Simultaneous Assessment In Vitro of Transporter and Metabolic Processes in Hepatic Drug Clearance: Use of a Media Loss Approach

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ABSTRACT

Hepatocyte drug depletion-time assays are well established for determination of metabolic clearance in vitro. The present study focuses on the refinement and evaluation of a “media loss” assay, an adaptation of the conventional depletion assay involving centrifugation of hepatocytes prior to sampling, allowing estimation of uptake in addition to metabolism. Using experimental procedures consistent with a high throughput, a selection of 12 compounds with a range of uptake and metabolism characteristics (atorvastatin, cerivastatin, clarithromycin, erythromycin, indinavir, pitavastatin, repaglinide, rosuvastatin, saquinavir, and valsartan, with two control compounds—midazolam and tolbutamide) were investigated in the presence and absence of the cytochrome P450 inhibitor 1-aminobenzotriazole and organic anion transporter protein inhibitor rifamycin SV in rat hepatocytes. Data were generated simultaneously for a given drug, and provided, through the use of a mechanistic cell model, clearance terms characterizing metabolism, active and passive uptake, together with intracellular binding and partitioning parameters. Results were largely consistent with the particular drug characteristics, with active uptake, passive diffusion, and metabolic clearances ranging between 0.4 and 777, 3 and 383, and 2 and 236 μl/min per milligram protein, respectively. The same experiments provided total and unbound drug cellular partition coefficients ranging between 3.8 and 254 and 2.3 and 8.3, respectively, and intracellular unbound fractions between 0.014 and 0.263. Following in vitro-in vivo extrapolation, the lowest prediction bias was noted using uptake clearance, compared with metabolic clearance or apparent clearance from the media loss assay alone. This approach allows rapid and comprehensive characterization of hepatocyte drug disposition valuable for prediction of hepatic processes in vivo.

Introduction

An array of in vitro methodologies have been developed to measure permeability and metabolic properties of drugs and new chemical entities; however, within the pharmaceutical industry there is more confidence in using the latter than the former for predicting in vivo pharmacokinetics (Jones et al., 2015). Predicting in vivo hepatic clearance resulting from metabolism alone has matured over the last two decades (Houston, 1994; Wood et al., 2017) and is routinely applied. Although the importance of transporters in controlling drug uptake and modulating metabolic enzyme interactions is widely appreciated (Shitara et al., 2006; Giacomini et al., 2010; Chu et al., 2013), standard in vitro and in silico approaches for assessment and extrapolation have yet to be broadly adopted (Zamek-Gliszczynski et al., 2013).

Uptake assays can be performed in vitro using multiple formats; however, methods are typically labor intensive. The classic oil-spin approach uses hepatocytes in suspension, requiring incubations to be centrifuged through a layer of oil to allow the separation of cells and media (Miyauchi et al., 1993; Hallifax and Houston, 2006). Monolayer and sandwich- cultured hepatocyte formats have also been developed that offer certain advantages, and can be used to generate similar estimates of uptake (Moench et al., 2012; De Bruyn et al., 2013). However, these systems require hours or days of culture time before drug assays can commence. It is argued that during this delay transporter expression at the membrane declines, which may lead to under prediction of clearance (Jigorel et al., 2005). The decline in cytochrome P450 (P450) activity with time is also well documented (Paine, 1990; Griffin and Houston, 2005) and must be another consideration for these formats. The “media loss” assay was developed (Soars et al., 2007) to provide a more rapid experimental option for determining drug uptake and retains many similarities to metabolic stability assays (subsequently referred to as the “conventional” assay). The course of drug depletion is monitored over time and from the simultaneous use of the media loss (involving the centrifugation of cells prior to sampling) and the conventional assay (involving direct sampling from the cell suspension) estimation of uptake and metabolism is achieved, making it possible to determine the probable rate-determining step in the hepatic clearance of the drug in question (Soars et al., 2007; Jigorel and Houston, 2012).

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DRUG METABOLISM AND DISPOSITION

Supplemental material is available at dmd.aspetjournals.org.

ABBREVIATIONS: ABT, 1-aminobenzotriazole; CL\textsubscript{uptake}, active uptake clearance; CL\textsubscript{int}, intrinsic clearance; CL\textsubscript{inst} in vivo, in vivo intrinsic clearance; CL\textsubscript{inst,ML}, media loss intrinsic clearance; CL\textsubscript{max}, metabolic intrinsic clearance; CL\textsubscript{plasma}, plasma clearance; CL\textsubscript{passive}, passive uptake clearance; CL\textsubscript{total}, total uptake clearance; GMFE, geometric mean fold error; KP, hepatocyte-to-medium partition coefficient for total drug; KP\textsubscript{ML}, hepatocyte-to-medium partition coefficient for unbound drug; LC-MS/MS, liquid chromatography with tandem mass spectrometry; Rfc, rifamycin-SV; RMSE, root mean squared error; V\textsubscript{cell,app}, apparent volume of the cell; WME, Williams’ medium E.
The primary restriction to the widespread use of uptake assays remains their labor intensive nature. The aim of this study, therefore, was to increase the throughput of the media loss assay, allowing the use of inhibitors of both uptake transporters and metabolic enzymes. Subsequently, a simple mechanistic model was used to determine several key pharmacokinetic parameters and to scale the in vitro parameters to determine the utility of this method for predicting in vivo hepatic clearance. Literature data for drug uptake data and metabolism rates in rat hepatocytes were used to identify a group of potential drugs for study. Figure 1 summarizes the parameters reflecting active transport function [assessed relative to passive transport with the parameter \(K_{pu}\) (hepatocyte-to-medium partition coefficient for unbound drug) and metabolic clearance (\(CL_{met}\) for 17 drugs). Ten compounds were selected for an investigation that covered a range of properties: atorvastatin, cerivastatin, clarithromycin, erythromycin, indinavir, pitavastatin, repaglinide, rosvastatin, saquinavir, and valsartan. An additional two drugs selected for study (midazolam and tolbutamide) intended to act as control compounds. For these two drugs the role of hepatic transporters is minimal and hence \(K_{pu}\) close to unity (Nicholls and Houston, 1996; Brown et al., 2007). Their differing rates of metabolism (tolbutamide being low-clearance and midazolam high clearance) provide useful checks for the temporal changes for both assay formats. The inhibitors selected for study were 1-aminobenzotriazole (ABT) for P450 (Mugford et al., 1992) and rifampycin-SV (Rfc) for Oatp1, Oatp2, and Ntcp (Fattinger et al., 2000).

### Materials and Methods

**Chemicals.** Atorvastatin, indinavir, pitavastatin calcium, rosvastatin and valsartan were purchased from Sequoia Research Products (Pangbourne, UK). Saquinavir and midazolam were purchased from Roche Products Ltd (Welwyn Garden City, UK). ABT, Rfc, clarithromycin, erythromycin, tolbutamide, and Bradenfeld reagent were purchased from Sigma-Aldrich (Dorset, UK). Cerivastatin and repaglinide were purchased from Carbosynth Limited (Berkshire, UK). Phenol Red Free Williams’ Media E (WME) was purchased from Lonza Ltd (Basel, Switzerland). All other reagents were obtained from Life Technologies (Paisley, UK).

**Animal Source, Housing, and Diet.** Male Sprague-Dawley rats (240–260 g) were obtained from the Biologic Sciences Unit, Medical School, University of Manchester (Manchester, UK). They were housed in groups of two in opaque boxes on a bed of sawdust in rooms maintained at a temperature of 20 ± 3°C, with a relative humidity of 40–70% and a 12-hour light/dark cycle. Animals were allowed free access to Chow rat and mouse diet and fresh drinking water. All animal protocols were approved by the University of Manchester review committee.

**Hepatocyte Isolation and Preparation.** Rat hepatocytes were isolated from the livers of male Sprague-Dawley rats weighing between 250 and 300 g (Charles River, Margate, Kent, UK). Rats were sacrificed using CO2 overdose followed by cervical dislocation. Hepatocytes were prepared using an adaptation of the two-step collagenase perfusion method, as described previously (Berry and Friend, 1969). After isolation, hepatocytes were suspended in WME, as supplied by Lonza Ltd with no protein present, at pH 7.4. Cell count and viability were determined using the Trypan blue exclusion method. Only preparations exceeding 85% viability were used. Cells were diluted to a density of 2 × 10^6 cells/ml in WME before being split into aliquots. Inhibitors were added to the appropriate cell aliquots to give final concentrations of 1 mM ABT and 100 µM Rfc.

**Conventional Depletion Assay.** Cell suspensions (125 µl) were transferred to a 96-well plate and preincubated for 10 minutes in a Heidolph Incubator 1000 (Heidolph, Schwabach, Germany) at 37°C and 900 rpm. Experiments were performed in duplicate, and the maximum organic solvent concentration in the incubation was 0.11% (v/v). To initiate the reaction, 125 µl of drug solution (2 µM) in WME was added to the cell suspension. The final incubation, therefore, had a cell density of 1 × 10^7 cells/ml and a nominal drug concentration of 1 µM. At nine specified time points, 75-µl aliquots were quenched in methanol containing relevant internal standard. Samples were stored at −20°C until analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Cell suspensions were frozen overnight to lyse cells, and a Bradford protein assay (Biorad, Hemel Hempstead, UK) was performed to determine protein concentrations in each well.

**Media Loss Assay.** The media loss assay, described previously (Soars et al., 2007; Jigorel and Houston, 2012), was performed simultaneously with the conventional depletion assay. Methodology remained identical in both depletion assays, except for the addition of a centrifugation step immediately prior to sampling of the media. Adaptations were made to the protocols outlined by Jigorel and Houston (2012) and Soars et al. (2007) to allow the assay to be performed in 96-well plates. Hepatocyte density and volume, drug volume, and shaking speed remained identical to that described by Jigorel and Houston (2012), and sampling volume was modified to 75 µl to maintain a sample-to-methanol ratio of 1:3, as described by Soars et al. (2007). Owing to the limitations of the plate centrifuge and to minimize the delay from the desired time point to sample quenching, while maintaining sufficient separation of cells from the media, other differences in both protocols included the centrifugation speed (3000g) and time (15 seconds), performed using an Eppendorf Centrifuge 5804 (Stevenage, UK).

**LC-MS/MS Analysis.** A Waters Alliance 2795 with a Micromass Quattro Ultima or Quattro Micro triple quadruple mass spectrometer (Waters, Milford, MA) was used for LC-MS/MS analysis. Analytes were centrifuged for 10 minutes at 2500 rpm and a 10-µl aliquot of the supernatant was analyzed by LC-MS/MS. Four mobile phases (A, B, C, and D) were used, the composition of each was as described by Soars et al. (2007). Owing to the limitations of the plate centrifuge and to minimize the delay from the desired time point to sample quenching, while maintaining sufficient separation of cells from the media, other differences in both protocols included the centrifugation speed (3000g) and time (15 seconds), except for the addition of a centrifugation step immediately prior to sampling of the media.

**Data Analysis and Modeling.** Data were fitted to a monophasic or biphasic exponential decay model, described in eqs. 1 and 2,

\[
C(t) = C_0 e^{-k_1 t}
\]

where \(C_0\) is the initial media substrate concentration and \(k\) is the elimination rate constant,

\[
C(t) = A e^{-k_2 t} + B e^{-k_3 t}
\]

where \(A\) and \(B\) represent the back-extrapolated drug concentration in the media in the first and second phases, respectively, and \(k_2\) and \(k_3\) are the elimination rate constants in the first and second phases, respectively. Following this, intrinsic clearance (\(CL_{int}\)) was calculated using eqs. 3 and 4 for monophasic and biphasic fits, respectively.
Where $V$ is the incubation volume and $P$ is the amount of protein (milligrams) in each incubation. A single-factor analysis of variance and a post hoc Scheffe's test were used to determine if $CL_{int}$ values were significantly different between conditions.

Figure 2 summarizes the experimental and data analysis methods used for data generated in the two assays. Figure 2A provides a schematic of the experimental steps taken and Fig. 2B illustrates the two-compartment mechanistic model used to describe drug uptake (active and passive), metabolism, and distribution.

The latter was adapted from Jigorel and Houston (2012), implemented in Matlab R2014a (The MathWorks, Inc., Natick, MA), and produced estimates for the clearance of active uptake ($CL_{active}$), passive uptake clearances ($CL_{passive}$), and metabolism ($CL_{met}$; see Fig. 2, A and B). The mechanistic model was found to be most successful when a step-wise approach was used and was not applied for drugs showing monophasic depletion under all conditions (midazolam and tolbutamide). In these cases active and passive uptake clearances in the media loss assay were practically unidentifiable.

The conventional assay was first modeled to obtain a measure of $CL_{met}$, as well as to obtain interaction terms for each inhibitor condition acting on $CL_{met}$ ($INT_{ABT}$, $INT_{Rfc}$, and $INT_{ABTRfc}$) described in eq. 5, $CL_{met,app} = CL_{met} \cdot INT_{ABT}^{0.1} \cdot INT_{Rfc}^{0.1} \cdot INT_{ABTRfc}^{0.1}$

Where $CL_{met,app}$ is the apparent metabolic clearance, and $ABT$, $Rfc$, and $ABTRfc$ are power constants of 0 (in the absence of inhibitor) or 1 (in the presence of inhibitor).

The default approach was to assume an additive effect of $ABT$ and $Rfc$ in the media loss assay; however, in cases where the fitting was poor the combined $ABTRfc$ term was implemented. $CL_{met}$ is the nonsaturable metabolic clearance term, with interaction terms acting as proportionality scalars to determine the overall effect in eq. 6. For the purpose of parameter identifiability, it was assumed that active transport was completely inhibited by $Rfc$ at the concentrations used in this study (Fattinger et al., 2000). Parameters estimated on the basis of the conventional assay were then fixed in the second step, in which the media loss concentration-time profiles were modeled to estimate rates of $CL_{active}$ and $CL_{passive}$ transport and the apparent cell volume ($V_{cell, app}$). Total uptake clearance ($CL_{uptake}$) was calculated from the summation of both $CL_{active}$ and $CL_{passive}$. All results were normalized to the amount of protein within each well.

Differential equations, eq. 6 (conventional assay) and eqs. 7 and 8 (media loss assay), were used to describe the concentrations in both the cell and media over time.

\[
\frac{dS_{med}}{dt} = \frac{-CL_{met} \cdot S_{med}}{V_{med}}
\]

\[
\frac{dS_{med}}{dt} = \frac{-S_{med} \cdot (CL_{active} + CL_{passive}) + (CL_{passive} \cdot S_{cell})}{V_{med}}
\]

\[
\frac{dS_{cell}}{dt} = \frac{S_{med} \cdot (CL_{active} + CL_{passive}) - S_{cell} \cdot (CL_{passive} + CL_{met})}{V_{app,cell}}
\]

Where $S_{med}$ is the concentration in the media, $V_{med}$ is the experimental volume of the media (set at 250 µl), and $S_{cell}$ is the concentration in the cell.

Other parameters were calculated as follows. $K_p$ is the hepatocyte-to-medium partition coefficient for total drug, and reflects the total drug within the cell, determined by passive permeation, active uptake, and intracellular binding (eq. 9).

A

Step 1

Conventional Assay

Control +ABT +Rfc +ABT,Rfc

Step 2

Media Loss Assay

Control +ABT +Rfc +ABT,Rfc

B

Media

$V_{med}$

Cell

$V_{cell, app}$

$CL_{active}$

$CL_{passive}$

$CL_{met}$

Fig. 2. Experimental and data analysis approaches. (A) Approach taken for generating and modeling of data from both the media loss and conventional assays. (B) Two-compartment mechanistic model used to describe drug uptake (active and passive), metabolism and apparent cell volume.
Fig. 3. Representative substrate depletion-time profiles in rat hepatocytes at 1 μM, with data fitted using eqs. 1 and 2. Media loss and conventional assay are displayed, respectively, for atorvastatin (A and B), pitavastatin (C and D), saquinavir (E and F), and tolbutamide (G and H). Data were generated using the media loss assay or the conventional depletion assay. Observed data (●) represents mean ± S.D. (n = 3).
clearance parameters were scaled to whole-body values of the rat, using
\( V_{cell} \) and \( CL_{passive} \) are estimated by the mechanistic model. Owing
to evidence of internalization of efflux transporters following hepatocyte isolation
(Bow et al., 2008), efflux was assumed to be negligible. Finally, \( f_{u,cell} \) was estimated
using eq. 11.

\[
K_{pu} = \frac{CL_{active} + CL_{passive}}{CL_{passive}}
\]

Prediction of In Vivo Clearance. For prediction of in vivo clearance, in vitro
clearance parameters were scaled to whole-body values of the rat, using
physiologic scaling factors of 200 mg protein/g liver (Seglen, 1976) and 40 g
liver/kg bodyweight (Davies and Morris, 1993). Data were scaled using protein
content rather than cell number as this could be measured experimentally more
accurately. For in vivo data, all blood clearance \( (CL_b) \) data were initially corrected
for renal clearance. In vivo \( CL_{int} \) was then calculated from in vivo
\( CL_b, \) fraction of drug unbound in the blood \( (f_{ub}) ,\) and hepatic blood flow \( (Q_H, \) set
at 100 ml/min per kilogram (Hsu and Houston, 2004)) using the well-stirred model
(eq. 12). Blood-to-plasma ratio \( (R_b) \) was used where necessary to calculate
\( CL_b \) and \( f_{ub} \) from plasma clearance \( (CL_p) \) or fraction of drug unbound in the plasma
\( (f_{up}) \) using \( CL_{p,R_b} = f_{ub} / f_{up} \) respectively. Mean values were used when multiple
sources were available. See Supplemental Table 2 for full list of values and
references.

\[
CL_{int, \text{ in vivo}} = \frac{CL_b}{f_{ub} \cdot \left(1 - \frac{CL_p}{CL_b}\right)}
\]

Bias and precision of in vitro values were assessed using the absolute geometric
mean fold error (GMFE, eq. 13) and the root mean squared error (RMSE, eq. 14),
respectively (Hallifax et al., 2010). Qualitative assessment of predictions were
judged as being well predicted when in vitro values fell within 2-fold of the
observed in vivo data. Values above or below this threshold were determined to be
overpredicted and underpredicted, respectively.

\[
GMFE = \sqrt[n]{\frac{\sum_{i=1}^{n} CL_{int, \text{ in vitro}}}{\sum_{i=1}^{n} CL_{int, \text{ in vivo}}}}
\]

\[
RMSE = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (\text{predicted} - \text{observed})^2}
\]

Results

The diversity of drugs selected in this study (Fig. 1) resulted in a range of
drug depletion-time profiles in both the media loss and conventional assays.
All conventional assay profiles were observed to be monophasic, whereas those from
the media loss assay were predominantly biphasic with a terminal phase parallel
to the conventional assay profile. All time profiles under control conditions are
displayed in Supplemental Fig. 1 and representative examples are shown
in Fig. 3. Indinavir, midazolam, and tolbutamide were exceptions
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\[
\begin{array}{ccc}
\text{Drug} & CL_{int} (\mu l/min/mg protein) & \% \text{ of Control} \\
\hline
\text{Control} & \text{ABT} & \text{Rfc} & \text{ABT + Rfc} & \text{ABT} & \text{Rfc} & \text{ABT + Rfc} \\
\hline
\text{Atovasstatin} & 120 \pm 18.9 & 45.9 \pm 7.0 & 7.1 \pm 4.8 & 4.6 \pm 1.0 & 38^* & 6^* & 4^* \\
\text{Cerivastatin} & 47.1 \pm 13.9 & 30.7 \pm 16.5 & 15.0 \pm 2.2 & 9.7 \pm 3.0 & 65 & 32 & 21^* \\
\text{Clarithromycin} & 16.2 \pm 3.4 & 7.2 \pm 2.4 & 17.8 \pm 3.0 & 5.0 \pm 3.4 & 45 & 110 & 31^* \\
\text{Erythromycin} & 16.2 \pm 6.7 & 5.1 \pm 1.4 & 13.1 \pm 4.4 & 3.9 \pm 1.5 & 32^* & 81 & 24^* \\
\text{Indinavir} & 80.4 \pm 17.5 & 12.2 \pm 3.3 & 56.4 \pm 22.1 & 10.9 \pm 4.6 & 15^* & 70 & 14^* \\
\text{Midazolam} & 231 \pm 67 & 32.3 \pm 6.5 & 288 \pm 28 & 33.5 \pm 12.2 & 14^* & 125 & 14^* \\
\text{Pitavastatin} & 32.5 \pm 1.3 & 15.1 \pm 9.0 & 2.1 \pm 1.8 & 1.9 \pm 1.6 & 46^* & 7^* & 6^* \\
\text{Repaglinide} & 44.2 \pm 11.3 & 11.8 \pm 0.4 & 17.1 \pm 6.8 & 7.6 \pm 3.2 & 27^* & 39^* & 17^* \\
\text{Rosuvastatin} & 65 \pm 5.2 & 6.5 \pm 3.4 & 1.3 \pm 0.9 & 1.3 \pm 0.4 & 99 & 19 & 20 \\
\text{Saquinavir} & 112 \pm 28 & 33.3 \pm 13.3 & 97.1 \pm 28.4 & 28.1 \pm 9.3 & 30^* & 87 & 25^* \\
\text{Tolbutamide} & 0.85 \pm 0.39 & 0.84 \pm 0.52 & 1.61 \pm 0.47 & 0.87 \pm 0.48 & 99 & 189 & 102 \\
\text{Valsartan} & 5.8 \pm 3.2 & 8.0 \pm 2.6 & 1.7 \pm 0.5 & 1.7 \pm 1.0 & 137 & 29 & 29 \\
\end{array}
\]

*P value of <0.05 using Scheffe’s test following analysis of variance.

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**Fig. 4.** Media loss \( CL_{int} (CL_{int, ML}) \) plotted against the ratio between \( CL_{int, ML} \) and \( CL_{int} \) measured in the conventional assay, calculated using eqs. 3 and 4 as appropriate. DRUGS are identified as follows: 1) atorvastatin, 2) cerivastatin, 3) clarithromycin, 4) erythromycin, 5) indinavir, 6) midazolam, 7) pitavastatin, 8) repaglinide, 9) rosuvastatin, 10) saquinavir, 11) tobutamide, 12) valsartan. Values close to unity denote no difference between assay formats and a clearance ratio of 2 is shown as a distinction for high and low ratios between assay formats.

\[
K_p = \frac{V_{cell, app}}{V_{cell}}
\]

where \( V_{cell, app} \) is the apparent volume of the cell, estimated using the mechanistic
model, and \( V_{cell} \) is the intracellular volume, calculated assuming 3.9 \( \mu l/10^6 \) cells
(Menochet et al., 2012) and a protein conversion of 1 \( \times 10^6 \) cells/mg protein
(determined in-house), multiplied by the amount of protein measured in each
assay. The ratio of unbound cytosolic drug concentrations, relative to the external
medium, is described by the hepatocyte-to-medium partition coefficient for
unbound drug \( (K_{pu}) \), and reflects the degree of active uptake (eq. 10).

\[
K_{pu} = \frac{CL_{active} + CL_{passive}}{CL_{passive}}
\]

where \( CL_{active} \) and \( CL_{passive} \) are estimated by the mechanistic model. Owing
to evidence of internalization of efflux transporters following hepatocyte isolation
(Bow et al., 2008), efflux was assumed to be negligible. Finally, \( f_{u,cell} \) was estimated
using eq. 11.

\[
f_{u,cell} = \frac{K_{pu}}{K_p}
\]
the relationship between these two clearances for the 12 drugs studied, with the clearance ratio plotted against the media loss CLint/CLint,ML.

Particularly high clearance ratios are evident for the four statins and valsartan. Both protease inhibitors, macrolides, and repaglinide showed lower clearance ratios, between 1.4 and 1.8, whereas for the two control compounds no difference was evident.

Inhibitors of both uptake and metabolism were investigated to determine the effect on depletion-time profiles and resulting CLint for each of the drugs. Representative profiles for four drugs displaying different characteristics are shown in Fig. 3, with CLint values and their change relative to control listed in Tables 1 and 2 for the media loss and conventional assays, respectively. A complete set of profiles for all drugs is displayed in Supplemental Figs. 2 and 3.

The inclusion of ABT had no effect on the initial uptake phase in the media loss assay, although a reduced rate of terminal decay caused by metabolism was evident. Generally this led to reduced CLint in both assay formats; however, effects tended to be greater in the conventional than the media loss assay. For compounds metabolized slowly or minimally (cerivastatin, rosuvastatin, and valsartan) no difference was observed, whereas drugs known to be rapidly metabolized (for example saquinavir, repaglinide, indinavir, and atorvastatin, each with metabolic clearances >100 ml/min per kilogram, see Fig. 1) showed substantial inhibition in both formats.

Inhibition of active uptake with the inclusion of Rfc is evident from the concentration-time profiles of drugs relying predominantly on active transport for hepatocyte uptake, which resulted in reduced depletion over time in comparison with control conditions. The effect was more pronounced for the media loss assay. Control compounds and those with lower clearance ratios (clarithromycin, indinavir, saquinavir, and erythromycin) were largely unaffected by the inclusion of Rfc. The anomalous result noted for tolbutamide was attributed to the low intrinsic clearance (<1 ml/min per milligram protein) and the limited accuracy in this low region. Combination of ABT and Rfc typically had an additive inhibitory effect, causing a reduction of CLint greater than observed for either Rfc or ABT alone to a similar extent in both formats.

Time profiles for all drugs in each assay format were analyzed using a mechanistic model to obtain values for CLactive, CLpassive, CLmet, and Vcell,app. The estimated values of these parameters are displayed in Table 3 (fitted concentration-time profiles are displayed in Supplemental Figs. 4 and 5). The model parameters generally matched the expected characteristics in terms of proportion of active transport and rate of metabolism. The range of CLactive and CLpassive values was 40- to 120-fold, with valsartan lowest (18.1 and 3.2 μl/mg per milligram protein, respectively) and saquinavir highest (777 and 383 μl/mg per milligram protein, respectively) for both clearances. In the case of tolbutamide and midazolam, profiles were monophasic in all experimental conditions and hence the mechanistic model was not applied. CLmet showed a 70-fold range of values, with valsartan (comparable with tolbutamide) and saquinavir (approaching midazolam) again being the extremes (2.2 and 139 μl/mg per milligram, respectively).

### Table 2

| Drug          | CLint (μl/min per milligram protein) | % of Control |
|---------------|-------------------------------------|--------------|
|               | Control | ABT | Rfc | ABT + Rfc | ABT | Rfc | ABT + Rfc |
| Atorvastatin  | 42.6 ± 7.9 | 9.8 ± 0.8 | 8.1 ± 2.1 | 2.3 ± 1.4 | 23* | 19* | 5* |
| Cervatstatin  | 4.9 ± 2.7 | 1.2 ± 1.2 | 5.7 ± 1.5 | 1.2 ± 1.3 | 25 | 118 | 26 |
| Clarithromycin| 9.1 ± 0.3 | 0.5 ± 0.5 | 8.2 ± 1.5 | 0.3 ± 0.3 | 6* | 90 | 4* |
| Erythromycin  | 5.4 ± 1.4 | 8.6 ± 1.4 | 38.6 ± 4.9 | 5.5 ± 1.3 | 11* | 67 | 10* |
| Midazolam     | 239 ± 14 | 7.1 ± 2.9 | 246 ± 36 | 8.0 ± 3.4 | 3* | 103 | 3* |
| Pitavastatin  | 6.3 ± 1.1 | 0.9 ± 0.2 | 1.5 ± 0.7 | 0.4 ± 0.2 | 14* | 25* | 6* |
| Repaglinide   | 24.8 ± 8.4 | 8.0 ± 2.6 | 13.5 ± 6.5 | 4.2 ± 1.7 | 32* | 54 | 17* |
| Rosuvastatin  | 2.2 ± 0.5 | 2.2 ± 0.4 | 0.8 ± 0.2 | 0.8 ± 0.6 | 97 | 36* | 36* |
| Saquinavir    | 37.8 ± 17.9 | 18.0 ± 3.5 | 53.2 ± 13.7 | 16.6 ± 3.1 | 24* | 72 | 22* |
| Tolbutamide   | 1.8 ± 0.40 | 0.81 ± 0.44 | 1.46 ± 0.56 | 0.44 ± 0.35 | 44 | 80 | 24* |
| Valsartan     | 1.5 ± 0.7 | 1.9 ± 1.7 | 0.5 ± 0.7 | 0.09 ± 0.02 | 125 | 33 | 6 |

*P value <0.05 using Scheffe’s test following analysis of variance.

### Table 3

Summary of uptake, metabolism, distribution and binding parameters calculated using a mechanistic model

Results are displayed as the mean ± S.D. (n = 3).

| Drug          | CLint | CLmet | CLactive | CLpassive | % of Active Transport | Vcell,app | Kp | Kpα | fαcell |
|---------------|-------|-------|----------|-----------|-----------------------|-----------|----|-----|--------|
|               | μl/mg protein | μl/mg protein | μl/mg protein | μl/mg protein | % | μl/mg protein | | | |
| Atorvastatin  | 34.0 ± 9.7 | 208 ± 34.2 | 183 ± 33.5 | 25.1 ± 7.1 | 88 | 1.0 ± 0.3 | 65.0 | 8.3 | 0.128 |
| Cervatstatin  | 4.8 ± 2.7 | 167 ± 67.8 | 136 ± 66.6 | 30.8 ± 12.6 | 82 | 386 ± 287 | 254 | 5.4 | 0.021 |
| Clarithromycin| 8.7 ± 0.5 | 46.3 ± 9.5 | 26.3 ± 8.7 | 20.0 ± 3.8 | 57 | 130 ± 8.8 | 73.5 | 2.3 | 0.031 |
| Erythromycin  | 12.4 ± 4.0 | 48.4 ± 8.6 | 28.9 ± 7.6 | 19.5 ± 4.0 | 60 | 24.8 ± 20.8 | 16.4 | 2.5 | 0.151 |
| Indinavir     | 52.5 ± 9.7 | 143 ± 41.2 | 87.0 ± 38.6 | 56.1 ± 14.6 | 61 | 3.6 ± 5.5 | 3.8 | 2.6 | 0.666 |
| Pitavastatin  | 6.7 ± 1.1 | 150 ± 56.4 | 125 ± 55.0 | 25.2 ± 12.5 | 83 | 60.9 ± 33.4 | 81.2 | 5.9 | 0.073 |
| Repaglinide   | 25.8 ± 10.6 | 95.8 ± 19.0 | 59.2 ± 17.9 | 36.7 ± 6.3 | 62 | 52.1 ± 31.5 | 35.1 | 2.6 | 0.074 |
| Rosuvastatin  | 2.3 ± 0.5 | 207 ± 64.3 | 161 ± 61.2 | 46.5 ± 19.8 | 78 | 54.8 ± 15.2 | 24.1 | 4.5 | 0.185 |
| Saquinavir    | 139 ± 40.9 | 1160 ± 919 | 777 ± 866 | 383 ± 307 | 67 | 256 ± 188 | 221 | 3.0 | 0.014 |
| Valsartan     | 2.2 ± 0.6 | 21.3 ± 3.3 | 18.1 ± 3.3 | 3.2 ± 0.5 | 85 | 31.1 ± 6.9 | 25.6 | 6.7 | 0.263 |
Linear regression analysis revealed three key relationships: a strong, significant relationship between Log $CL_{\text{passive}}$ and LogD$_{7.4}$ ($r^2 = 0.69$; $P < 0.01$; Fig. 5A), and $CL_{\text{met}}$ and LogD$_{7.4}$ ($r^2 = 0.79$; $P < 0.01$; Fig. 5B). These relationships could be valuable for an initial estimation of the importance of metabolism and passive permeability using the LogD$_{7.4}$. Also, unexpectedly, $CL_{\text{active}}$ and $CL_{\text{passive}}$ were found to be positively related ($r^2 = 0.71$; $P < 0.01$; Fig. 5C).

Parameters $K_p$, $K_{pu}$, and $f_{\text{u,cell}}$ are also listed in Table 3. $K_p$ and $K_{pu}$ ranged between 3.8 and 254 and 2.3 and 8.3, respectively (excluding tolbutamide and midazolam), and are indicative of the range of intracellular binding and active transport properties of the drugs selected in this study. From these values, estimates of $f_{\text{u,cell}}$ ranged between 0.014 and 0.263 for saquinavir and valsartan, respectively. Linear regression analysis indicated no relationship between either $CL_{\text{passive}}$, $CL_{\text{active}}$, or $K_{pu}$ and the $f_{\text{u,cell}}$ for the 12 drugs investigated, confirming that intracellular binding was independent of uptake characteristics. Likewise, Fig. 5D illustrates the lack of any relationship between $V_{\text{cell,app}}$ and $K_{pu}$.

To assess the utility of the current methodology as a predictor of in vivo clearance, parameters from both the direct cellular assay ($CL_{\text{int, ML}}$, see Table 1) and following mechanistic modeling ($CL_{\text{uptake}}$ and $CL_{\text{met}}$, see Table 3) were evaluated. Clearance values were scaled to the level of the whole body using standard physiologic scaling factors. Clearance values (Table 4) showed similar ranges: 6–1851, 15–1887, and 26–3587 ml/min per kilogram for $CL_{\text{int, ML}}$, $CL_{\text{met}}$ and $CL_{\text{uptake}}$, respectively.

In terms of in vivo clearance predictions, $CL_{\text{uptake}}$ was seen to have least bias and precision according to the GMFE and RMSE values compared with $CL_{\text{met}}$ and $CL_{\text{int, ML}}$ (Table 4). However, it is evident that the success of each parameter is drug-dependent (Fig. 6). Four of the five drugs with high clearance ratios (all of which were statins) were best predicted by $CL_{\text{uptake}}$; valsartan, the other high-clearance-ratio drug, was not well predicted by any of the clearance terms. For the five lower-clearance-ratio drugs, $CL_{\text{int, ML}}$ was the most successful predictor; however, in the case of repaglinide $CL_{\text{uptake}}$ was comparable and for saquinavir and erythromycin $CL_{\text{met}}$ was comparable. Indinavir, the other member of this group, was not well predicted by any of the clearance
terms. The control drugs were also best predicted by the CL\text{int}, Mt or the CL\text{met} parameters. This highlights the difficulty in the application of a generic cellular extravasation approach if the properties of the compound are not considered. Of the three terms, CL\text{int}, Mt produced the highest frequency of predictions within 2-fold of the observed in vivo clearance (58% compared with 50 and 33% for CL\text{uptake} and CL\text{met}, respectively). Values and trends for CL\text{int} from the conventional assay were almost identical to CL\text{met}, and so are not presented.

### Discussion

In this study the methodology of the media loss assay previously proposed (Soars et al., 2007; Jigorel and Houston, 2012) was adapted to increase throughput and also to be more informative through the use of inhibitors. It was anticipated that this approach would allow implementation of a simple mechanistic model to estimate values for several clearance processes (active, passive, and metabolic), as well as provide information on intracellular binding and partitioning of the drug. The use of multi-well plates not only reduces the demands of the required reagents and operator time but also increases the feasibility of simultaneously investigating multiple experimental conditions, as exemplified here with the use of various inhibitor conditions.

Drugs in this study were selected to represent a range of metabolic clearances and relative contributions of active and passive transport, as determined from previous in vitro data (see Fig. 1). Comparison of time profiles obtained from the conventional and media loss assay served to highlight the clear differences between assays. Although all conventional depletion assays were monophasic, the majority of media loss profiles were biphasic, indicating that uptake occurred at a different rate to that of the subsequent metabolic metabolism. Indinavir and the control compounds (midazolam and tolbutamide) were exceptions, with monophasic profiles observed in both assays under basal conditions. Although it was expected for both midazolam and tolbutamide, selected as low and high clearance drugs with minimal transporter involvement, it was unexpected for indinavir, which had been shown previously to be transported predominantly by an active process (De Bruyn et al., 2016). Drugs with a small passive permeability component displayed high clearance ratios (higher CL\text{eq}, in the media loss, compared with the conventional depletion), and were also the most affected by Rfc, confirming the importance of uptake to their hepatic clearance.

In contrast those with lower clearance ratios (CL\text{eq} comparable across formats) reflected higher passive permeabilities and indicated that metabolism was the key determinant of their respective hepatic clearance.

The inclusion of inhibitors was intended to allow the estimation of individual clearance processes using a simple mechanistic model. ABT, a broad spectrum P450 inhibitor, was used in an attempt to prevent the majority of phase I metabolism, leaving the total rate of uptake as the key determinant of depletion rate. In both assay formats ABT reduced the depletion rate, indicating that it successfully inhibited a large portion of metabolism. However, there is the obvious caveat that non-P450 enzymes would not be susceptible to ABT. Inhibition caused by ABT only affected the terminal phase in the media loss assay, leaving the initial uptake phase unhindered. This method may be suitable as a standalone assay if a measure of total uptake alone was desired through the initial depletion phase. Rfc, a potent OATP inhibitor, was used to prevent active transport of drugs into the hepatocytes. The concentration used in this study has been shown previously to extensively inhibit rat Oatp1, Oatp2, and Nrtp (Fattinger et al., 2000). For the highly transported compounds (with high clearance ratios), Rfc greatly reduced the uptake phase in the media loss assay, whereas lower assay clearance ratio compounds appeared to be less affected. For indinavir, clarithromycin, and repaglinide, it is unclear why Rfc had little effect, as previous data would suggest that these are subjected to active transport (Yabe et al., 2011; Menochet et al., 2012; De Bruyn et al., 2016). It is possible that activity of other transporters not inhibited by Rfc may enable continued uptake of these particular drugs. The control compounds were largely unaffected by the inclusion of Rfc, consistent with the expectation that they enter the cell via passive diffusion. The effect of Rfc was also evident in the conventional depletion assay, which saw reduced clearance for several transported compounds. This is a secondary effect, since drug is prevented from entering the cells, thereby limiting the sequential metabolism. As anticipated, the use of both inhibitors together generally led to an increase in inhibition compared with use of inhibitors individually. It should be noted that the current methodology would require modification for compounds that are non-P450 or non-OATP substrates, for example, compounds that undergo extensive glucuronidation. In such cases, additional or alternative inhibitors would be required; however, the same principles and model would apply.

### Table 4

| Drug      | CL\text{int}, Mt | CL\text{met} | CL\text{uptake} | CL\text{int, in vivo} | Predicted/Observed |
|-----------|------------------|--------------|------------------|-----------------------|--------------------|
| Atravastatin | 960 ± 151       | 272 ± 77     | 1664 ± 274       | 1593                  | 0.60 0.17 1.04     |
| Cerivastatin | 377 ± 111       | 38 ± 22      | 1333 ± 542       | 1517                  | 0.25 0.03 0.88     |
| Clarithromycin | 129 ± 28       | 70 ± 3.7     | 371 ± 76         | 121                   | 1.07 0.58 3.07     |
| Erythromycin | 129 ± 54        | 100 ± 32     | 387 ± 69         | 115.5                 | 1.12 0.87 3.35     |
| Indinavir   | 643 ± 140        | 420 ± 78     | 1145 ± 330       | 50                    | 12.9 8.41 22.94    |
| Midazolam  | 1851 ± 536       | 1887 ± 144   | 1331             | 1331                  | 1.39 1.42 —        |
| Pitavastatin | 260 ± 10         | 54 ± 8.7     | 1201 ± 451       | 1165                  | 0.22 0.05 1.03     |
| Repaglinide | 353 ± 90         | 206 ± 85     | 767 ± 152        | 496                   | 0.71 0.42 1.55     |
| Rosuvastatin | 52 ± 42         | 19 ± 4.0     | 1658 ± 514       | 1412                  | 0.04 0.01 1.17     |
| Saquinavir  | 895 ± 221        | 1110 ± 327   | 9282 ± 7351      | 911                   | 0.98 12.10 10.19   |
| Tolbutamide | 6.8 ± 3.1        | 15 ± 4.4     | —                | 7.4                   | 0.92 1.96 —        |
| Valsartan  | 47 ± 26          | 17 ± 5.1     | 171 ± 26         | 1554                  | 0.03 0.01 0.11     |

*Data calculated from conventional depletions assay using eq. 1.*
Media Loss Assay to Assess Metabolic and Transport Clearance

Fitting of the mechanistic hepatocyte model followed a stepwise approach. First, data from the conventional depletion assay was modeled to give estimates of metabolic clearance and the interaction of inhibitors. This was then entered into the mechanistic model to allow the estimation of active transport, passive diffusion, and an apparent volume of distribution using data from the media loss assay. Data typically followed the expected characteristics on the basis of previous discussion, in terms of the proportions of active transport and metabolism. Once again the outliers were indinavir, repaglinide, and clarithromycin. It was also observed that in the case of midazolam and tolbutamide, where profiles remained monophasic under all experimental conditions, the mechanistic model became highly insensitive to changes in uptake parameters, and hence were excluded from detailed analysis. These findings are consistent with active transport being a negligible contributor to the overall uptake clearance of the two compounds.

A strong correlation existed between the LogD_{7.4} and the log of the estimated CL_{passive} and CL_{met}. This relationship has been noted previously by Yabe et al. (2011) for CL_{passive} and may serve as a useful tool for providing initial estimates of both passive diffusion and metabolic clearance for novel compounds, should the LogD_{7.4} be known. A significant relationship was also observed between CL_{active} and CL_{passive}; a similar trend was reported by Yabe et al. (2011), although it did not reach statistical significance. These relationships could prove useful in assay design and data modeling for novel compounds for which little information is known.

Using the uptake terms (CL\textsubscript{active} and CL\textsubscript{passive}), along with the V\textsubscript{cell, app} estimated in the mechanistic model, it was possible to indirectly determine the ratios of total and unbound drug in hepatocytes to that in the medium (Kp and Kpu, respectively) and therefore the extent of intracellular binding. Kp values varied by >60-fold and reflects the difference in both active transport and intracellular binding that occurs for each drug. Kpu, which reflects the degree of active transport, had much less variation, with a >3-fold range. The difference between these two parameters is accounted for by the extent of intracellular binding, reflected by the almost 20-fold range in f\textsubscript{cell}. Values and rank order of f\textsubscript{cell} were in good agreement with that published previously (Yabe et al., 2011; Menochet et al., 2012). These terms provide a detailed understanding of the specific intracellular processes that govern the hepatic disposition of each drug.

In vivo hepatic clearance predictions were performed to determine the utility of CL\textsubscript{int, ML}, CL\textsubscript{uptake}, and CL\textsubscript{met} as predictors of in vivo clearance, with bias and precision assessed by GMFE and RMSE, respectively. Overall, CL\textsubscript{uptake} had the lowest bias compared with CL\textsubscript{int, ML} and CL\textsubscript{met} (GMFE = 2.96, 3.13 and 6.40, respectively); however, it was evident that the accuracy of each term was linked to drug clearance ratio. High ratio compounds were more accurately predicted when CL\textsubscript{uptake} was used, since this appeared to be the predominant clearance process, whereas lower clearance ratio drugs benefitted from the use of CL\textsubscript{int, ML} and CL\textsubscript{met}. In both ratio groups there was one example (indinavir and valsartan) where no accurate prediction was obtained from any clearance term. It must also be considered that the CL\textsubscript{int, ML} term, although producing slightly greater bias than modeled uptake data, can be estimated using only the media loss assay (without the requirement for a simultaneous conventional assay). This method may therefore be more suitable if a quantitative prediction of CL\textsubscript{int, in vivo} is the sole reason for performing the assay, since fewer reagents and analysis is required, at the expense of producing estimates of CL\textsubscript{int} only.

Further investigations would be needed if the methodology is to be extended from fresh to rat cryopreserved hepatocytes or to other species, particularly human. Although highly adaptable in terms of practical aspects, it may prove challenging to accurately determine clearance parameters for species when rates of either metabolism or uptake are

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**Fig. 6.** Observed in vivo intrinsic clearance (CL\textsubscript{int, in vivo}) plotted against CL\textsubscript{int, ML} (A), CL\textsubscript{met} (B), and CL\textsubscript{uptake} (C) scaled using standard physiologic scaling factors of 200 mg protein/g liver and 40 g liver/kg bodyweight. Drugs are identified as follows: 1) atorvastatin, 2) cerivastatin, 3) clarithromycin, 4) erythromycin, 5) indinavir, 6) midazolam, 7) pitavastatin, 8) repaglinide, 9) rosuvastatin, 10) saquinavir, 11) tolbutamide, 12) valsartan. Line of unity (solid line), and twofold under and over-prediction (dashed line) are displayed.
significantly lower. Additional applications of the methodology may also include determination of \( K_m \) values, as demonstrated previously by Jigorel and Houston (2012), and drug interaction studies, allowing estimation of \( IC_{50} \) or \( K_i \) values.

In conclusion, this study adapted the media loss assay into a higher throughput format, allowing the inclusion of several inhibitor conditions to split the observed \( CL_{\text{tot}} \) into individual clearance parameters. Using a mechanistic model, it was possible to directly estimate the rates of active transport, passive diffusion, and metabolism, as well as the apparent volume of distribution of the cell, and subsequently \( K_p \), \( K_{p,t} \), and the \( f_{\text{int}} \). Together, these provide a detailed account of the parameters governing drug hepatic disposition. Clearance data from the media loss assay, metabolism, and uptake data were assessed as a predictor of in vivo clearance. It was found that the accuracy of each clearance term was strongly linked to the particular drug characteristics. \( CL_{\text{tot}} \) values from the media loss assay alone remains a viable option for quantitative predictions of in vivo clearance, if no additional information is required.

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Authorship Contributions

Participated in research design: Harrison, De Bruyn, Darwich, Houston.

 Conducted experiments: Harrison.

 Performed data analysis: Harrison.

 Wrote or contributed to the writing of the manuscript: Harrison, De Bruyn, Darwich, Houston.

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