Utility of drug depletion – time profiles in isolated hepatocytes for accessing hepatic uptake clearance: Identifying rate limiting steps and role of passive processes

Emilie Jigorel and J Brian Houston

Centre for Applied Pharmacokinetic Research, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, M13 9PT, UK
Running title: Drug depletion and hepatic uptake clearance

Corresponding Author:

J Brian Houston  
Centre for Applied Pharmacokinetic Research  
School of Pharmacy and Pharmaceutical Sciences  
University of Manchester  
Manchester  
M13 9PT  
UK  
Tel: +44 (0) 161 275 2358  
Fax: +44 (0) 161 275 8349  
Email: brian.houston@manchester.ac.uk

Number of text pages: 27  
Number of tables: 3  
Number of figures: 5  
Number of references: 31

Words in abstract: 286  
Words in introduction: 709  
Words in discussion: 1297

Abbreviations used are:

\( \text{CL}_{\text{active}} \), clearance by active uptake; \( \text{CL}_{\text{met}} \), clearance by metabolism; \( \text{CL}_{\text{obs}} \), observed in vitro clearance (either by uptake or metabolism); \( \text{CL}_{\text{uptake}} \), total uptake clearance by active and passive processes; \( \text{fu}_{\text{cell}} \), fraction of unbound drug in the hepatocyte; \( k_{\text{dep}} \), initial depletion rate constant; \( Kp_{\text{total}} \), tissue-to-medium total drug concentration ratio; \( Kp_{\text{u}} \), hepatocyte-to-medium unbound drug concentration ratio; OATP, organic anion transporter polypeptide; \( P_{\text{diff}} \), passive uptake clearance.
Abstract

Drug depletion-time profiles in isolated hepatocytes, as well as microsomes, have become a standard method of assessing hepatic metabolic clearance in vitro. Soars et al. (Drug Metab Dispos. 35: 859-65, 2007) have described an adaptation of the depletion approach to allow determination of hepatic uptake by transporters in addition to metabolism. Dual incubations are carried out where one set of incubations undergo conventional methodology whereas for the second set cells and media are separated for determination of drug loss from the media. The utility of this dual incubation approach has been assessed using eight drugs (atorvastatin, clarithromycin, erythromycin, fexofenadine, pitavastatin, repaglinide, rosuvastatin and saquinavir) with a range of active uptake, passive permeability, cell binding and metabolic characteristics. Four of these compounds (fexofenadine, rosuvastatin, pitavastatin and atorvastatin) show a biphasic time profile when assessing drug loss from media indicative of hepatic uptake prior to elimination within the hepatocyte which is distinct from the time profile in a conventional incubation and show higher clearances. The four other compounds (clarithromycin, saquinavir, erythromycin and repaglinide) show an identical depletion-time profiles (and clearances) in both sets of incubation. Whether or not the biphasic nature (and higher clearance) is evident, indicating transporter activity for a particular drug appears to be dependent upon its passive permeability. Using the parameter $K_{pu}$ to reflect the relative importance of hepatic transporters versus passive diffusion, a value of 10 was identified as a cut off for whether the biphasic nature was evident or not; those compounds in excess of 10 show this characteristic clearly. There appears to be no relationship between the presence of the biphasic nature and any other parameter, including cellular binding, extent of metabolism or the magnitude of active uptake.
**Introduction**

The use of isolated hepatocytes, either in suspension or as a mono-layer, has allowed many aspects of qualitative and quantitative drug metabolism to be assessed (Soars et al., 2007a; Hewitt et al., 2007). Investigations carried out as early as the mid-70s (Inaba et al., 1975; Yih and Van Rossum, 1977) demonstrated the use of the substrate depletion-time profile within an incubation of isolated hepatocytes to estimate of clearance and to characterize the nonlinear nature of this process, in an analogous way to an in vivo pharmacokinetic study. The popularity of in vitro systems to make quantitative predictions of in vivo clearance has led to similar investigations using hepatic microsomes (Obach 2001; Riley et al., 2005). This substrate depletion approach is widely used because formal kinetic characterization and metabolite quantification are not required, allowing the rapid screening of compounds.

The basis of predicting in vivo clearance values from in vitro kinetic parameters is the use of Michaelis-Menten kinetics and experiential data obtained under initial rate conditions (Houston, 1994). For a number of drugs with simple metabolic pathways it has been demonstrated that drug depletion and metabolite formation can provide equivalent clearance terms (Jones and Houston, 2004; Stringer et al., 2009; Sjogren et al., 2009). However Jones and Houston have also indicated the importance of enzyme stability for longer incubation times as instability may generate biphasic depletion time profiles. The drug depletion method assumes that the concentrations of drug in media and cells are the same which may not be true due to transporter protein activity. The most common approach to measure hepatic uptake is analogous to the Michaelis-Menten approach involving the measurement of cellular uptake under initial rate conditions.
conditions following an “oil-spin” separation of cells from medium (Petzinger and Fuckel 1992; Ishigami et al., 2005). A range of substrate concentrations are usually investigated and the kinetic parameters $V_{\text{max}}$, $K_m$, CL$_{\text{active}}$ ($V_{\text{max}}/K_m$) and $P_{\text{diff}}$ obtained (Yabe et al., 2011).

Soars et al., 2007b have described an adaptation of the depletion approach to allow determination of hepatic uptake in addition to hepatic metabolism. Dual incubations are carried out where one set of incubations undergo conventional methodology (drug depletion assessed from samples of the incubation matrix of cells and buffer) and in the second set of incubations the cells and media from samples of the incubation matrix are separated by centrifugation to determine drug loss from the media alone (media loss methodology). The second set of incubations yield a clearance reflecting total loss from the media (that is both uptake and subsequent metabolism) whereas the conventional incubations provide a measure of metabolic (irreversible) clearance (see Fig. 1). By adopting this dual approach Soars et al., 2007b were able to demonstrate for 36 proprietary compounds that much closer (higher) estimates of in vivo clearance could be obtained than were achieved adopting the conventional approach. A similar improvement was also reported by Gardiner and Paine (2011) for seven marketed drugs. This experimental design is similar to that used previously by others for various investigations including intracellular binding of lipophilic amines and the impact of saturable events within the lysosomes (Hallifax and Houston, 2006). The methodology offers a more pragmatic, if arguably less robust, approach to identifying and characterizing hepatic uptake by transporters than the oil-spin method. As with other drug depletion approaches, clearance is derived from only one substrate
concentration and there are the usual assumptions regarding linearity (with time and cell number) to allow scaling of the CL_{obs} parameter.

In order to test the general utility of the dual incubation approach to substrate depletion, eight drugs (atorvastatin, clarithromycin, erythromycin, fexofenadine, pitavastatin, repaglinide, rosuvastatin and saquinavir) known to be OATP substrates (Shitara et al., 2006; Kalliokoski and Niemi 2009; Giacomini et al., 2010) have been selected. They show a range of active uptake, passive permeability, cellular binding and metabolic characteristics. All eight compounds are actively transported into hepatocytes (range of CL_{act} 100-fold); however due to differences in passive permeability (range of P_{diff} 40-fold) they show differing degrees of importance for active uptake (Yabe et al., 2011). These characteristics can be seen in Table 1 together with the intracellular binding metric f_{cell} which covers a range of 50-fold. These drugs have been investigated using a protocol suggested by Soars et al., (2007b) to obtain clearance values from the slopes of the depletion-time profiles. Three initial substrate concentrations are investigated in order to establish whether saturation can be identified. The data have also been analysed by a simple pharmacokinetic model (see Fig. 2) to obtain an alternative set of parameters to describe hepatocellular events including uptake and metabolism.
Materials and Methods

Chemicals: Atorvastatin calcium, pitavastatin calcium, rosuvastatin calcium, saquinavir were obtained from Sequioia Research Products (Pangbourne, UK); erythromycin, clarithromycin, repaglinide and fexofenadine were from Sigma-Aldrich (Buchs, Switzerland).

Animal source, housing and diet: Male Sprague-Dawley rats (240-260 g) were obtained from the Biological Sciences Unit, Medical School, University of Manchester (Manchester, UK). They were housed in groups of two to four, in opaque boxes on a bedding of sawdust in rooms maintained at a temperature of 20 ± 3°C, with a relative humidity of 40 to 70% and a 12 h light/dark cycle. The animals were allowed free access to Chow Rat Mouse diet and fresh drinking water. All animal protocols were approved by University of Manchester review committee.

Hepatocyte studies: Anaesthetized rats were sacrificed by cervical dislocation and hepatocytes were prepared using an adaptation of the collagenase perfusion method as described previously (Hayes et al., 1995). Hepatocyte viability was determined using the trypan blue exclusion test and only those hepatocyte preparations with viabilities greater than 85% were used. All hepatocyte studies were performed using 3 independent hepatocyte preparations.

Depletion studies in rat hepatocytes. Conventional assay: Drug diluted in Williams’ medium E (125 μl, final concentration range: 0.1 – 10 μM) was preincubated for 5 minutes in an Eppendorf Thermomixer (37°C, 900rpm). To initiate the reaction 125 μL pre-warmed (37°C) hepatocyte suspension (final concentration range: 0.1-1 x 10⁶ cells/mL) was added, giving a final incubation volume of 250 μL. Experiments were performed in duplicate and the organic solvent concentration (methanol) in the incubation was 0.5%. At ten specified time points (up to 90 min) reactions were
terminated by snap-freezing in liquid nitrogen. Samples were then thawed, and ice-cold acetonitrile containing an appropriate internal standard for LC-MS/MS analysis was added.

Media loss assay: The media loss assay (based on the methods by Soars et al., 2007b) was carried out in parallel to the conventional assay in each case (n=3 for each drug). The only difference between the protocols was that at the specific time points (up to 90 min) in the media loss assay the incubation was centrifuged at 7000g for 30 sec, and 80 μL of supernatant was snap-frozen in liquid nitrogen to terminate the reaction.

LC-MS/MS analysis: The LC-MS/MS system used consisted of a Waters 2790 with a Micromass Quattro Ultima triple quadruple mass spectrometer (Waters, Milford, MA). Hepatocyte samples were vortexed and centrifuged for 10 min at 11,600g (Eppendorf centrifuge 5413) and an aliquot (10 µl) of the supernatant was analyzed by LC-MS/MS. Conditions used have been described in detail by Yabe et al., 2011.

Data analysis: All depletion data were fitted to either a monoexponential decay model or a biexponential decay model as shown in equation 1 and 2

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

(1)

where \( C_0 \) is the substrate concentration in the incubation media at time 0 and \( k_{dep} \) is initial depletion rate constant.

\[ C(t) = A e^{-k_1 t} + B e^{-k_2 t} \]  

(2)

where A and B represent the back-extrapolated substrate concentration in the incubation media for the first and the second phase, respectively, and \( k_{dep} \) and \( k_2 \) are the initial depletion rate constants for the first phase and the second phase respectively.
k_{dep} values obtained for the three drug concentrations were used to determine the theoretical depletion constant at low substrate concentration \( k_{dep} ([S] \to 0) \) using equation 3 (Obach and Reed-Hagen, 2002).

\[
K_{dep} = k_{dep}[S] \rightarrow 0 \left(1 - \frac{[S]}{[S] + K_m}\right) \tag{3}
\]

The observed in vitro clearance (CL) was determined using equation 4 where V was the incubation volume and normalized for number of cells.

\[
CL = k_{dep}[S] \rightarrow 0 \cdot V \tag{4}
\]

Data were also modelled by a 2 compartment model (Fig. 2) to obtain specific clearance terms – \( CL_{uptake} \), \( CL_{met} \) and \( P_{diff} \) (reverse transport out of the cell was assumed to occur only by passive permeability as efflux transporters are known to be internalized following isolation (Bow et al., 2008)). The relationships between the modelling parameters and the experimentally determined parameters are shown in Fig. 1.

For comparative purposes in vitro estimates of the same parameters \( CL_{uptake} \) and \( P_{diff} \) obtained from the oil-spin method (Yabe et al., 2011) were used. Also, the hepatocyte/medium partition coefficient for unbound drug \( (K_{pu}) \), and the fraction unbound in the hepatocyte \( (f_{ucell}) \) were calculated from equations 5 and 6 respectively (Yabe et al., 2011).

\[
K_{pu} = \frac{CL_{active} + P_{diff}}{P_{diff}} \tag{5}
\]
\[ f_{u_{cell}} = \frac{K_{pu}}{K_{ptotal}} \]  

(6)

where \( K_{ptotal} \) is the ratio of the total cellular concentration relative to the external medium.

\( K_{pu} \) provides a measure of the cytosolic cellular concentration relative to the external medium and hence reflects the importance of active uptake. The parameter \( K_{ptotal} \) reflects intracellular binding in addition to active uptake processes. These two partitioning parameters are related by intracellular binding (Parker and Houston, 2008) and hence \( f_{u_{cell}} \) can be calculated. The experimental derived parameters used in Equations 5 and 6 were taken from Yabe et al., 2011 and were based on the oil-spin method.

**Results**

Both mono and biphasic depletion-time profiles were observed in the conventional and media loss experiments. Whilst six of the eight drugs studies showed biphasic profiles, in only four of the eight cases (atorvastatin, fexofenadine, pitavastatin and rosuvastatin) was this unique to the media loss approach (Type A behaviour). Clarithromycin and saquinavir were biphasic in both types of experiments. Monophasic depletion time profiles were observed for erythromycin and repaglinide in both the media loss and conventional experiments. This latter type of behaviour (no difference between approaches) was designated as Type B.
A characteristicType A profile obtained with fexofenadine is shown in Fig. 3A where the fraction remaining over time is shown for 0.1µM fexofenadine in the two systems. With fexofenadine, the clearance is clearly biphasic and faster using the media loss assay with a parallel decline for both systems seen at the later time points. Fexofenadine CLint is 17 µl/min/10^6 cells using the conventional assay and 117 µl/min/10^6 cells using the media loss assay (Table 2). This higher in vitro CLint in the media loss assay, compared to the conventional assay, may be explained by the characterization of the uptake which is faster than metabolism; hence, fexofenadine accumulates into the cells with subsequent metabolism/excretion. Similar profiles were obtained with rosuvastatin, pitavastatin and atorvastatin.

A second characteristic (Type B) profile was exemplified by clarithromycin (see Fig. 3B). The profiles obtained with the two methods are similar (Fig. 1B) with CLint values of 46 and 68 µl/min/10^6 cells using the conventional assay and the media loss assay respectively (Table 2). In this case, no conclusion can be made concerning the importance of active uptake from the present data. Although there is good evidence for active uptake of clarithromycin in rat hepatocytes, uptake is probably not the rate limiting step for its hepatic clearance; once the drug is actively transported into cells it may or may not undergo intracellular accumulation prior to metabolism (depending on whether metabolism is the rate limiting step). Similar characteristics were obtained with saquinavir, erythromycin and repaglinide.

The apparent clearance values seen in the conventional approach ranged from 3.3 to 1675 µl/min/10^6 cells whereas there is a smaller range in the media loss experiments (35 to 1657 µl/min/10^6 cells). Extreme values were obtained for rosuvastatin and
saquinavir, and erythromycin and rosuvastatin, respectfully. As can be seen in Table 2, the rank order across the eight drugs was different for the two methods. Also shown in Table 2 is the ratio of the two methods which varied from one to almost 500.

Concentration dependence was seen over the range of 0.1 to 10 µM for five of the eight drugs either in both systems (atorvastatin, erythromycin and saquinavir) or one system - conventional (fexofenadine) or media loss (pitavastatin) approaches. In all cases, $K_m$ values obtained for the two systems were not statistically different and were in the low µM region (see Table 2). For clarithromycin, repaglinide and rosuvastatin there was no evidence of concentration dependence over the initial concentration range studied. Fig. 4 shows examples of the concentration dependence for atorvastatin and fexofenadine. In the latter case the $K_m$ is similar to that reported by us using the oil-spin method, however for atorvastatin this value was an order of magnitude lower (Yabe et al., 2011).

The data for the four drugs (atorvastatin, fexofenadine, pitavastatin and rosuvastatin) that showed a clear difference between the two methods were modelled according to the two compartment approach described (Fig. 2) and the parameter values obtained are shown in Table 3. Five parameters were obtained to describe clearances for uptake and metabolism, ($CL_{uptake}$, $CL_{met}$) and passive transport ($P_{diff}$) together with the media and the cell volume terms - $V_{media}$ and $V_{cell}$ (Table 3). The clearance due to metabolism showed good agreement with the apparent clearance observed in the conventional method for all four compounds. However greater variability was evident in these parameters (Table 3). This is larger than previously reported for the
oil-spin method (Yabe et al., 2011) and for comparable data analysis with experiments using monolayers of hepatocytes (Menochet et al., 2011).

In the case of the uptake clearance, good agreement was seen between the model parameter and the media loss slope only for fexofenadine. For atorvastatin (where there is extensive metabolism) the model value was reasonably similar but in the case of pitavastatin and rosuvastatin a lower value was obtained using the model. $P_{\text{diff}}$ values were also obtained from the model and was lowest for fexofenadine (13 µl/min/10^6 cells) but similar for the other three compounds in (162-234 µl/min/10^6 cells). The $V_{\text{media}}$ values were similar for all four compounds and close to the actual volume of the incubation (250 µl). The model parameters showed no marked difference when this volume term was restrained to 250 µl. $V_{\text{cell}}$ showed a 10-fold range over the four compounds, the rank order being rosuvastatin < pitavastatin < fexofenadine < atorvastatin which is consistent with the $f_u_{\text{cell}}$ values previously determined (see Table 1).

When compared with published values using the oil spin method (Yabe et al., 2011) $P_{\text{diff}}$ were considerably greater and CL$_{\text{uptake}}$ were lower for atorvastatin and fexofenadine whereas they were higher for rosuvastatin and pitavastatin. Also the rank order did not coincide however it should be noted that the spread of the parameter values are quite minimal for these four drugs.

Fig. 5 illustrates the relationship between CL$_{\text{uptake}}$ and $P_{\text{diff}}$ in defining the ratio of cellular to media concentrations ($K_{p_u}$) based on equation 4. Each of the eight drugs studied are identified on this three dimensional surface, it is clear that the four drugs
corresponding to Type A fall within the top half with $K_{pu}$ values in excess of ten. In contrast Type B compounds have combinations of $P_{diff}$ and $CL_{uptake}$ which gave $K_{pu}$ values $< 10$. 
Discussion

The under-prediction of in vivo clearance from both hepatic microsomal and isolated hepatocyte studies has been evident by several recent analyses of published reports (Soars et al., 2007a; Hallifax et al., 2010). While it has been acknowledged that in vitro methods underperform kinetically relative to in vivo, there is also a need to ensure that all clearance processes are considered, so the scaling of in vitro parameters is not compromised (Houston 1994). Attention has been drawn to the impact of hepatic transporters as a mechanism for underprediction and hence the need to incorporate this phenomenon into in vitro-in vivo extrapolation (Soars et al., 2007b). Examples of successful scale up of OATP transported drugs from in vitro kinetic studies using the oil-spin method have been reported (Watanabe et al., 2009 and 2010).

There is a need for methodology for routine assessment of hepatic uptake that has faster throughput than the established oil-spin approach. The use of a monolayer of hepatocytes which allows successive washing prior to cellular drug determination offers one alternative approach (Menochet et al., 2011). Another methodology has been proposed by Soars et al., 2007b who have adapted the standard depletion approach, widely used for metabolic clearance determination, to allow assessment of hepatic uptake. The notion of dual incubations to determine hepatic uptake in addition to hepatic metabolism is attractive. However certain criteria need to be established regarding the rate limiting processes operating in this system for particular drugs, namely uptake or metabolism/efflux. If uptake clearance exceeds metabolic clearance
a difference in the two methods would be anticipated, i.e. when the rate limiting step is not the initial uptake process.

In the current investigations with eight drugs known to be OATP substrates, comparison of the conventional assay and the media loss assay has led to the identification of two groups. The first group (Type A - atorvastatin, fexofenadine, pitavastatin and rosuvastatin) is characterized by a higher predicted CL_{int} using the media loss assay than the conventional assay and this can be explained by a markedly faster uptake clearance than metabolic clearance. In the second group, (Type B - clarithromycin, erythromycin, repaglinide and saquinavir) the same profiles were obtained with both methods and this may be explained by several situations including either the lack of active transport, an uptake rate limitation for metabolic clearance or relatively similar clearance values; further experimentation would be needed to discriminate between these options.

P_{diff} in addition to CL_{active} appeared to play a strong role in defining whether Type A or Type B behaviour was observed in this particular series of eight compounds where P_{diff} ranged 40-fold. For Type A compounds P_{diff} was minor (<10% of CL_{active}), whereas for Type B compounds P_{diff} was at least 20% of CL_{active} and in two cases (erythromycin and saquinavir) the two parameters were comparable. K_{pu} is a valuable metric reflecting the relative values of these two terms. The combined role of passive and active process in controlling drug uptake has been recently reviewed (Sugano et al., 2010).
Binding within the cell ($f_{u_{\text{cell}}}$) appears to be independent of the type of behaviour observed. For example, atorvastatin and rosuvastatin have widely different $f_{u_{\text{cell}}}$ values but yet are both Type A and saquinavir and erythromycin, both Type B, also have quite different $f_{u_{\text{cell}}}$ values (Table 1). There is good agreement between the $f_{u_{\text{cell}}}$ values previously reported and the $V_{\text{cell}}$ values obtained from modelling the current data. While cellular binding might be expected to result in biphasic depletion-time profiles, these would not be unique to the media loss assay, as exemplified by clarithromycin and saquinavir in this study.

The $CL_{\text{met}}$ values for the eight compounds differ significantly. In the case of saquinavir, metabolic clearance is known to be uptake rate limited (Parker and Houston, 2008) whereas clarithromycin (Brown et al., 2010) and repaglinide (Menochet et al., 2011) are known to be metabolism rate limited. It would be expected that erythromycin would show similar behaviour to clarithromycin; atorvastatin also appears to show similar characteristics (Paine et al., 2008). Thus whether a compound falls into the Type B category or the Type A category appears not to be related to its rate limiting step in hepatic clearance.

Three of the four Type A drugs are known to show minimal metabolism and the slow metabolic clearance from the conventional method is to be expected. Thus the biphasic behaviour using the media loss methodology indicating rapid uptake prior to metabolism/excretion is consistent. Although efflux transporters are internalised during the isolation of fresh hepatocytes (Bow et al., 2008) the clearance term designated as metabolism may include some cellular efflux activity. Atorvastatin also showed Type A behaviour despite its extensive and rapid metabolism. It would
appear that the uptake process for atorvastatin is faster than its metabolism. For Type A compounds there will be accumulation within the cell as metabolism/efflux is the rate limiting step governing the intracellular unbound concentration for these drugs.

There is some ambiguity in the transporter literature regarding the terms rate limiting and rate determining in describing the role of transport in hepatocellular uptake (Parker and Houston, 2008; Brown et al., 2010; Watanabe et al., 2010). The rate limiting step in kinetic terms reflects the slowest step in a catenary sequence, thus only when the uptake by transporters into the cell has a slower clearance than the metabolism will hepatic disposition be uptake rate limited. However, use of the terminology rate determining is more descriptive and can be applied to the overall loss of drug from the plasma (external media) and should be viewed differently. Thus the action of transporters can influence hepatocellular uptake without being the rate limiting step. There are examples where biliary excretion clearance is less than hepatic uptake clearance, for example napsagatrán (Poirer et al., 2009) as well as examples where metabolic clearance is slower than uptake clearance as in the case of saquinavir and nelfinavir (Parker and Houston, 2008). For drugs with metabolism/efflux as the rate determining step for hepatic disposition, extensive intracellular accumulation of unbound drug would be expected. This is not always the case as passive permeability also contributes to the parameter \( K_{pu} \) (see equation 5); this parameter appears to be a particularly valuable guide to the hepatocellular kinetic behaviour.

The use of a range of initial drug concentrations allowed saturation of uptake as well as metabolism to be identified. This was evident for atorvastatin and pitavastatin, but no indication of nonlinearity was seen for clarithromycin, repaglinide or rosuvastatin.
For erythromycin, saquinavir and fexofenadine there are inconsistencies between the two methods, suggesting that a more robust methodology (oil-spin or monolayer methods where a substantial amount of kinetic data are generated) is required for more detailed analysis. The high imprecision evident in the modelling of Type A drugs would support this notion as the use of biexponential equations (eq. 2) requires a reasonable number of data points in both phases and practically this is difficult to achieve, as illustrated in Figure 3A.

It is concluded from these investigations that the dual incubation approach cannot be relied upon to identify the importance of hepatic transporters for a given drug. In the present study this approach proved to be successful in 50% of the eight cases where a biphasic decline was evident with the media loss but not conventional methodology (Type A behaviour). However, whilst transporter activity may not always be identified in the dual incubation approach, an accurate estimate of hepatic clearance from the media is still obtained and this may reflect uptake or metabolism. Hence if the outcome is Type B behaviour, and the same clearance value is obtained in both methods. The media loss rather than conventional approach may prove to be the method of choice for drugs where transporters are anticipated and this safer option may provide more reliable predictions. However detailed studies involving more robust methods such as the oil-spin or monolayer methods will be required subsequently to improve precision of parameter estimation and to provide mechanistic information reflected in the parameters $V_{\text{max}}$, $K_m$, $P_{\text{diff}}$ and $K_p$. 
Acknowledgements

The Authors would like to thank Sue Murby and Dr David Hallifax (University of Manchester) for valuable assistance with the LC-MS/MS and Dr Aleksandra Galetin for useful discussions.

Authorship Contributions

Participated in research design: Jigorel and Houston

Conducted experiments: Jigorel

Contributed new reagents or analytical tools: N/A

Performed data analysis: Jigorel

Wrote or contributed to the writing of the manuscript: Jigorel and Houston
References


DMD#45732


Sjögren E, Lennernäs H, Andersson TB, Gråsjö J and Bredberg U (2009) The multiple depletion curves method provides accurate estimates of intrinsic clearance (CL_{int}), maximum velocity of the metabolic reaction (V_{max}), and Michaelis Constant (K_{m}): Accuracy and robustness evaluated through experimental data and Monte Carlo simulations. *Drug Metab Dispos* **37**:47-58.


Yih TD and van Rossum JM (1977) Isolated rat hepatocytes and 9000g rat liver supernatant as metabolic systems for the study of the pharmacokinetics of barbiturates. *Xenobiotica* **7**:573-582.
Footnotes

The work was funded by a consortium of pharmaceutical companies (GlaxoSmithKline, Lilly, Pfizer and Servier) within the Centre of Applied Pharmacokinetic Research at the University of Manchester.

Parts of this work were previously presented at the 11th European Regional ISSX Meeting, Lisbon, Portugal, May 17-20, 2009 – Jigorel E and Houston JB, 2009. Involvement of drug uptake in hepatic clearance. Drug Metab Rev 41: (Suppl 1) 66.

Current address: Emilie Jigorel, UCB Pharma SA, Drug Metabolism & Pharmacokinetics, Braine l’Alleud, Belgium
Figure Legends

FIG. 1: *Relationship between the parameters obtained by modelling, conventional and media loss assays*

FIG. 2: *The 2-compartment model used to describe drug uptake and clearance*

FIG. 3: *Substrate depletion time profiles in rat hepatocytes for fexofenadine (A) and clarithromycin (B) at 0.1 µM.*

Fraction remaining-time data were generated using either a conventional assay (black symbols) or a media loss assay (white symbols). Each value represents the mean of three determinations in rat hepatocytes.

FIG. 4: *Concentration dependence of in vitro CL_{int} obtained using conventional assay and media loss assay involving data obtained with atorvastatin (A) and fexofenadine (B).* The solid line represents the line obtained using equation 3.

FIG. 5: *Relationship between passive permeability (P_{diff}), uptake (CL_{uptake}) and unbound liver to plasma concentration ratio (K_{pu})*.

Shading shows progressive increases in K_{pu} from 0.01 to 10,000 and the critical values of 10 and 100 uL/min/10^6 cells for P_{diff} and CL_{active}, respectively for defining K_{pu} of 10. Specific drugs with K_{pu} values are shown: atorvastatin, A (494); rosuvastatin, R (58); fexofenadine, F (25); pitavastatin, P (23); repaglinide, Re (8); clarithromycin, C (6); erythromycin, E (2) and saquinavir, S (2).
Table 1 - Hepatic uptake and metabolism characteristics of 8 drugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>CL_{active} (µL/min/10^6 cells)</th>
<th>P_{diff}</th>
<th>K_{pu}^a</th>
<th>f_{u,cell}^a</th>
<th>Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>1500</td>
<td>5.35</td>
<td>494</td>
<td>0.056</td>
<td>Extensive</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>50.7</td>
<td>10.9</td>
<td>6.05</td>
<td>0.024</td>
<td>Moderate</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>11.4</td>
<td>12.2</td>
<td>2.00</td>
<td>0.05</td>
<td>Moderate</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>167</td>
<td>6.75</td>
<td>25.5</td>
<td>0.10</td>
<td>Minor</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>197</td>
<td>18.8</td>
<td>22.9</td>
<td>0.028</td>
<td>Minor</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>299</td>
<td>58.2</td>
<td>7.94</td>
<td>0.036</td>
<td>Extensive</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>418</td>
<td>7.08</td>
<td>58.2</td>
<td>0.48</td>
<td>Minor</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>239</td>
<td>191</td>
<td>2.34</td>
<td>0.0075</td>
<td>Extensive</td>
</tr>
</tbody>
</table>

Values are taken from Yabe et al., 2011

^a Calculated from equations 5 and 6
Table 2 - Comparison of clearance and $K_m$ values obtained by the conventional and media loss methods

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conventional Method$^a$</th>
<th>Media Loss Method$^a$</th>
<th>Ratio of clearance by two Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$CL_{int}$ ($\mu l/min/10^6$ cells)</td>
<td>$K_m$ ($\mu M$)</td>
<td>$CL_{int}$ ($\mu l/min/10^6$ cells)</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>113 ± 38</td>
<td>1.3 ± 0.7</td>
<td>459 ± 233</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>46 ± 11</td>
<td>-</td>
<td>68 ± 11</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>26 ± 15</td>
<td>3.5 ± 0.9</td>
<td>35 ± 20</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>17 ± 1.7</td>
<td>4.7 ± 2.1</td>
<td>117 ± 16</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>7.1 ± 2.3</td>
<td>-</td>
<td>1705 ± 466</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>49.9 ± 3.5</td>
<td>-</td>
<td>49.3 ± 14</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>3.3 ± 1.9</td>
<td>-</td>
<td>1657 ± 849</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>1675 ± 69</td>
<td>2.1 ± 1.2</td>
<td>1469 ± 208</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SD for $n = 3$
Table 3 – Modelling the depletion-time profiles for the conventional and media loss methods at 0.1 µM initial substrate concentrations

<table>
<thead>
<tr>
<th>Compound</th>
<th>CL&lt;sub&gt;met&lt;/sub&gt;</th>
<th>CL&lt;sub&gt;uptake&lt;/sub&gt;</th>
<th>P&lt;sub&gt;diff&lt;/sub&gt;</th>
<th>V&lt;sub&gt;cell&lt;/sub&gt;</th>
<th>V&lt;sub&gt;medium&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µL/min/10⁶ cells</td>
<td>µL/10⁶ cells</td>
<td>µL</td>
<td>µL</td>
<td>µL</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>77.4 ± 5.1</td>
<td>393 ± 576</td>
<td>234 ± 303</td>
<td>2164 ± 370</td>
<td>198 ± 29</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>13.5 ± 2.2</td>
<td>115 ± 136</td>
<td>42.6 ± 23.6</td>
<td>912 ± 384</td>
<td>221 ± 25</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>7.2 ± 3.7</td>
<td>716 ± 255</td>
<td>162 ± 21</td>
<td>555 ± 185</td>
<td>211 ± 29</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>3.1 ± 1.2</td>
<td>627 ± 236</td>
<td>190 ± 24</td>
<td>192 ± 22</td>
<td>186 ± 37</td>
</tr>
</tbody>
</table>
Figure 1

\[ CL_{int,cube} = CL_{met} \cdot \frac{CL_{up} - CL_{int,cube}}{CL_{met} + P_{diff}} \]
Figure 2

![Diagram showing CL_{uptake}, V_{media}, P_{diff}, Cell, V_{cell}, CL_{met} relationships]
Figure 4

A

\[ K_{d_{ep}} \text{ (min}^{-1} \text{)} \]

\[ \text{[Atorvasatin] (µM)} \]

\[ 0.01 \ 0.1 \ 1 \ 10 \ 100 \]

\[ 0 \ 0.02 \ 0.04 \ 0.06 \ 0.08 \ 0.1 \ 0.12 \ 0.14 \ 0.16 \ 0.18 \ 0.2 \]

B

\[ k_{d_{ep}} \text{ (min}^{-1} \text{)} \]

\[ \text{[Fexofenadine] (µM)} \]

\[ 0.01 \ 0.1 \ 1 \ 10 \ 100 \]

\[ 0.002 \ 0.004 \ 0.006 \ 0.008 \ 0.01 \]