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RESEARCH ARTICLE

Determination of low intrinsic clearance *in vitro*: the benefit of a novel internal standard in human hepatocyte incubations

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ABSTRACT

1. A novel method utilizing an internal standard in hepatocytes incubations has been developed and demonstrated to decrease the variability in the determination of intrinsic clearance (CL_{int}) in this system. The reduced variability was shown to allow differentiation of lower elimination rate constants from noise.
2. The suggested method was able to compensate for a small but systematic error (0.5 μL/min/10^6 cells) caused by an evaporation of approximately 15% of the volume during the incubation time.
3. The approach was validated using six commercial drugs (ketoprofen, tolbutamide, phenacetin, etodolac and quinidine) which were metabolized by different pathways.
4. The suggested internal standard, MSC1815677, was extensively characterized and the acquired data suggest that it fulfills the requirements of an internal standard present during the incubation. The proposed internal standard was stable during the incubation and showed a low potential to inhibit drug metabolizing enzymes and transporters. With MSC1815677 we propose a novel simple, robust and cost-effective method to address the challenges in the estimation of low clearance in hepatocyte incubations.

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KEYWORDS
Word; hepatocyte; low clearance; internal standard

Introduction

Human hepatic clearance is an important parameter for a drug candidate as it influences oral bioavailability as well as elimination half-life. Extrapolation of intrinsic clearance (CL_{int}) from *in vitro* systems have been established (Houston, 1994; Obach, 1999). Several *in vitro* systems such as microsomes, S9, liver slices and isolated hepatocytes have been shown to be useful in the extrapolation to *in vivo* (Ito & Houston, 2004). These advancements have now made it routine to determine *in vitro* CL_{int} values in drug discovery so that it can act as a compound selection criterion as well as an aid in drug design. Frequently, successful drug design produce compounds with CL_{int}s below the limit of quantification in standard *in vitro* metabolism systems. New cellular systems with higher and long-lasting expression of drug metabolizing enzymes allowing estimation of lower clearance *in vitro* have been utilized to address such situations (Chan et al., 2013; Schaefer et al., 2016). The characterization of these novel systems has shown variations in the expression of drug metabolism enzymes over time and changes in the relative importance (Chan et al., 2013; Schaefer et al., 2016). This suggested that additional complexities relevant to the *in vitro* to *in vivo* translation may be present in these systems. An effort to qualify some of those systems with prototypical substrates for a wide range of drug metabolism enzymes displayed that human cryopreserved hepatocytes express all the tested enzyme to levels comparable or higher levels compared to other systems considered (Kratochwil et al., 2017). These uncertainties and factors, combined with the elevated cost for the novel systems may explain why the human primary hepatocytes still represent the most popular tool to assess the hepatic clearance in drug discovery. We have earlier reported modifications to standard protocols and demonstrated that low CL_{int} values (<1 μL/min/10^6 cells) could be estimated in such a modified system with maintained extrapolation accuracy (Yamagata et al., 2017).

Regardless of the nature of the *in vitro* system used, it is of interest to obtain high accuracy and precision over the widest possible dynamic range. A common way to reduce errors in the quantification by LC-MS is to use a generic internal standard (Hop et al., 2008). This standard is mixed into an organic solvent used to stop the incubation. In this work, we compare a new methodology employing a metabolically stable internal standard used in the incubation mixture versus and internal standard added after the incubation employing six different commercial drugs. These drugs are metabolized by different phase I and phase II enzymes. We hypothesized that employing this approach could help compensate for potential systematic errors caused by evaporation and random errors caused by liquid handling in the experiment. We further examined if a lower detection limit for *in vitro* CL_{int} could be obtained as a consequence of the reduced variability.

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Materials and methods

Materials

Ketoprofen, tolbutamide, phenacetin, etodolac, furafylline, tranylcypromine, sulfaphenazole, ketoconazole and quinidine were purchased from Sigma-Aldrich Corporation, LLC. (St. Louis, MO). The compounds used as internal standards, dextromethorphan-d3 and MSC1815677 were synthesized in the medicinal chemistry laboratories of Merck KGaA (Darmstadt, Germany). The structure of MSC1815677 has been reported in Loannis et al. (2009). Solvents for chromatography were purchased from Merck KGaA (Darmstadt, Germany). All other solvents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Pooled cryopreserved human hepatocytes (Product No. X008005/Lot No. GOZ/pool of 50 donors with mixed gender), the thawing medium (Z99019/C31056B) and Krebs-Henseleit buffer (Z99074/C14056A) were purchased from Bioreclamation IVT (Brussels, Belgium). Caco-2 cells (TC-7 clone) were obtained from INSERM U505, Paris, France. Human serum was obtained from Merck internal blood donation program.

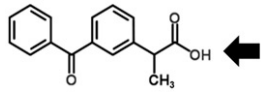
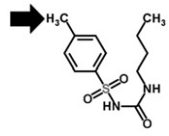
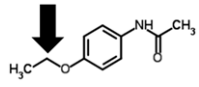
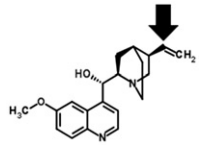
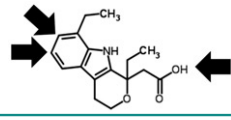
LC-MS/MS analysis

LC-MS/MS analyses were performed with an AB Sciex API 4000 triple quadrupole (AB Sciex LLC, Framingham, MA) coupled to Waters Acquity I-Class UPLC (Waters Corporation, Milford, MA), equipped with a 20 µL sample loop. The software Analyst version 1.6.3 (AB Sciex LLC) was used to control the LC system and the mass spectrometer. An Acquity UPLC BEH C18 1.7 µm, 2.1 mm × 50 mm (Waters Corporation, Milford, MA) was used as the analytical chromatography column. Chromatography was performed using a generic gradient at a flow rate of 0.8 mL/min. The mobile phases consisted of A: 70 mM ammonium formate buffer containing 0.1% (v/v) formic acid and B: acetonitrile. The gradient conditions were as follows: 0–0.1 min 0% B, 0.1–0.8 min 0–100% B, 0.8–1.0 min 100% B, 1.0–1.4 min 0% B. The total time between injections was 2 min. The mobile phase was eluted to waste for the first 0.3 min, whereas data collection occurred between 0.3 and 1.4 min. Electrospray ionization in both positive and negative mode with multiple reaction monitoring (MRM) was used. The capillary voltage was set to 5.0 kV and the desolvation temperature was set at 600 °C. Curtain gas and collision gas were set to 30 and 4 units, respectively. Ion source gas 1 and ion source gas 2 were set to 50 and 70 units, respectively (nitrogen purity 5.0/99.9990%). The entrance potential was set to 10 V. MRM transitions, Declustering potential, Collision Energy and Collision Cell Exit Potential were optimized for each compound using MultiQuant version 3.0.2 software (AB Sciex LLC, Framingham, MA). The dwell time for each transition was 20 ms.

Human hepatocyte incubations

Each vial of cryopreserved hepatocytes was thawed according to the manufacturer's instructions. Hepatocyte viabilities after thawing were determined by trypan blue exclusion. Hepatocyte viability was typically near 95% immediately after thawing. In dedicated experiments viability was also measured at 60, 120, 180, 240 and 300 min. All hepatocyte incubations were performed in 96-well plate (Multiwell plate, round bottom, VWR International GmbH, Darmstadt, Germany). The test

Table 1. Reference compounds used in cocktail incubations in human hepatocytes.

Name	Hepatic metabolism pathway	
Ketoprofen	Glucuronidation (UGT 2B4, 2B7 and 1A3 mainly)[a]	
Tolbutamide	Hydroxylation (CYP 2C9)[b]	
Phenacetin	Dealkylation (CYP 1A2)[c]	
Quinidine	3′-Hydroxylation (CYP 3A4)[d]	
Etodolac	6- and 7-Hydroxylation (CYP 2C9 on R-) Glucuronidation (UGT 1A9 on S-)[e]	

[a]Kuehl et al. (2005).
[b]Srivastava et al. (1991).
[c]Tassaneeyakul et al. (1993).
[d]Kenworthy et al. (1999).
[e]Tougou et al. (2004).

items, metabolized different phase I and phase II enzymes, were incubated (250 nM each) as a cocktail (Table 1).

A 10 mM DMSO solution of MSC1815677 (ISTD1) was dissolved in the incubation buffer to obtain a final concentration of 50 nM in all incubations as well as alone without additional compounds. The final concentration of total organics solvents was below 0.2%. The incubations (final volume 50 μL) were performed with hepatocytes (10^6 cells/mL) suspended in Krebs–Henseleit buffer (pH 7.4) containing 5% human serum. Incubations were carried out in duplicate under gentle agitation, at 37 °C under an atmosphere of 5% CO_2 and 100% humidity. The hepatocytes were incubated for 0, 10, 20, 30, 60, 90, 120, 180, 240 and 300 min, and the reactions were stopped by transferring 30 μL of the sample to 90 μL ice-cold acetonitrile (1:3) containing 250 nM dextromethorphan-D3 (ISTD2). The plates were then centrifuged (2800 rpm/room temperature) and 10 μL of the supernatant was analyzed by LC-MS/MS. The experiment was performed eight times on different days.

Determination of CL_{int}

CL_{int} was calculated based on the disappearance of the compound assessed. For quantification, all samples were analyzed using LC-MS/MS, using ISTD1 (included in the incubation) as well as using ISTD2 (included in the stop solution). The concentration of remaining compound after the period of incubation was determined by calibration curves, separate for each internal standard, in the same matrix. Calibrations samples within 10% of the nominal concentration were accepted. Only depletion greater than or equal to 10% over the entire incubation was used to calculate half-life and CL_{int} values. This corresponds to a limit of quantification for the CL_{int} measured with this method of 0.35 μL/min/10^6 cells. This limit covers only the analytical variability. The disappearance of the compounds was fitted to the first-order elimination equation using GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA). The CL_{int} normalized by the number of hepatocytes in the incubation was calculated as follows:

$$CL_{int} = \frac{K_{et} \times V}{n} \quad (1)$$

where V is the volume of the incubation and n is the number of hepatocytes.

Statistical analysis

Significance of difference between the regression coefficient r^2 of the fitting using data normalized with ISTD1 or with ISTD2 was performed by Student's t test for paired data. The same procedure was adopted to evaluate the significance of the difference in CL_{int} using the two ISTDs.

The observed concentrations were used to fit two models: one corresponding to an exponential decay model and one corresponding to a horizontal line. The latter representing random sampling of the initial concentration of the compound under assessment. The probability of each model to be correct was assigned by the Akaike's Information Criterion (AIC) adjusted for low sample size as calculated by GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA). When the exponential decay model displayed a probability higher than 75% to be different from the horizontal line, the estimated CL_{int} was considered valid. It is worth to note that these acceptance criteria do not translate in a threshold for the CL_{int} value although it tends to be more frequently fulfilled when higher clearance is observed.

Volume shift estimation

The ratio between ISTD1 (present during the incubation) and ISTD2 (present in the quenching solution) was regarded as an accurate measure of the volume shift occurring during the incubation.

Characterization of ISTD1: apparent permeability and efflux in Caco-2 cells

Apparent permeability coefficients were determined with and without the presence of cyclosporine A as a transporter inhibitor as previously described (Mallinger et al., 2015). In short, the experiment was performed at 1 μM concentration at 37 °C during 2 h of agitation at 150 rpm with and without cyclosporine. Aliquots from the receiver and donor sides were analyzed by LC-MS/MS.

Characterization of ISTD1: Cytochrome P-450 inhibition for ISTD1

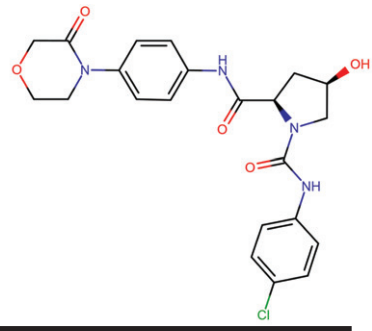
CYP450 inhibition was performed as previously described (Crespi et al., 2002). Briefly, a 10 mM DMSO solution of the test compound was prepared and tested at different concentration ranging from 0.2 to 20 μM at a final concentration of DMSO of 0.2%. The CYP450 activity of cDNA expressed CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 was assessed by luciferin fluorescence. Reagents and instructions were obtained from Promega P450-GloTM kits (Promega Corporation, Madison, WI). The fluorescence was quantified using a TECAN Magellan fluorescence reader (Tecan, Zürich, Switzerland). Furafylline, sulfaphenazole, tranylcypromine, and quinidine were used as positive controls for inhibition of CYP1A2, 2C9, 2C19, and 2D6, respectively, while ketoconazole was used for 2C8 and 3A4.

Results and discussion

The structure and physicochemical properties of the ISTD1 are summarized in Figure 1. No turnover was observed for ISTD1 in hepatocyte incubations. ISTD1 did not show any inhibition of major P450 enzymes. Furthermore, ISTD1 had an excellent response in mass spectrometry which allowed a low concentration (50 nM) to be used. Inhibition of transporters in this concentration range is a rare finding especially as the suggested standard has a low lipophilicity (Ahlin et al., 2008; De Bruyn et al., 2013; Matsson et al., 2007, 2009). Finally, low passive permeability (0.75×10^{-6} cm/min)

MSC1815677

Cytochrome P-450 Inhibition
No inhibition observed at 20 µM for any tested enzyme (1A2, 2C8, 2C9, 2C19,2D6,3A4)



Physicochemical properties	
Ion Class	Neutral
Molecular Weight	458.8 Da
TPSA	111.2
Clog P	1.7
Aqueous solubility	0.23 mg/mL

Caco-2 Permeability		
$P_{app,A-B}$	0.2	10^{-6} cm/min
Efflux ratio ($P_{app,B-A}/P_{app,A-B}$)	44	
$P_{app,A-B}$ + P-gp Inhibitor	0.7	10^{-6} cm/min
Efflux ratio ($P_{app,B-A}/P_{app,A-B}$) + P-gp Inhibitor	1	
Recovery	83 %	

Stability
No disappearance in human hepatocytes (5 hr)

Figure 1. Structure, physiochemical and *in vitro* ADME properties of MSC1815677 (ISTD1). Topological polar surface area (TPSA) was calculated using the MOE (Chemical Computing Group Inc., Montreal, Quebec, Canada). CLogp was calculated using ACDLabs version (Advanced Chemistry Development Inc., Toronto, Ontario, Canada).

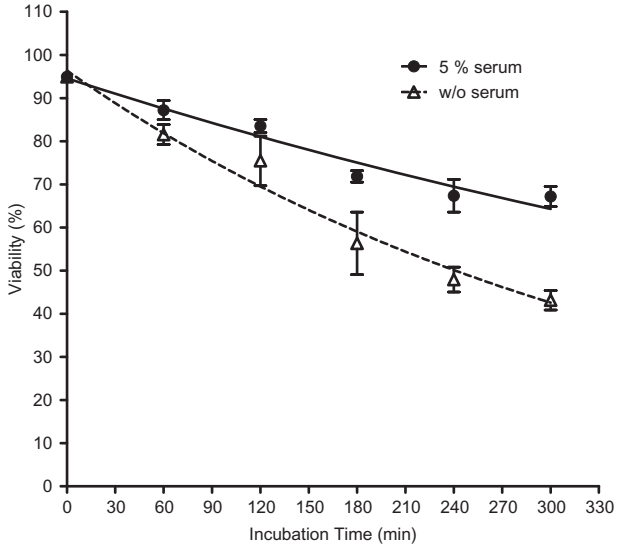


Figure 2. Assessment of cell viability during incubation. The cell viability was measured by trypan blue exclusion test every hour after the start of the incubations. Data are the average ± standard deviation of five independent experiments.

estimated in Caco-2 cells in the presence of cyclosporine A and an efflux ratio of 44 in the same cell line was observed. Hence, ISTD1 was found to be metabolically stable, did not inhibit any Cytochrome P-450 and the low concentration applied indicated that inhibition of transporters and phase II enzymes was unlikely, making it an appropriate internal standard for use in hepatocyte incubations. Incubation times beyond 2 h frequently result in low viability of suspended hepatocytes which makes determinations of especially low CL_{int} values challenging. In this study, we showed residual viability above 70% up to 5 h using an addition of 5% human serum (Figure 2). This suggested that longer incubation times can produce valid results using this modification and that the loss of viability during the incubation could be disregarded when normalizing for the matrix concentration. This finding was supported by the observation that no change in CL_{int} values was observed when the last time points were removed (data not shown). Furthermore, clearance extrapolation performed based on incubations containing 5% human serum has been shown to result in a sound *in vitro–in vivo* correlation with an absolute average fold error of 1.7 (Yamagata et al., 2017). The paper by Yamagata et al. describes the required compensation to obtain the free concentration in the incubation compensating for the binding to hepatocytes as well as for the binding to human serum proteins. This publication focus on the CL_{int} determination per se rather than extrapolation to *in vivo*. Hence, mathematical transformations aiming at human extrapolation were not used here.

The use of the ISTD1 during the incubation resulted in a significantly improved r^2 for the elimination rate constant compared to ISTD2 which was added in the stop solution (Table 2). A trend suggesting a more pronounced effect for compounds with lower CL_{int} values was observed. The improved r^2 allowed the determination of CL_{int} values for the references with low turnover. This was indicated by a higher frequency of a statistically significant exponential decay, i.e. having a p value >75% compared to a horizontal line (Table 3). Furthermore, the probability of the exponential decay was always higher when using the ISTD1 during the incubation in comparison to ISTD2 (Data not shown). An example demonstrating the difference between internal standard during and after the incubation is shown in Figure 3.

The improved r^2 when using an internal standard in the incubation was expected to be accompanied by of reduced variability in the determined CL_{int} values. A strict comparison of the variability was difficult as the number of valid

Table 2. Statistical differences between r^2 values for the exponential decay estimated by internal standards in incubation versus stopping solution.

		r^2		Probability of same r^2	
Compound	ISTD used for quantitation	(average)	SD	Same drug [%]	All drugs [%]
Ketoprofen	ISTD1	0.92	0.06	<5	
	ISTD2	0.68	0.20		
Tolbutamide	ISTD1	0.74[a]	0.14[a]	<5	
	ISTD2	0.37[a]	0.30[a]		
Phenacetin	ISTD1	0.99	0.01	<5	<0.1
	ISTD2	0.97	0.03		
Quinidine	ISTD1	0.96	0.02	<1	
	ISTD2	0.78	0.09		
Etodolac	ISTD1	0.94	0.04	<5	
	ISTD2	0.78	0.19		

Comparison of the correlation coefficients (R^2) of the exponential decay estimated using MSC1815677 (ISTD1) present in the incubation and dextromethorphan-D3 (ISTD2), added with the stopping solution. Significance in the difference of the precision of the fitting was assessed by Student's *t* test for paired data. All calculations were based on eight replicates.
[a]$n = 5$, in three experiment the clearance was below the detection limit.

Table 3. CL_{int} values estimated by internal standards in incubation versus stopping solution.

	ISTD	Average CL_{int} (μL/min/10^6 cells ± SD)	Exponential decay model ($p > 75\%$) (n)	ΔCL_{int} of pairs (μL/min/10^6 cells)
Ketoprofen	ISTD1	2.2 ± 0.4	6	0.7
	ISTD2	1.7 ± 0.1	3	
Tolbutamide	ISTD1	1.1 ± 0.3	2	NA
	ISTD2	NA[a]	0	
Phenacetin	ISTD1	11.5 ± 3.0	8	0.1
	ISTD2	12.2 ± 1.9	7	
Quinidine	ISTD1	3.8 ± 0.8	7	0.1
	ISTD2	3.7 ± 0.6	7	
Etodolac	ISTD1	2.8 ± 0.7	6	0.3
	ISTD2	2.4 ± 0.9	4	

Dextromethorphan-D3 (ISTD2) and MSC1815677 (ISTD1), present in the incubation and in the stopping solution, respectively. Metabolism was identified if the exponential decay model was preferred over the horizontal line according to AIC corrected parameter ($p > 75\%$). Difference in CL_{int} (ΔCL_{int}) where exponential decay model was preferred with both internal standards.
[a]Not applicable.
No CL_{int} value observed with exponential decay preferred for tolbutamide with ISTD2.

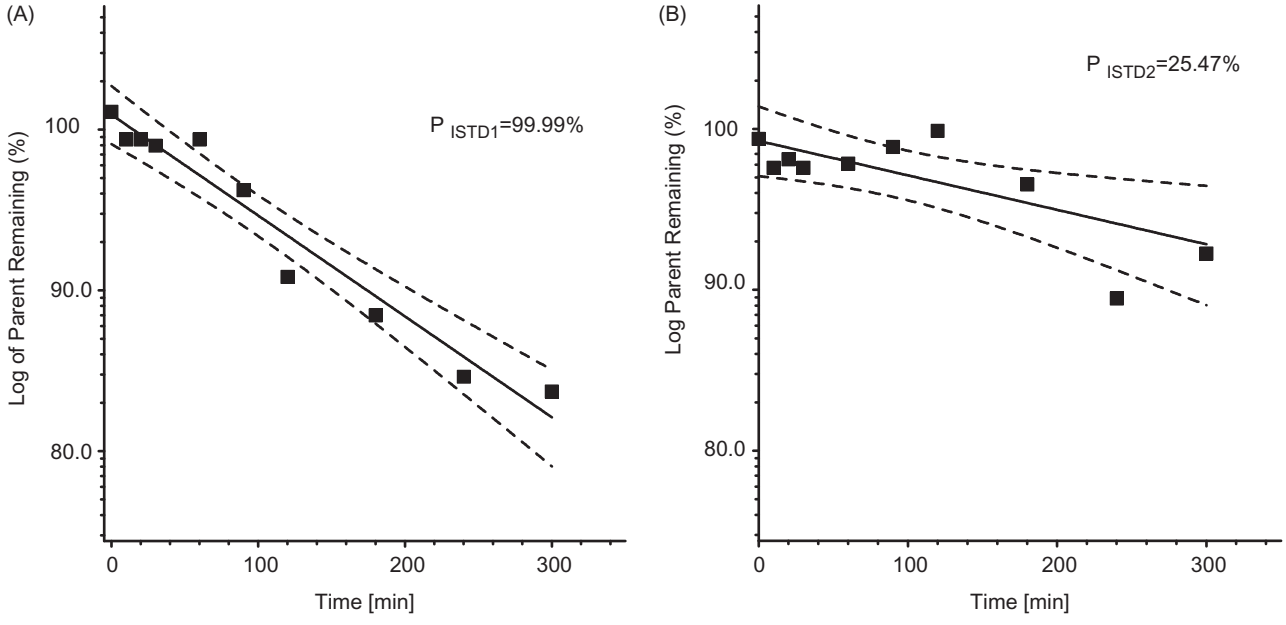


Figure 3. Example of the impact of adding the internal standard in the incubation (A) or at the end of the incubation (B) in the determination of intrinsic clearance of tolbutamide. The concentrations versus time plot of tolbutamide (■) were calculated on the same data adopting either MSC1815677 (ISTD1), used as internal standard during the incubation or dextromethorphan-D3 (ISTD2) added with the stopping solution. Each point is the average of two determinations. Both series of data of concentration/time profiles were fitted to an exponential decay function (bold line) and 95% confidence interval (dashed lines) was calculated. *p* is the probability assigned by AIC method corrected for small sample size, to the exponential decay model used to fit the data for CL_{int} computation.

experiments differ between the two standards, however (Table 3). Still, the data did not indicate a clear advantage in terms of variability in CL_{int} values when ISTD1 was used. This suggests that other factors such as thawing of hepatocytes, operator differences, etc. were more important to the inter-day variability compared to sampling and evaporation which were compensated for by the use of ISTD1.

The CL_{int} values calculated using ISTD1 present in the incubation were higher than the CL_{int} values calculated using ISTD2 added with the quenching solution (Table 3). This difference in the CL_{int} values using the different standard was reflected in about 15% difference in peak area of the ratio of ISTD1 and ISTD2 over the course of the experiment. The differences in peak area and the associated difference in CL_{int} were attributed to evaporation during the incubation. The evaporation was observed despite of the application of humid atmosphere and an optimized sampling procedure (one incubation per time point). This evaporation and thereby the under estimation of CL_{int} values is likely to be larger without such measures. A variation of 15% of the volume during the incubation corresponds to an underestimation of clearance equal to 0.54 µl/min/10^6 cells. Under estimation of CL_{int} values (ΔCL_{int}) in this magnitude represented a significant part of the overall disappearance of the substances with CL_{int} values in the lower range (etodolac, ketoprofen and tolbutamide). The effect was not apparent for the references with higher CL_{int} values where other sources of variability may be more important (Table 3). It has been recognized previously that low clearance compounds in long-term incubations benefits from special adaptations. Kratochwil et al. suggested the use of non-linear mixed effect modeling approach (Kratochwil et al., 2017). Interestingly, the suggested model included a volume term to increase accuracy and precision in the estimation of CL_{int} values. Hence, this approach mathematically fits a theoretical volume to obtain the best fit thereby indirectly confirming that volume shift as an important factor. By contrast, the use of an internal standard in the incubation allows for experimental control of the volume which is free of uncertainties associated with the theoretical estimation.

This study indicates that the addition of internal standard in hepatocyte incubations allows the detection of lower CL_{int} values as more shallow slopes could be quantified. The new approach also demonstrates that the inclusion of an internal standard in the incubation was able to compensate for the small systematic error caused due to the evaporation of the media during the incubation. This error may be negligible for compounds with higher clearance, but will become more important for low clearance compounds. The possibility to include an internal standard in the actual incubation could be of use in some alternative low clearance assays, particularly the so called relay method (Di et al., 2012). This assay uses larger incubation volumes than this in the study which may impact the evaporation but the incubation times are long (4 h) and multiple which may increase evaporation. In addition, several additional liquid handling steps required which may introduce additional errors which would be compensated for by using an internal standard in the incubation.

In conclusion, our data and method provide an efficient and robust tool to accurately assess low CL_{int} values *in vitro* during drug discovery and may be a useful approach to increase the prediction of human PK parameters in early phases of drug development and well as provide the possibility to further optimize compounds *in vitro* pharmacokinetic properties by increasing the sensitivity of *in vitro* CL_{int} determinations.

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Disclosure statement

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