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DIRECT DETERMINATION OF UNBOUND INTRINSIC DRUG CLEARANCE IN THE MICROSOMAL STABILITY ASSAY

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Running title: Direct determination of unbound intrinsic clearance

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List of non-standard abbreviations used in the paper:

CL_{in}: *In Vitro* intrinsic clearance
$CL_{in,u}$: intrinsic clearance of unbound drug
f_u: unbound fraction
HLM: human liver microsomes
LESA: Linear Extrapolation in the Stability Assay
LC-MS/MS: liquid Chromatography tandem mass spectrometry

ABSTRACT:

The microsomal stability assay is commonly used to rank compounds according to their metabolic stability. Determination of the unbound intrinsic clearance ($CL_{in,u}$) is essential for the accurate comparison of compounds, as nonspecific binding to microsomes can lead to an underestimation of the microsomal clearance. In this study, a new method (Linear Extrapolation in the Stability Assay, LESA) was established, which allows direct calculation of $CL_{in,u}$ from microsomal stability data, without the need to independently determine the fraction of free (unbound) drug. The method was validated using nine drugs with different chemical structures and physicochemical properties. The $CL_{in,u}$ of these compounds was extrapolated from the intrinsic clearance values obtained at different concentrations of human liver microsomes and compared to that calculated by the conventional method, using microsomal intrinsic clearance values and the free fraction of drug determined by equilibrium dialysis, ultracentrifugation or ultrafiltration. A good agreement was observed between the data generated by the LESA method vs. those determined by conventional procedures. The method was further evaluated using a published dataset for 10 additional drugs and found to yield intrinsic clearance data comparable to the previously reported values. LESA provides a convenient and rapid method to determine the influence of microsome binding on intrinsic clearance in a single assay.

Introduction

During the drug discovery process, *in vitro* drug metabolism data are widely used in the pharmaceutical industry as criteria to select new chemical entities for further development (Rodrigues, 1997). An important parameter, which is used to rank compounds on the basis of their metabolic stability, is the intrinsic clearance (CL_{in}), determined using hepatic microsomes (Obach, 1999; McGinnity and Riley, 2001).The metabolite formation method has been used for measurement of *in vitro* CL_{in} (Madan et al., 2002; Jones and Houston, 2004). Here, the initial rate of metabolite production is measured using hepatic microsomes over a range of substrate concentrations under linear conditions with respect to protein concentration and time (Houston and Galetin, 2003). Alternatively, the substrate depletion approach has been adopted, where the consumption of the parent drug is monitored over time (Obach, 1999). This method is particularly popular in the pharmaceutical industry, as formal kinetic characterization of the enzymes involved and quantification of metabolites formed are not required, allowing rapid screening of compounds with automated and semi-automated methodologies. Normally at least 20% of the substrate must be metabolized within the incubation period, so that any substrate depletion can be distinguished from baseline variability (Jones and Houston, 2004). For this reason higher microsome concentrations and longer incubation times are used than in studies utilizing the metabolite formation approach.

Many drugs are lipophilic organic compounds that can bind non-specifically to the lipid-protein milieu of the microsomal membrane. The result of nonspecific binding is a reduction in the free concentration of drug that is available for interaction with

microsomal drug metabolizing enzymes. Depletion of unbound drug by extensive membrane partitioning leads to an underestimation of CL_{in}. The "true" CL_{in}, i.e. the value that would be observed in the absence of binding to microsomes, is termed unbound intrinsic clearance ($CL_{in,u}$). Unbound intrinsic clearance can be calculated by determining the free fraction of compound in microsomal incubations (f_u) according to the relationship:

$CL_{in,u} = CL_{in}/f_u$ equation 1

Three different experimental methods are commonly used to determine f_u and consequently $CL_{in,u}$, namely equilibrium dialysis, ultracentrifugation and ultrafiltration. Of these, equilibrium dialysis is the most widely used method to determine f_u as it is experimentally easy and can be performed in a 96 well format (Kariv et al., 2001). In all three methodologies there is the possibility that nonspecific drug adsorption to equipment surfaces (dialysis membrane, ultrafiltration device, etc.) may distort the values obtained, leading to an underestimation of the $CL_{in,u}$ (Lin et al., 1987). In addition, these methods are relatively laborious and time-consuming. The aim of the present work was to establish a methodology for the direct determination of $CL_{in,u}$, without the need for separate measurement of f_u. The new method is based on the assumption that compounds bind to or partition into microsomes in a nonspecific fashion, i.e. with low affinity, and that binding sites are not saturated at the concentrations used in microsomal stability assays. Under these conditions, the $CL_{in,u}$ and f_u can be directly extrapolated from the microsomal stability data obtained at different microsome concentrations. The method was validated using a series of structurally diverse compounds that are subject to

oxidative metabolism and are known to exhibit significant nonspecific binding to hepatic microsomes (Obach, 1999).

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Materials and Methods

Materials. All chemicals were obtained from Sigma Aldrich (Milan, Italy). Stock solutions of all compounds were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. From these, working solutions containing 200 µM of compound were prepared in 50 % methanol. The internal standard used in all LC-MS/MS analyses was a proprietary compound. Solvents and other reagents were from common sources and of HPLC grade or higher. Human liver microsomes (HLM Pool, Lot 24) were purchased from Gentest (Woburn, MA).

Microsomal Incubations. All incubations were conducted in quadruplicate. The incubation mixtures were prepared in 96 well cluster tubes (1.2 ml, Corning Life Sciences, Acton, MA) and contained 1 µM test compound, HLM (0.1 – 2 mg microsomal protein/ml), 3 mM $MgCl_2$, and 25 mM potassium phosphate buffer pH 7.4, in a final volume of 1 ml. Reactions were initiated by the addition of NADPH (final concentration 1 mM) and kept in a shaking water bath at 37° C. Reactions were terminated by adding 100 µl of the incubation mixture to 100 µl of acetonitrile/0.1 % formic acid containing 1 µM of internal standard. Immediately after the addition of NADPH the sampling point for t = 0 min was taken, and further sampling points were taken at 5, 10, 30, 60 and 90 minutes. For incubations with the rapidly metabolized compounds diclofenac and midazolam, samples were taken at 0, 2, 4, 6, 8, 10, 15, 30 and 60 minutes. The samples were centrifuged for 10 minutes at 4000 x g to pellet precipitated microsomal protein and

the supernatant subjected to LC/MS-MS analysis without further treatment. CL_{in} was calculated according to:

$$CL_{in} = \frac{Dose}{AUC_{\infty}} \qquad \text{equation 2}$$

where Dose is the initial amount of drug in the incubation mixture (unit of moles/mg microsomal protein), and AUC_{∞} is the area under the concentration vs. time curve, extrapolated to infinity (unit of M * h). The unit for CL_{in} is L/h/mg protein. For all compounds tested, turnover was greater than 20%/hour at the lowest concentration of microsomes. All CL_{in} were also calculated using the half-life derived from fitting of the concentration time course data to a first order kinetic model. No significant differences in CL_{in} were observed between the 2 calculation methods (data not shown).

Equilibrium dialysis. Dialysis mixtures contained 1 µM of test compound, HLM (0.2 and 1 mg/ml), 3 mM $MgCl_2$ and 25 mM potassium phosphate buffer pH 7.4, in a final volume of 200 µl. Control mixtures did not contain microsomal proteins. Triplicate mixtures were subjected to equilibrium dialysis against 200 µl of phosphate -$MgCl_2$ buffer using a 96 well DispoDialyzer (Harvard Apparatus, Holliston, MA). The dialyzing unit consists of two chambers separated by an ultra-thin membrane with a molecular weight cut-off of 10 kDa. The plate was rotated for 12 h at 37° C in the perpendicular direction of the well orientation to ensure a constant contact between the two chambers, using a plate rotator (Harvard Apparatus, Holliston, MA). The solvent volumes in the 2 chambers did not change significantly during the course of the experiment. Upon completion of the dialysis, 100 µl of the samples from the microsome and buffer sides

were processed as outlined for the metabolic stability samples and analyzed by LC/MS-MS. Recovery was found to be between 75 % and 100% for all compounds, with the exception of chlorpromazine where recovery was 61 %. The free fraction was calculated according to equation 3:

f_u [%] = 100 x C_b / C_m equation 3

where C_b and C_m denote the concentrations of compound in the dialysis chambers containing buffer and microsomes, respectively.

Ultrafiltration. Mixtures were prepared as outlined for the equilibrium dialysis method. Aliquots of 200 µl were subjected to ultrafiltration using Centrifree® filter devices (Millipore, Bedford, MA). The assembled filter unit was centrifuged for 1 h at 863 x g at 37° C. Upon completion of the filtration, 100 µl of ultrafiltrate were processed as outlined for the microsomal stability samples and analyzed by LC/MS-MS. Recovery was determined by analysis of filtered control samples prepared in the absence of microsomes and was found to be between 70 % and 100% for all compounds, with the exception of chlorpromazine where recovery was 17 %. Results were expressed as the concentration ratio of sample vs. control samples:

f_u [%] = 100 * C_{sample} / $C_{control}$ equation 4

Ultracentrifugation. Mixtures were prepared as outlined for the equilibrium dialysis method. Aliquots of 200 µl were placed in polycarbonate centrifuge tubes (8 x 34 mm, Beckman, Palo Alto, CA) and centrifuged for 3 h at 356000 x g at 37° C (Optima TL ultracentrifuge, Beckman). One hundred µl of the resulting supernatant were processed as

outlined for the metabolic stability samples and analyzed by LC/MS-MS. Recovery was determined by analysis of centrifuged control samples prepared in the absence of microsomes and was found to be betweeen 75 % and 100% for all compounds. Results were expressed as the concentration ratio of sample vs. control samples:

f_u [%] = 100 * C_{sample} / $C_{control}$ equation 5

LC/MS-MS Analysis. The LC/MS-MS system consisted of a Agilent 1100 series gradient HPLC pump (Agilent, Palo Alto, CA), a CTC HTS PAL Autosampler (CTC Analytics, Zwingen, Switzerland) and an Applied Biosystems/PE Sciex API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a turbo ionspray interface. Analytes in incubation mixtures were separated by reverse phase HPLC using an Ace Act RP C18 50 X 4.6 mm column (Mac Mod Analitica Inc., Chadds Ford, PA) A generic gradient elution program was used at a flow rate of 2 ml/min with a mobile phase of acetonitrile/0.1 % formic acid (10 % v/v) in water/0.1 % formic acid for 0.2 min, after which time the acetonitrile concentration was increased to 90 % over 1.7 min before restoring it back to 10 % for the remaining 0.7 min. The injection volume was 20 µl. Approximately 10 % of the eluent was introduced into the mass spectrometer source. The source temperature of the mass spectrometer was maintained at 450° C and other source parameters (e.g. collision energy, declustering potential, curtain gas pressure etc.) were individually optimized for each compound. The most prominent fragment of the molecular ion ($M + H^+$) was followed for each compound and the internal standard in the multiple reaction monitoring mode. Quantitation of each compound was achieved by

comparison of the analyte/internal standard peak area ratios to those of a calibration curve ranging from 0.01 µM to 2 µM.

LESA Model. Two models for drug binding to microsomes have been proposed. The first model (McLure et al., 2000) assumes saturable association of drug to defined microsomal binding sites, according to the relationship:

$$B = \frac{F \times B_{max}}{K_D + F} \qquad \text{equation 6}$$

where B and F are the concentrations of bound and free drug, respectively, B_{max} the concentration of binding sites, and K_D the equilibrium binding constant.

The second model (Austin et al., 2002) treats microsomal binding as a non-saturable phase equilibrium process governed by a membrane partition coefficient K_P:

$$K_p = \frac{B}{F} \qquad \text{equation 7}$$

Mathematically, this model is equivalent to the particular case of the defined binding site model where binding is non saturable, i.e. $F << K_D$.

In this case, $B = (B_{max}/K_D) \times F$, with $K_P = B_{max}/K_D$.

It should be noted that the membrane partition coefficient K_P defined in this way is directly proportional to the total number of membrane binding sites and subsequently to the total membrane protein concentration M, i.e.

$$K_p = K' \times M \qquad \text{equation 8}$$

with K' denoting the proportionality constant.

As pointed out by Austin and coworkers (Austin et al., 1995), microsomal binding is normally independent of compound concentration, and saturation does not occur at the

low micromolar concentrations used in microsomal stability assays. It is therefore appropriate to use equation 7 to describe this process.

The free fraction of drug, f_u, is given by:

$$f_u = \frac{F}{B+F} \qquad \text{equation 9}$$

Substituting equations 8 and 9 into equation 7 and rearranging, we obtain:

$$f_u = \frac{1}{1+K'\times M} \qquad \text{equation 10}$$

If intrinsic clearance is determined in the presence of drug binding to microsomes, the relationship between the observed clearance CL_{in} and the "true" clearance of unbound drug, $CL_{in,u}$ is calculated according to equation 1:

$$CL_{in} = f_u \times CL_{in,u} \qquad \text{(equation 1)}$$

Substituting equation 10 into equation 1 and rearranging, we obtain:

$$\frac{1}{CL_{in}} = \frac{1}{CL_{in,u}} + \frac{K'}{CL_{in,u}} \times M \qquad \text{equation 11}$$

According to equation 11, plotting the reciprocal of CL_{in} against the microsome concentration M should result in a straight line intersecting the y axis at $1/CL_{in,u}$. $CL_{in,u}$ can thus be calculated without independently determining f_u. Values of $CL_{in,u}$ obtained using this method were compared to those calculated using equation 8, with f_u values determined by dialysis, ultrafiltration or ultracentrifugation.

Statistical Analysis. Linear regression analysis and associated standard errors were determined using Sigma Plot 9.0 (Systat Software Inc., Chicago, IL).

Results

The LESA model (described in Materials and Methods) was applied to calculate the $CL_{in,u}$ of nine drugs. The *in vitro* CL_{in} of chlorpromazine, desipramine, amitriptyline, imipramine, verapamil, diltiazem, propafenone, midazolam and diclofenac was determined at five different concentrations of pooled HLM, ranging from 0.1 to 2 mg/ml. A typical concentration-time curve is reported in Figure 1 for NADPH-dependent desipramine consumption in HLM. According to the LESA model, when $1/CL_{in}$ is plotted against the concentration of HLM, a straight line intersecting the y-axis at $1/CL_{in,u}$ should be obtained (see equation 11). As shown in Figure 2 for desipramine, $1/CL_{in}$ was directly proportional to the concentration of HLM. Similar linear plots were obtained for the other eight compounds investigated, with correlation coefficients (r^2) ranging from 0.88 to 0.99. Table 1 summarizes the CL_{in}, the statistics of the linear correlations as well as the values of $CL_{in,u}$ extrapolated from the data.

In order to investigate whether the results obtained with the LESA method reflected the true $CL_{in,u}$, the unbound fractions (f_u) of the nine drugs investigated were determined by equilibrium dialysis, ultracentrifugation and ultrafiltration at two different microsome concentrations, 0.2 mg/ml and 1 mg/ml (Table 2). For chlorpromazine, f_u could not be determined by ultrafiltration, since the compound displayed very low mass balance in this system (see Materials and Methods). $CL_{in,u}$ was then calculated by the conventional method using equation 1 ($CL_{in,u} = CL_{in}/f_u$, Table 3). For all the nine drugs the results for $CL_{in,u}$ obtained by direct measurement with the LESA method were in

good agreement with those obtained with the other three (or two in the case of chlorpromazine) methodologies (Table 3).

In Fig. 3, $CL_{in,u}$ obtained by the LESA method is compared for the nine compounds investigated with that determined by direct determination of f_u, using the average value from equilibrium dialysis, ultracentrifugation and ultrafiltration. It should be noted that the nine compounds differ in their structures, physico-chemical properties and $CL_{in,u}$. Furthermore they have greatly differing degrees of nonspecific binding to microsomes with f_u ranging from 25 to near 100 %. The correlation obtained was excellent at both microsome concentrations, with $r^2 = 0.92$ and $r^2 = 0.96$ for 1 mg/ml and 0.2 mg/ml of microsomal protein, respectively.

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Discussion

The determination of $CL_{in,u}$ is essential for an accurate comparison of the metabolic stability of compounds, as nonspecific binding to microsomes can introduce an error, leading to underestimation of the microsomal clearance (Obach, 1997; Austin et al., 2002; Jones and Houston, 2004). Furthermore, knowledge of $CL_{in,u}$ is necessary for an accurate prediction of human pharmacokinetic parameters from *in vitro* results (Obach et al., 1997). The aim of this work was to establish a new methodology for the direct determination of the $CL_{in,u}$, by extrapolation from in vitro metabolic stability studies performed with varying amounts of microsomal protein.

The methodologies most frequently used (equilibrium dialysis, ultrafiltration and ultracentrifugation) determine $CL_{in,u}$ indirectly via measurement of f_u (eq. 1). Equilibrium dialysis (Lin et al., 1987) is technically simple, a variety of apparatus are commercially available, and using 96 well plates it is possible to determine the f_u of several compounds in a single experiment. However, equilibrium time can be long, and unstable drugs or proteins may degrade during long equilibration times. Drug adsorption to the dialysis membrane or dialysis device tends to be greater than drug adsorption to ultracentrifugation tubes, and recovery of the parent compound is not always quantitative. Another problem that can increase the error in the measurement of f_u by equilibrium dialysis is the potential for volume shift due to the Donnan effect (Lin et al., 1987). However, the methodology is widely applied and yields satisfactory results if appropriate controls are included.

Ultrafiltration is faster than equilibrium dialysis, but an increased protein concentration during filtration, as well as a potential decrease in the filter pore size due to protein accumulation may cause errors in the measurement of f_u. Ultracentrifugation is not affected by membrane or Donnan effects. However, the technique is of low throughput and potentially subject to artifacts due to surface adsorption and variation of the protein concentration during centrifugation.

All the drugs selected for the present study are mainly subject to hepatic oxidative metabolism (Obach, 1999). Desipramine, amitriptyline, imipramine, verapamil, diltiazem, propafenone, chlorpromazine are basic compounds, midazolam is neutral and diclofenac is acidic. Seven basic compounds were selected because compounds with a $pK_a > 7.4$ generally show greater nonspecific binding than neutral and acidic compounds (Austin et al., 1995). This is expected because basic compounds exhibit enhanced affinity for membrane phospholipids, as demonstrated by liposome binding studies (Austin et al., 1995; Kramer et al., 1998). Furthermore, all of the drugs used were reported to display appreciable binding to hepatic microsomes (Obach, 1999). The substrate depletion approach was used because formal kinetic characterization and metabolite quantification are not required. The CL_{in} was calculated as Dose/AUC_∞ rather than with the more rigorous approach that uses enzyme kinetic data (i.e., maximum enzyme velocity V_{max} and Michaelis-Menten constant K_M). This simplified approach is appropriate, since the substrate concentration employed (1 μM) is below the apparent K_M for substrate turnover and no significant product inhibition, or mechanism-based inactivation of the enzyme is present (Obach, 1999). All the drugs selected were metabolized in HLM with $CL_{in,u}$ ranging between 38 μl/min/mg for diltiazem and 344 μl/min/mg for diclofenac (Table 1).

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In spite of the experimental issues associated with the traditionally used techniques, the results were in good agreement within the three methods and in comparison with LESA. In addition, the standard errors associated with each method were in the same range for the four methodologies As shown in Table 3, the values of $CL_{in,u}$ obtained for the different compounds using either experimental determination of f_u or the LESA method are comparable.

Austin et al. (Austin et al., 2002) measured the CL_{in} and f_u for 13 drugs at three different concentrations of rat liver microsomal protein, 0.25, 1 and 4 mg/ml and determined the $CL_{in,u}$ from these data. This set of 13 compounds includes five neutral, four acidic and four basic drugs covering a wide range of lipophilicity. We applied the LESA method to calculate $CL_{in,u}$ from the reported values of CL_{in} (Austin et al., 2002). The LESA method could not be applied to isradipine, as only two experimental CL_{in} values were reported (Austin et al., 2002). As shown in Table 4, there was a generally good agreement between $CL_{in,u}$ extrapolated by LESA and that calculated using the experimentally measured f_u values (Austin et al., 2002). For 10 out of the 12 compounds analyzed, the difference between the results obtained with the 2 methods was less than 2-fold. The two outliers were amiodarone and astemizole. Both compounds were reported to bind extensively to microsomes even at the lowest concentration tested (0.25 mg/ml), with f_u of 0.006 and 0.076 respectively (Austin et al., 2002), which may introduce a significant error in the calculation of $CL_{in,u}$ by either method. Since $CL_{in,u}$ is the ratio between CL_{in} and f_u, compounds with high CL_{in} and very low f_u and will yield very high estimates of $CL_{in,u}$ (16000 and 10000 μl/min/mg for amiodarone and astemizole respectively), associated with an amplified statistical error. Obviously, the reciprocal

value 1/ $CL_{in,u}$ will be close to zero, posing a practical limit to the applicability of the LESA method. Thus, for amiodarone the extrapolation yielded a negative intercept, which has no physical meaning. On the other hand, extrapolation of the data for astemizole yielded a significantly lower $CL_{in,u}$ than that calculated by the conventional method (Austin et al., 2002), raising the possibility that the latter was biased by an underestimation of f_u for that compound. Further studies will be needed to clarify this point. Excluding amiodarone, astemizole and isradipine from the comparison, a good correlation was obtained between $CL_{in,u}$ values calculated with LESA vs. the conventional method, with a linear regression coefficient (r^2) of 0.96.

The main limitation of LESA is due to the fact that it is utilizing the substrate depletion approach. For this reason, the $CL_{in,u}$ can only be calculated with sufficient accuracy in the case of appreciable turnover of the substrate (at least 20%) (Jones and Houston, 2004). On the other hand, the $CL_{in,u}$ in LESA is extrapolated linearly from a range of CL_{in}'s obtained at different microsome concentrations. This increases the confidence in the experimental data. Notably, in the other three methodologies, $CL_{in,u}$ is usually obtained from the f_u at a single microsome concentration. Another potential limitation of the LESA method is that it is based on the assumption that drug binding to microsomes is truly nonspecific, i.e. of low affinity. The method would not be valid for compounds whose binding is saturated at the concentrations used in the microsomal stability assay. However, as discussed by Austin et al. (Austin et al., 2002), this is unlikely to occur at the low micromolar concentrations used in modern metabolic assays. Notwithstanding these potential limitations, the LESA method provides a convenient and rapid method to determine the influence of microsome binding on intrinsic clearance,

without the need for separate determination of the unbound fraction. The method should be particularly useful in cases where the unbound fraction cannot be determined by conventional methods due to technical limitations such as nonspecific adsorption to dialysis apparatus or compound solubility (Walsky et al., 2005). It may also be applicable also to studies of kinetic parameters of drug interaction with microsomal enzymes (e.g. cytochrome P450 inhibition) (Margolis and Obach, 2003; Walsky et al., 2005) and to other *in vitro* systems, such as hepatocytes, where clearance can be influenced by cellular accumulation (Jones and Houston, 2004)

In summary, LESA was shown to accurately determine the $CL_{in,u}$ in the microsomal stability assay by comparison with three traditionally used methods. Furthermore, LESA could be applicable to investigate the influence of nonspecific binding of drugs to protein or lipids in enzyme inhibition/induction studies (Tran et al., 2002).

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Footnotes

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Figure legends

FIG. 1. *Typical depletion profile for desipramine in pooled HLM.*

The compound (1 μM) was incubated in the presence of NADPH with the indicated concentrations of HLM. Each point represents the mean ± SD of triplicate determinations.

FIG. 2. *Linear correlation between $1/CL_{in}$ and HLM concentration for desipramine.*

Data were fitted by linear regression ($y = 0.0931x + 0.0244$, $r^2 = 0.98$). The y axis intercept corresponds to $1/CL_{in,u}$ (equation 11). Each point represents the mean ± S.D. of quadruplicate determinations.

FIG. 3. *Correlation between $CL_{in,u}$ values for 9 drugs obtained by LESA vs. that calculated using experimentally determined values of f_u.*

The value of $CL_{in,u}$ used was the average of the values determined by equilibrium dialysis, ultrafiltration and ultracentrifugation at two different concentrations of HLM, 1 mg/ml (plot A, $r^2 = 0.92$) and 0.2 mg/ml (plot B, $r^2 = 0.96$). Standard errors of $CL_{in,u}$ determined by LESA were derived from those of $1/CL_{in,u}$ as calculated by the curve fitting software.

FIG. 4. *Correlation between published $CL_{in,u}$ values with those extrapolated by LESA*

Data are from Table 4. Each points represents the mean ± SE of triplicate determinations.

TABLE 1

Microsomal CL_{in} determined at different HLM concentrations and extrapolation of $CL_{in,u}$

Compounds (1 μM) were incubated in the presence of NADPH with 5 different concentrations of pooled HLM (0.1, 0.2, 0.5, 1, 2 mg/ml) and intrinsic clearance values (mean ± S.D., n = 4) were obtained as described in Materials and Methods. Data were fitted by linear regression analysis according to equation 11 and $1/CL_{in,u}$ was calculated by extrapolation to zero microsome concentration. r, correlation coefficient of the linear fit. Standard errors of $CL_{in,u}$ were derived from those of $1/CL_{in,u}$ as calculated by the curve fitting software.

Drug	Cl_{in}					Linear Regression	r^2	$CL_{in.u}$
	(2 mg/ml)	(1 mg/ml)	(0.5 mg/ml)	(0.2 mg/ml)	(0.1 mg/ml)			
			μl/min/mg					μl/min/mg
Desipramine	5 ± 1	10 ± 1	14 ± 1	21 ± 2	29 ± 1	y = 0.0931x + 0.0244	0.98	41 ± 12
Amitryptiline	17 ± 1	34 ± 3	48 ± 2	71 ± 5	76 ± 3	y = 0.0238x + 0.0092	0.99	108 ± 16
Imipramine	14 ± 2	25 ± 3	32 ± 3	44 ± 3	59 ± 2	y = 0.0271x + 0.0167	0.99	59 ± 6
Verapamil	73 ± 2	118 ± 9	153 ± 4	185 ± 3	187 ± 6	y = 0.0045x + 0.0045	0.99	222 ± 15
Diltiazem	18 ± 2	31 ± 2	28 ± 2	33 ± 1	36 ± 2	y = 0.0133x + 0.0262	0.88	38 ± 4
Propafenone	70 ± 4	87 ± 3	132 ± 4	168 ± 7	172 ± 1	y = 0.0054x + 0.0047	0.96	185 ± 21
Chlorpromazine	27 ± 1	52 ± 1	84 ± 1	113 ± 3	128 ± 3	y = 0.0154x + 0.0052	0.99	191 ± 33
Midazolam	196 ± 9	238 ± 6	287 ± 9	319 ± 22	318 ± 21	y = 0.0011x + 0.0030	0.99	333 ± 11
Diclofenac	194 ± 10	225 ± 7	294 ± 8	349 ± 45	320 ± 21	y = 0.0012x + 0.0029	0.94	344 ± 24

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TABLE 2

Comparison of the f_u measured by equilibrium dialysis, ultracentrifugation and ultrafiltration at two different concentrations of microsomal protein

Values represent the mean ± S.D. of triplicate determinations.

Drug	f_u					
	Equilibrium dialysis		Ultracentrifugation		Ultrafiltration	
	1 mg/ml	0.2 mg/ml	1 mg/ml	0.2 mg/ml	1 mg/ml	0.2 mg/ml
Desipramine	0.21 ± 0.03	0.66 ± 0.15	0.29 ± 0.01	0.63 ± 0.03	0.31 ± 0.02	0.60 ± 0.04
Amitryptiline	0.53 ± 0.26	0.82 ± 0.08	0.25 ± 0.02	0.71 ± 0.04	0.31 ± 0.02	0.60 ± 0.04
Imipramine	0.48 ± 0.18	0.83 ± 0.28	0.75 ± 0.08	1.12 ± 0.11	0.50 ± 0.05	0.76 ± 0.04
Verapamil	0.53 ± 0.06	0.72 ± 0.19	0.67 ± 0.10	1.09 ± 0.11	0.60 ± 0.02	0.88 ± 0.06
Diltiazem	1.09 ± 0.42	0.96 ± 0.09	0.92 ± 0.02	1.14 ± 0.02	0.98 ± 0.04	1.10 ± 0.06
Propafenone	0.77 ± 0.08	0.98 ± 0.17	0.75 ± 0.08	1.12 ± 0.11	0.69 ± 0.07	0.98 ± 0.09
Chlorpromazine	0.15 ± 0.02	0.40 ± 0.12	0.27 ± 0.02	0.68 ± 0.01	ND	ND
Midazolam	0.65 ± 0.08	0.98 ± 0.17	0.75 ± 0.08	1.12 ± 0.11	0.69 ± 0.08	0.98 ± 0.10
Diclofenac	0.89 ± 0.05	0.95 ± 0.03	0.62 ± 0.06	0.88 ± 0.06	0.61 ± 0.06	0.84 ± 0.04

ND, not determined

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TABLE 3

Comparison of the $Cl_{in,u}$ determined by LESA and the $Cl_{in,u}$ calculated at two different microsomal protein concentrations using f_u values determined by equilibrium dialysis, ultrafiltration and ultracentrifugation

Clearances are expressed as µl/min/mg microsomal protein. Values represent the mean ± S.E. of triplicate determinations.

Drug	$CL_{in,u}$						
	LESA	Equilibrium dialysis		Ultracentrifugation		Ultrafiltration	
		1 mg/ml	0.2 mg/ml	1 mg/ml	0.2 mg/ml	1 mg/ml	0.2 mg/ml
Desipramine	41 ± 12	47 ± 4	32 ± 5	34 ± 1	34 ± 3	32 ± 2	35 ± 3
Amitryptiline	108 ± 4	63 ± 18	86 ± 6	131 ± 8	99 ± 5	107 ± 7	119 ± 6
Imipramine	59 ± 6	54 ± 12	53 ± 11	35 ± 2	39 ± 3	52 ± 3	58 ± 4
Verapamil	222 ± 15	223 ±18	256 ± 38	175 ± 16	170 ± 10	197 ± 10	210 ± 12
Diltiazem	38 ± 4	28 ± 6	34 ± 2	34 ± 2	29 ± 1	32 ± 2	30 ± 1
Propafenone	185 ± 21	114 ± 7	171 ± 18	116 ± 7	150 ± 10	127 ± 8	171 ± 11
Chlorpromazine	191 ± 33	338 ± 27	353 ± 52	195 ± 9	167 ± 3	ND	ND
Midazolam	333 ± 11	364 ± 26	325 ± 35	316 ± 19	285 ± 20	347 ± 24	325 ± 23
Diclofenac	344 ± 24	252 ± 9	366 ± 28	363 ± 21	395 ± 34	372 ± 22	415 ± 35

ND, not determined

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TABLE 4

Comparison of $CL_{in,}$ by the conventional method with that calculated by LESA

Values represent the mean ± S.E. of triplicate determinations.

Drug	$CL_{in,u}$	
	LESA method[a]	conventional method (literature data)[b]
	µl/min/mg	µl/min/mg
2-Ethoxybenzamide	56 ± 6	45 ± 10
Albendazole	164 ± 33	200 ± 24
Amiodarone	ND[c]	15721 ± 3521
Astemizole	450 ± 308	10327 ± 2112
Betaxolol	78 ± 5	84 ± 6
Bumetanide	143 ± 19	147 ± 11
Cerivastatin	34 ± 5	21 ± 7
Clozapine	588 ± 17	922 ± 89
Glyburide	230 ± 10	178 ± 34
Indapamide	32 ± 12	32 ± 7
Isradipine	ND[d]	139 ± 10
Metyrapone	72 ± 9	38 ± 14
Oxaprozin	106 ± 2	96 ± 21

[a] $CL_{in,u}$ calculated by LESA method using published CL_{in} values obtained at 0.25, 1 and 4 mg/ml of rat liver microsomes (Austin et al., 2002).

[b] Average $CL_{in,u}$ calculated from published CL_{in} and f_u values at 0.25, 1 and 4 mg/ml of rat liver microsomes (Austin et al., 2002).

[c]Not determined; Intercept of the $1/CL_{in}$ vs. microsome concentration plot yielded a negative value.

[d]LESA is not applicable as only two experimental CL_{in} were reported .

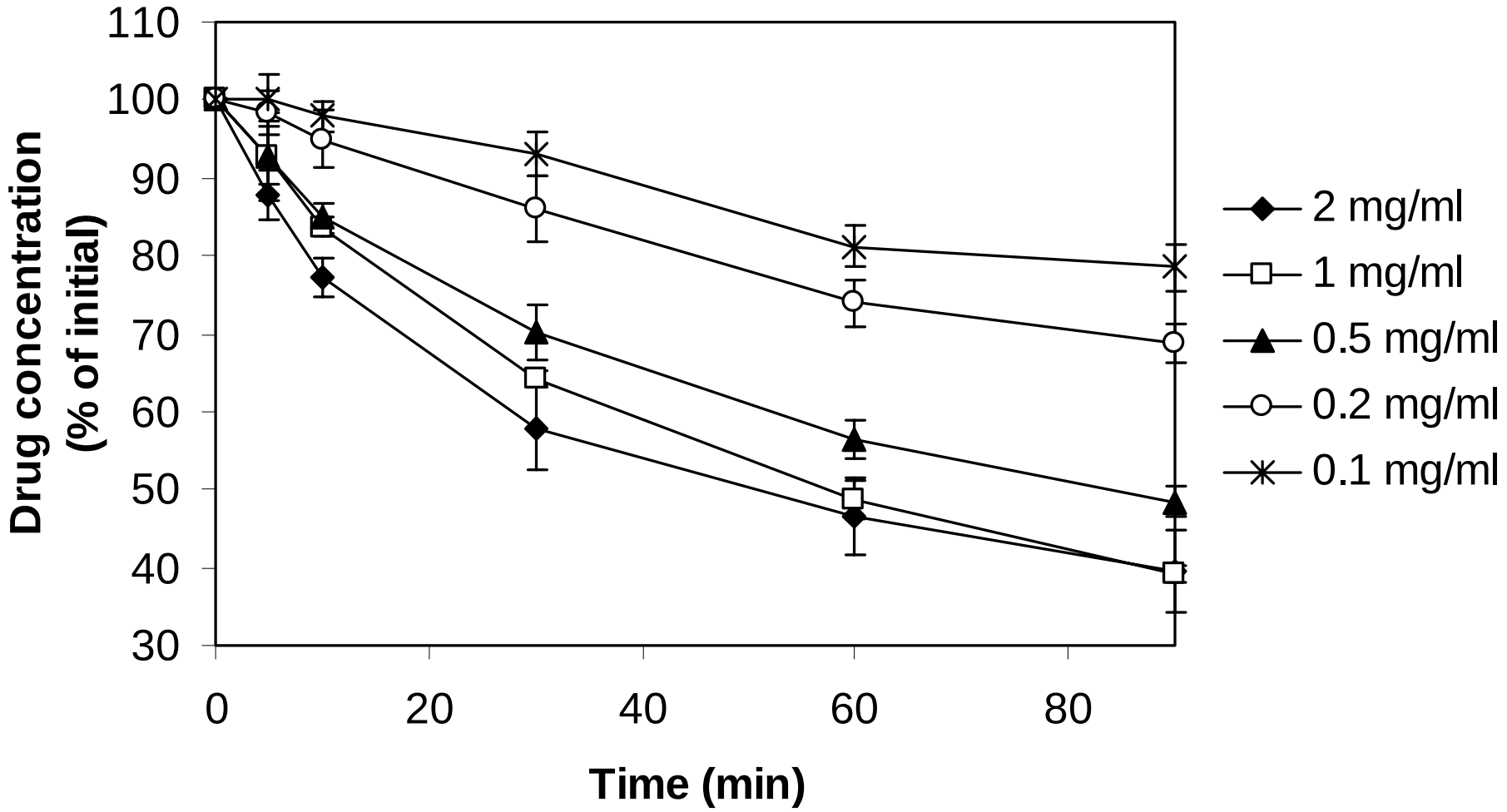

Fig. 1

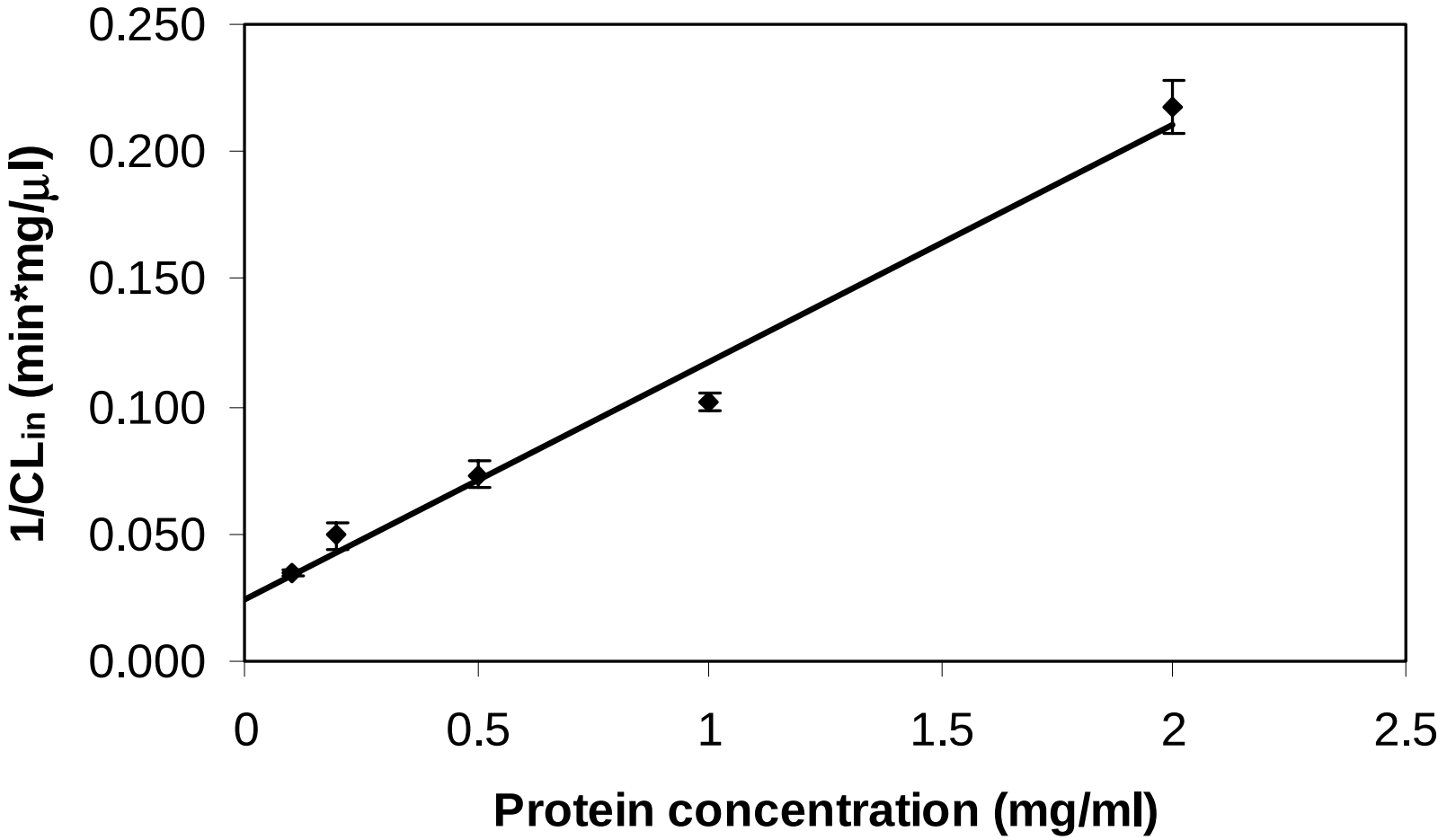

Fig. 2

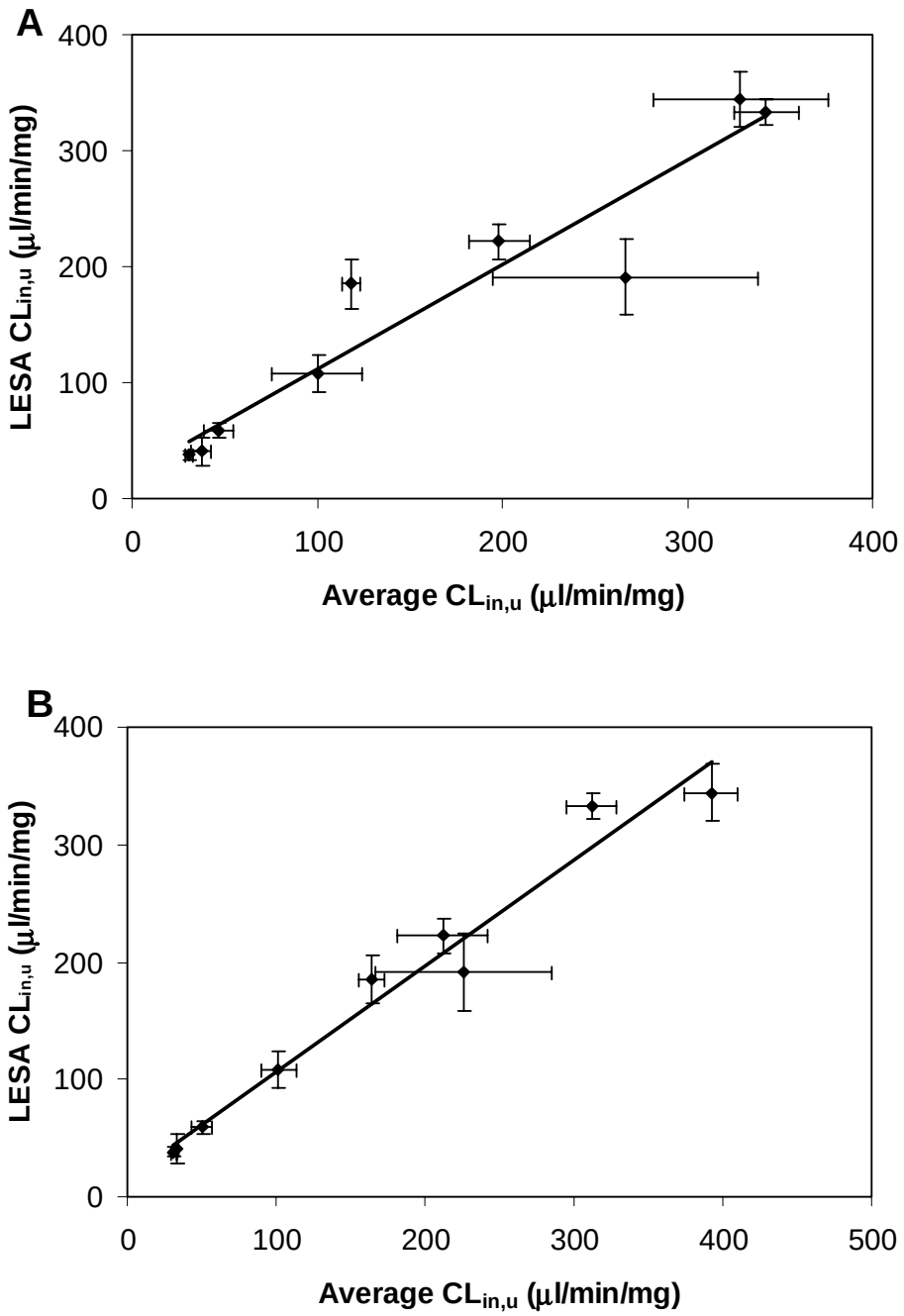

Fig. 3

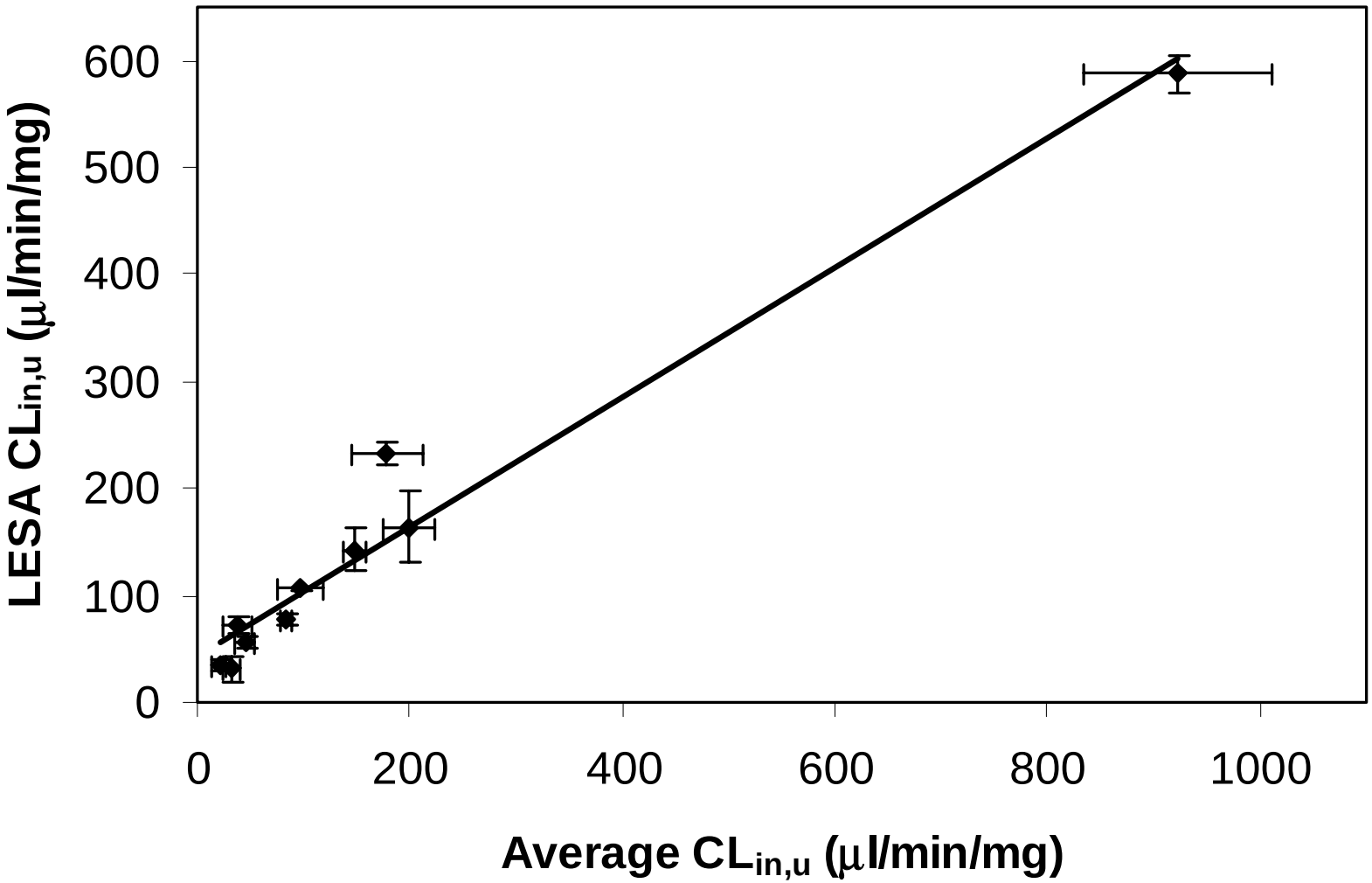

Fig. 4