beads stock. The protein content in each sample was quantified by Bradford assay at 595 nm. Each time point represents a mean of three determinations ± SD.

**Figure 5.** Time course of compound binding to HLM-beads. The progress of diazepam, clozapine and imipramine binding to HLM-beads (1.0 mg/mL) was determined via monitoring the free compound in supernatant by LC/MS/MS. The free compound at each given time point after the initiation of incubation was normalized to the compound content prior the exposure to HLM-beads. Each time point represents a mean of three determination ± SD.
**Figure 6.** Regression and correlational analysis of $f_{u,mic}$ values obtained using HLM-beads and equilibrium dialysis. Panels A), B) and C) represent the analysis at HLM concentrations of 0.025, 0.50 and 1.0 mg/mL. Blue, green and red symbols represent basic, neutral and acid compounds, respectively. Insertions in panels B) and C) are the expansions of the regions of the graphs associated with low $f_{u,mic}$ values. Each value represents the mean ± SD of 3 replicates.
### Table 1. \( f_{u,mic} \) values obtained by HLM-beads and RED methods

<table>
<thead>
<tr>
<th>Compound</th>
<th>HLM (mg/mL)</th>
<th>HLM-beads ( f_{u,mic} )</th>
<th>RED ( f_{u,mic} )</th>
<th>HLM-beads ( f_{u,mic} )</th>
<th>RED ( f_{u,mic} )</th>
<th>HLM-beads ( f_{u,mic} )</th>
<th>RED ( f_{u,mic} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.025</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>Acidic</td>
<td>0.94 (0.09) (^a)</td>
<td>0.98 (0.02)</td>
<td>0.96</td>
<td>0.92 (0.03)</td>
<td>0.93 (0.04)</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Warfarin</td>
<td>1.02 (0.01)</td>
<td>0.97 (0.05)</td>
<td>1.1</td>
<td>1.02 (0.04)</td>
<td>0.94 (0.03)</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Tolbutamide</td>
<td>0.93 (0.07)</td>
<td>0.98 (0.02)</td>
<td>0.96</td>
<td>1.01 (0.04)</td>
<td>0.98 (0.03)</td>
<td>1.0</td>
</tr>
<tr>
<td>Neutral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alprazolam</td>
<td>Neutral</td>
<td>0.81 (0.06)</td>
<td>0.98 (0.14)</td>
<td>0.82</td>
<td>0.78 (0.06)</td>
<td>1.0 (0.1)</td>
<td>0.77</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Neutral</td>
<td>1.0 (0.1)</td>
<td>0.96 (0.01)</td>
<td>1.1</td>
<td>0.82 (0.07)</td>
<td>0.77 (0.02)</td>
<td>1.1</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Neutral</td>
<td>0.24 (0.04)</td>
<td>0.29 (0.07)</td>
<td>0.84</td>
<td>0.10 (0.01)</td>
<td>0.036 (0.005)</td>
<td>2.8</td>
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<tr>
<td>Midazolam</td>
<td>Neutral</td>
<td>1.02 (0.01)</td>
<td>0.98 (0.14)</td>
<td>1.0</td>
<td>0.76 (0.02)</td>
<td>0.67 (0.06)</td>
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<tr>
<td>Basic</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine(^b)</td>
<td>Basic</td>
<td>0.79 (0.13)</td>
<td>0.71 (0.02)</td>
<td>1.1</td>
<td>0.11 (0.02)</td>
<td>0.084 (0.005)</td>
<td>1.4</td>
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<tr>
<td>Clozapine</td>
<td>Basic</td>
<td>0.96 (0.06)</td>
<td>0.87 (0.06)</td>
<td>1.1</td>
<td>0.48 (0.01)</td>
<td>0.42 (0.05)</td>
<td>1.1</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>Basic</td>
<td>0.99 (0.04)</td>
<td>0.96 (0.03)</td>
<td>1.0</td>
<td>1.2 (0.1)</td>
<td>0.77 (0.09)</td>
<td>1.5</td>
</tr>
<tr>
<td>Imipramine(^b)</td>
<td>Basic</td>
<td>0.94 (0.04)</td>
<td>0.94 (0.04)</td>
<td>0.99</td>
<td>0.35 (0.01)</td>
<td>0.40 (0.03)</td>
<td>0.88</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Basic</td>
<td>1.0 (0.1)</td>
<td>1.0 (0.1)</td>
<td>1.0</td>
<td>0.59 (0.04)</td>
<td>0.56 (0.05)</td>
<td>1.1</td>
</tr>
<tr>
<td>BIRT2584(^b)</td>
<td>Basic</td>
<td>0.43 (0.04)</td>
<td>0.42 (0.05)</td>
<td>1.0</td>
<td>0.064 (0.021)</td>
<td>0.024 (0.003)</td>
<td>2.7</td>
</tr>
</tbody>
</table>

\(^a\) Values represent the mean (standard deviation) from triplicate determination; \(^b\) Data obtained using pre-soaked RED membranes.
HLM + Bead = HLM-Beads

Test compound

Magnet

Take supernatant for LC/MS analysis

Remove supernatant

HLM-beads

Remove supernatant

Add test compound

Incubate at 37°C for 5 mins

Take supernatant for LC/MS analysis
Diclofenac
LogP = 4.51
pKa = 4.2

Tolbutamide
LogP = 3.13
pKa = 5.27

Warfarin
LogP = 3.15
pKa = 5.00

Chlorpromazine
LogP = 5.18
pKa = 9.30

Diazepam
LogP = 2.25
pKa = N/A

Diltiazem
LogP = 2.29
pKa = 7.70

Midazolam
LogP = 3.12
pKa = 6.6

Verapamil
LogP = 4.10
pKa = 8.90

BIRT 2584
LogP = 4.30
pKa = 8.2

Acidic

Neutral

Basic
Figure 3
Beads stock solution (μl) vs. Fraction of unbound protein

Figure 4
Figure 5

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Diazpem
Clozapine
Imipramine

Time (min)
Compound free fraction

0.0 0.25 0.50 0.75 1.00
0.0 0.25 0.50 0.75 1.00
0.0 0.25 0.50 0.75 1.00

Diazpem
Clozapine
Imipramine

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A.

The figure shows a set of data points with corresponding compounds. The equation $R^2 = 0.92$ and $r = 0.96$ indicates a strong correlation. The compounds included in the figure are:

- Alprazolam
- Clozapine
- Diazepam
- Diclofenac
- Diltiazem
- Imipramine
- Midazolam
- Tolbutamide
- Verapamil
- Warfarin

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Figure 6

B.

R² = 0.87
r = 0.93
Figure 6

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