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HLM-beads: Rapid Assessment of Nonspecific Binding to Human Liver Microsomes Using Magnetizable Beads[S]

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ABSTRACT

In early drug development, drug-drug interaction risk is routinely assessed using human liver microsomes (HLMs). Nonspecific binding of drugs to HLMs can affect the determination of accurate enzyme parameters (K_m, K_i, K_I). Previously, we described a novel in vitro model consisting of HLMs bound to magnetizable beads [HLM-magnetizable-beads system (HLM-beads)]. The HLM-beads enable rapid separation of HLMs from incubation media by applying a magnetic field. Here, HLM-beads were further characterized and evaluated as a tool to assess HLM nonspecific 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 with 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 10 minutes and compound apparatus adsorption.

SIGNIFICANCE STATEMENT

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

Introduction

The potential for drug-drug interactions (DDIs) 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 (HLMs) are the most widely used in vitro tool for this purpose because of their inclusion of the most relevant drug-metabolizing enzymes at physiologic 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, HLMs 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 the tenet that only free drug is available to 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 HLMs (Obach, 1997; Austin et al., 2002; Hallifax and Houston, 2006). Accounting for nonspecific binding of drugs to HLMs 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 in which $f_{u,mic}$ 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 nonspecific binding of drugs to HLMs is equilibrium dialysis, wherein compounds diffuse across a semipermeable membrane separating a suspension of HLMs 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 HLMs are able to bind to silica-coated magnetizable beads (Horspool et al., 2020). The HLMs could not be released from the beads by multiple washing steps, indicating strong binding. Furthermore, the HLM-magnetizable-beads system (HLM-beads) retained functional drug-metabolizing enzyme activities and could be rapidly removed from the

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ABBREVIATIONS: ACN, acetonitrile; DDI, drug-drug interaction; FA, formic acid; $f_{u,mic}$, free fraction; HLM, human liver microsome; HLM-beads, HLM-magnetizable-beads system; IS, internal standard; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PB, 50 mM of KPO_4 buffer pH 7.4; RED, rapid equilibrium dialysis; RPM, revolutions per minute.

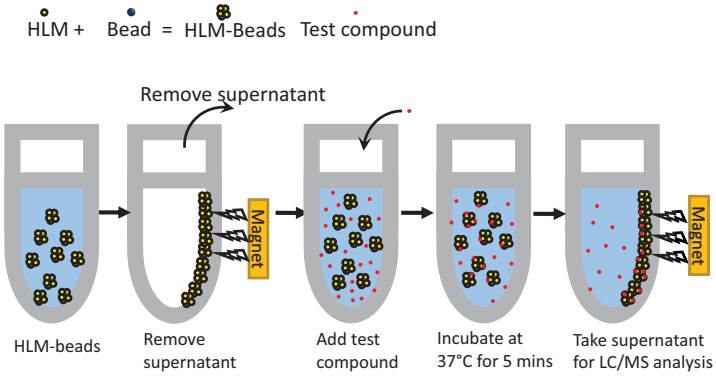

Fig. 1. General procedure for HLM-beads microsomal binding study.

incubation media using a strong magnet. In this work, we have developed a method to assess nonspecific binding of compounds to HLMs 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). Alprazolam, chlorpromazine, diazepam, and midazolam were purchased from Cerilliant Corp. (Round Rock, TX). BIRT2584 was synthesized in-house (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Diclofenac-d_4, (+/−)-verapamil-d_3 and warfarin-d_5 were purchased from CDN Isotopes (Point-Claire, Quebec, Canada). HLMs (lot 38291, mixed-sex 150 donors) were acquired from Corning Inc. (Glendale, AZ). Silica-coated magnetizable beads (501036426) were obtained from G-Biosciences (St. Louis, MO). Coomassie Plus – The Better Bradford Assay Reagent and Pre-Diluted Protein Assay Standards: Bovine Serum Albumin set were purchased from Thermo Fisher Scientific (Waltham, MA). Rapid equilibrium dialysis (RED) device was obtained from Thermo Fisher Scientific Pierce Laboratories (Waltham, MA).

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 Supplemental Table S1, respectively. Diclofenac-d_4, nevirapine, (+/−)-verapamil-d_3, and warfarin-d_5 were used as internal standards (ISs) for liquid chromatography–tandem mass spectrometry (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 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 HLMs (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 track, 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 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

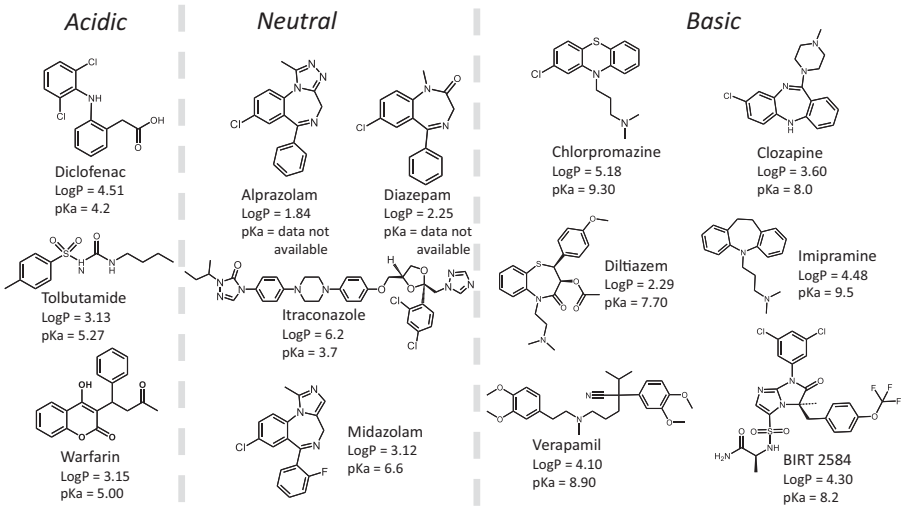

Fig. 2. Structures of the compounds tested.

compared with 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 minutes 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 minutes 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 HLMs 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 HLMs (20 mg/ml, 50 μl) and PB (906 μl). The suspension was gently mixed and incubated at 4°C for 5 minutes. 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 minutes, and then the supernatant was removed. Prewarmed (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 rpm. At 1.0, 2.5, 5.0, 10, 15, and 30 minutes 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 rpm (RPM), 4°C for 10 minutes. The compound solution in the absence of HLM-beads was taken as t_0. 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

$$Compound\ free\ fraction = \frac{[Compound]_t}{[Compound]_{t0}} \quad (1)$$

in which $[Compound]_t$ represents the compound concentration in the supernatant at any given incubation time point, and $[Compound]_{t0}$ 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 HLMs) 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 and then incubated for 5 minutes 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), and this was followed by centrifugation at 3500 RPM at 4°C for 10 minutes. 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.

$$f_{u,mic} = \frac{[Compound]_{+HLM-beads}}{[Compound]_{PB}} \quad (2)$$

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

Microsomal Binding Study Using 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 (molecular weight cutoff, 8 kDa). Test compound (200 μl) and HLMs (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 6 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 HLMs (free of test compound) to the receiver samples. An aliquot of each sample was diluted 4-fold with ACN (0.1% FA, IS), which was followed by centrifugation at 3500 RPM at 4°C for 10 minutes. The test compounds remaining in the supernatant were then analyzed by LC-MS/MS. The $f_{u,mic}$ was calculated according to eq. 3

$$f_{u,mic} = \frac{[Compound]_{receiver}}{[Compound]_{donor}} \quad (3)$$

in which $[Compound]_{donor}$ and $[Compound]_{receiver}$ 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 presoaked 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 preincubated with the test compound in PB for 30 minutes. 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 high-performance liquid chromatography using a Waters Acquity or an Agilent 1290 series ultrahigh performance pump. A Waters Acquity UPLC BEH, C18, 1.7 μm, 2.1 × 50 mm, 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 multiple reaction monitoring transitions of the analytes are compiled in Supplemental 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. When 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-minute incubation time was used to ensure that the binding of HLMs 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 minute 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 minutes. Incorporation of more automated techniques could reduce the incubation time even further.

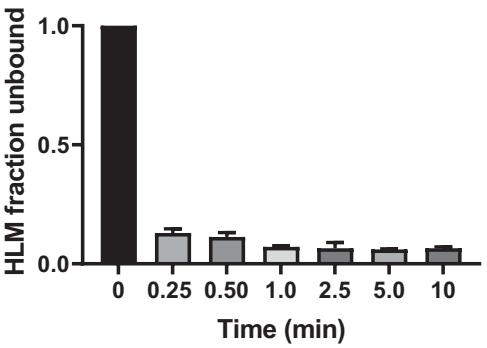

Fig. 3. Time course of HLM binding to beads. The progress of HLM binding to beads was monitored by through determining the amount of protein remaining in the supernatant. The free protein content in the supernatant at each given incubation time point was normalized to the protein content prior to the exposure of HLMs 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 ± S.D.

To determine the HLM-binding capacity of the beads, washed beads were titrated into HLMs (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 HLMs (without beads), Fig. 4. It was determined that 185 ± 13 μl of commercially supplied bead suspension is capable of binding 1.0 mg of HLMs.

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

Time Course of Test Compound Binding to HLM-beads. The time required for compound to reach binding equilibrium to HLMs (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 minutes after the initiation of the incubation. A 5-minute incubation time was then used in subsequent experiments to ensure that the of binding equilibrium achieved.

Microsomal Binding Study Using HLM-beads and 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 Table 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 BIRT2584 and itraconazole are exceptions in that their $f_{u,mic}$ values are up to 2.9-fold higher using HLM-beads versus RED because of their extensive adsorption onto the RED device.

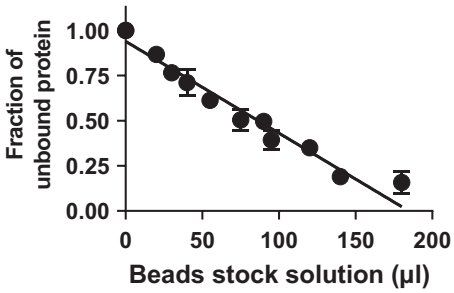

Fig. 4. Capacity of beads to capture HLMs. The stoichiometry of HLM binding to beads was monitored by determining the amount of protein remaining in the supernatant at each addition of beads. The free protein content in the supernatant at each addition of beads was normalized to the protein content prior to the exposure of HLMs to beads. Interception of the straight line to *x*-axis represents the stoichiometry of the beads to bind 1.0 mg of HLMs. 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 ± S.D.

For these three compounds, the microsomal binding studies using RED were conducted with and without presoaking the apparatus with 1.0 μM of compound. A comparison of the $f_{u,mic}$ values obtained with and without presoaking are listed in Supplemental 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 BIRT2584 when the dialysis membrane of RED device was not presoaked and presoaked with the test compound.

Discussion

HLMs 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 HLMs 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 HLMs to beads is a fast event, reaching completion within 1 minute after combining HLMs 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 HLMs (Fig. 4). The capacity of the beads to capture HLMs 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 HLMs.

Nonspecific 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 (K_m), reversible inhibition constant (K_i), or inactivation constant (K_I) (Obach, 1997; Tran et al., 2002; Venkatakrishnan et al., 2003). Several different methods to measure the nonspecific binding of compounds to HLMs have been described that use principles of centrifugation or filtration (Niwa et al., 2005; Brown et al., 2006; Ballard and Rowland, 2011). However, equilibrium dialysis remains the most commonly used method to determine the $f_{u,mic}$ values (Kurz et al., 1977; Vuignier et al., 2010). Although greater efficiency of determining $f_{u,mic}$ values using equilibrium dialysis has 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 HLMs, diazepam, clozapine, and imipramine were chosen to evaluate the time required for compounds to bind to HLMs on HLM-beads. As shown in Fig. 5, binding of these compounds to HLMs reached completion within 5 minutes regardless of the compound tested. Thus, compared with 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 10 minutes as opposed to at least several hours for the previously mentioned methods.

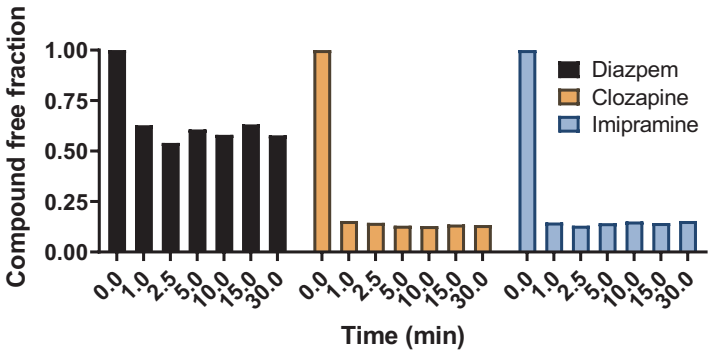

Fig. 5. Time course of compound binding to HLM-beads. The progress of diazepam, clozapine, and imipramine binding to HLM-beads (1.0 mg/ml) was determined via monitoring the free compound in supernatant by LC-MS/MS. The free compound at each given time point after the initiation of incubation was normalized to the compound content prior the exposure to HLM-beads. Each time point represents a mean of three determination ± S.D.

To determine whether HLM-beads can be used to assess microsomal binding of small molecules, the $f_{u,mic}$ 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 physiologic pH. The structure and the pKa, LogP, and $LogD_{7.4}$ values of the compounds are listed in Fig. 2 and Supplemental Table S1, respectively. Previous studies have highlighted the relative propensity of basic compounds to bind nonspecifically to HLMs 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 HLMs demonstrated a high concordance of $f_{u,mic}$ values between methods for compounds with $LogD_{7.4}$ 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 nonspecific 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 ($cLogD_{7.4}$ = 7.31) (Treyer et al., 2019) and BIRT2584 ($cLogD_{7.4}$ = 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 HLMs.

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 nonspecific adsorption of compound to the RED apparatus on $f_{u,mic}$ values (see Supplemental Information for detailed discussion). Presoaking the RED device with test compound reduced the ratios of $f_{u,mic}$ obtained by HLM-beads and RED of chlorpromazine, itraconazole, and BIRT 2584 down to 1.3, 2.9, and 1.9 folds compared with non-soak, which were 5.1, 26, and 7.6 folds. As the data shows, that $f_{u,mic}$ values obtained for these three high binders have a better agreement between the HLM-beads and RED device with reduced apparatus nonspecific binding (i.e., presoak). The results indicate that the HLM-beads have much lower extent of apparatus adsorption and thus could provide more accurate $f_{u,mic}$ 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 (unpublished data). Furthermore, we previously showed that phospholipids can block HLMs 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 HLMs would have the same effect of blocking other compounds from binding to the beads. Further investigations on the nonspecific 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 HLMs 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 $f_{u,mic}$ (e.g., silanization of glass tubes). Since HLM-beads enable the quick separation of HLMs from solvent, the sample analysis process has been simplified compared with RED. Procedures, such as developing separated bioanalytical methods required for RED to assess donor or recipient compartments or preparing crossmatrix normalization of samples and having to account for differences in bioanalytical matrix-dependent sensitivity due to different concentrations of HLMs, are no longer required, which reduces the effort associated with downstream liquid chromatography–mass spectrometry 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 depend 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 nonmembrane 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 that utilizes binding of plasma proteins to nonmagnetizable beads (Schuhmacher et al., 2004). We do not think that it would be difficult to adapt such a product into a magnetizable bead format.

TABLE 1

$f_{u,mic}$ values obtained by HLM-beads and RED methods

	HLMs (mg/ml)	0.025			0.50			1.0		
		$f_{u,mic}$			$f_{u,mic}$			$f_{u,mic}$		
	Compound	HLM-beads	RED		HLM-beads	RED		HLM-beads	RED	
Acidic	Diclofenac	0.94 (0.09)[a]	0.98 (0.02)	0.96	0.92 (0.03)	0.93 (0.04)	0.99	0.87 (0.17)	0.83 (0.08)	1.0
	Warfarin	1.02 (0.01)	0.97 (0.05)	1.1	1.02 (0.04)	0.94 (0.03)	1.1	0.97 (0.03)	0.86 (0.08)	1.1
	Tolbutamide	0.93 (0.07)	0.98 (0.02)	0.96	1.01 (0.04)	0.98 (0.03)	1.0	0.92 (0.04)	0.97 (0.03)	0.95
Neutral	Alprazolam	0.81 (0.06)	0.98 (0.14)	0.82	0.78 (0.06)	1.0 (0.1)	0.77	0.68 (0.07)	1.0 (0.1)	0.66
	Diazepam	1.0 (0.1)	0.96 (0.01)	1.1	0.82 (0.07)	0.77 (0.02)	1.1	0.66 (0.03)	0.64 (0.02)	1.0
	Itraconazole	0.24 (0.04)	0.29 (0.07)	0.84	0.10 (0.01)	0.036 (0.005)	2.8	0.073 (0.004)	0.025 (0.001)	2.9
	Midazolam	1.02 (0.01)	0.98 (0.14)	1.0	0.76 (0.02)	0.67 (0.06)	1.1	0.53 (0.02)	0.52 (0.08)	1.0
Basic	Chlorpromazine[b]	0.79 (0.13)	0.71 (0.02)	1.1	0.11 (0.02)	0.084 (0.005)	1.4	0.056 (0.006)	0.0437 (0.0003)	1.3
	Clozapine	0.96 (0.06)	0.87 (0.06)	1.1	0.48 (0.01)	0.42 (0.05	1.1	0.26 (0.02)	0.30 (0.02)	0.85
	Diltiazem	0.99 (0.04)	0.96 (0.03)	1.0	1.2 (0.1)	0.77 (0.09)	1.5	0.97 (0.03)	0.76 (0.12)	1.3
	Imipramine[b]	0.94 (0.04)	0.94 (0.04)	0.99	0.35 (0.01)	0.40 (0.03)	0.88	0.19 (0.01)	0.26 (0.01)	0.71
	Verapamil	1.0 (0.1)	1.0 (0.1)	1.0	0.59 (0.04)	0.56 (0.05)	1.1	0.32 (0.03)	0.43 (0.06)	0.74
	BIRT2584[b]	0.43 (0.04)	0.42 (0.05)	1.0	0.064 (0.021)	0.024 (0.003)	2.7	0.035 (0.004)	0.019 (0.002)	1.9

[a]Values represent the mean (standard deviation) from triplicate determination.
[b]Data obtained using presoaked RED membranes.

Fig. 6. Regression and correlational analysis of $f_{u,mic}$ values obtained using HLM-beads and equilibrium dialysis. (A–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 (B and C) are the expansions of the regions of the graphs associated with low $f_{u,mic}$ values. Each value represents the mean ± S.D. of three replicates.

In summary, we have demonstrated that HLM-beads can be used to assess the nonspecific 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 nonspecific binding to other subcellular fractions from other sources, such as animals, plants, and cellular systems.

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