UPTAKE AND INTRACELLULAR BINDING OF LIPOPHILIC AMINE DRUGS
BY ISOLATED RAT HEPATOCYTES AND IMPLICATIONS FOR PREDICTION OF IN
VIVO METABOLIC CLEARANCE

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Running title: Predicting metabolic clearance from isolated hepatocyte data

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Abbreviations: ABT, aminobenzotriazole; CL_{int}, intrinsic clearance; f_u, fraction unbound; K_p, cell to medium concentration ratio; WME, Williams’ Medium E.
Abstract

The hepatic uptake of imipramine and propranolol was investigated following incubation with isolated rat hepatocytes over a wide concentration range (0.04 - 400 µM). The cell-to-medium concentration ratio ($K_p$) was concentration-dependent and could be described using a two-site binding model incorporating a high affinity/low capacity site and a linear component for a site which was apparently not saturated. Maximum (at 0.04 µM) and minimum $K_p$ values (at 400 µM) were 360 and 280 and 110 and 70 for imipramine and propranolol, respectively. During these incubations, metabolism was inhibited using aminobenzotriazole (an irreversible inhibitor of P450). Pre-treatment of cells either by freeze-thawing or with saponin (which permeabilises the plasma membrane) eliminated the saturable process. The saturable uptake process of imipramine was also inhibited by 18 other lipophilic amine drugs (including propranolol). This uptake component may involve membrane transporter(s) whereas the non-saturable component probably represents partition into the phospholipid component of membranes. Propranolol was further investigated to determine the impact of high $K_p$ values on hepatocellular clearance. The area under the curve for propranolol concentrations in the total incubate (medium including the cells) from the depletion-time profile was substantially greater than the corresponding area under the curve for the drug concentration in the extracellular medium and this difference approximated to the non-saturable uptake component. It is concluded that the clearance of propranolol in isolated hepatocytes is not rate limited by hepatic uptake and is directly proportional to unbound drug concentration being independent of the higher $K_p$ value.
Introduction

The ability to predict in vivo drug clearance from metabolite kinetic parameters generated in vitro is valuable for the selection, and ultimately for the a priori design, of new pharmacological agents based on pharmacokinetic properties (Lin and Lu, 1997; McGinnity and Riley, 2001; Obach, 2001). Studies with rat hepatocytes have been instrumental in the acceptance of simple scaling factors (such as hepatocellularity) to express in vitro kinetic parameters in terms of the whole liver rather than per unit of enzyme (Houston, 1994). There is excellent concordance between in vitro predictions from freshly isolated hepatocytes and in vivo values for both $CL_{int}$ and hepatic clearance in rat (Ito and Houston, 2004).

Furthermore human cryopreserved hepatocytes are becoming more widely used and their utility in predicting human clearance is encouraging (Lau et al., 2002; McGinnity et al., 2004; Hallifax et al., 2005).

While an analogous scaling strategy is applicable for hepatic microsomes, there are additional complications implicit with the use of this subcellular system and these have become evident in the prediction of highly cleared drugs. For several drugs it has been clearly demonstrated in rat that in vivo predictions are less accurate from microsomes than from freshly isolated hepatocytes (Jones et al., 2005; Houston and Carlile, 1997). This situation is of concern for human prediction studies as, due to the limited availability of fresh human tissue of high quality, there is a heavy reliance on human liver microsomal preparations. A clearer understanding of the relationships between $CL_{int}$ and other kinetic measurements determined in isolated cells and in microsomal preparations would allow priorities to be assigned for the most appropriate use of cell or microsomal incubations and ensure optimal use of valuable fresh human tissue.
A key question is: what drug concentration is available to the enzyme? As accumulation of drugs in hepatocytes may occur via active transport processes and/or intracellular binding, a concentration difference may exist for hepatocytes and microsomes and this may impact on the determination of hepatic clearance. An important consideration for hepatocyte drug accumulation is whether the drug within the cell is in the form of bound or free drug. Intracellular binding to sites not involved in the metabolic process may be of little consequence, as the free concentration in the cell will be in equilibrium with the external incubation media concentration (Austin et al., 2005). In the case of uptake transporters however raising the cellular concentration in excess of the incubation medium concentration will result in more drug available to the enzyme. Resolution of this situation is of importance not only for the assessment of clearance but also for prediction of drug-drug interactions resulting from inhibition of drug clearance (Rostami-Hodjegan and Tucker, 2004; Yao and Levy, 2002).

Lipophilic amine drugs are of particular interest for hepatic uptake, as many have been widely reported to extensively partition into this tissue (Fichtl et al., 1991; Rodgers et al., 2004; Austin et al., 2005). The first aim of these investigations was to characterize drug uptake into isolated rat hepatocytes for two prototypic drugs, imipramine and propranolol, over a wide concentration range. In these studies metabolism was blocked using a nonspecific and irreversible inhibitor of P450-mediated metabolism, ABT (Ortiz de Montellano and Correia, 1995), that is not hepatotoxic (Shiba and Shimanato, 1999) and the rapid separation of cells from the incubation medium was achieved by centrifugation through silicone oil (Miyauchi et al., 1993). To explore the relative importance of the processes of transport and binding, the uptake was also investigated under conditions where the structural
and functional integrity of the cell was altered, namely freeze-thawing and by pre-incubation with saponin (a plasma membrane permeabiliser (Mick et al., 1988). In order to characterize the process further, the uptake was also investigated in the presence of 24 potential competitors possessing a range of physico-chemical characteristics. Our second aim was to explore the impact of hepatic accumulation of drug on metabolic clearance in hepatocytes. Propranolol was selected as the most appropriate lipophilic basic drug for further investigation as it undergoes several independent parallel routes of metabolism (Bargar et al., 1983; Masubuchi et al., 1993). In contrast imipramine has been documented to show complex kinetics due to product inhibition from its demethylated metabolites (Chiba et al., 1990a and b). A time course study of propranolol depletion in a suspension of freshly isolated hepatocytes was undertaken by sampling from various incubation matrices (intact cells, medium and total incubation).
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Materials and Methods

Chemicals. [3H]Imipramine HCl (733 GBq/mmoll), (S)-[3H]propranolol HCl (555 GBq/mmoll), [3H]water (37.0 MBq/ml) and [14C]sucrose (25.0 GBq/mmoll) were obtained from GE Healthcare (Amersham, UK). Imipramine (non-radiolabelled), (S)-propranolol, (R)-propranolol, clomipramine, trimipramine, amitriptyline, nortriptyline, metoprolol, atenolol, chlorpromazine, promethazine, quinine, quinidine, chloroquine, verapamil, diltiazem, diazepam, warfarin, nifedipine, caffeine, serotonin (5-hydroxytryptamine), aminobenzotriazole (ABT), saponin, rotenone, Williams’ Medium E cell culture medium (WME), trypan blue and Folin and Ciocalteu’s phenol reagent were obtained from Sigma-Aldrich (Poole, UK). Collagenase A was obtained from Boehringer Ltd. (Lewes, UK). Silicone oil 510/50 (density 0.99) and 550 (density 1.07) were obtained from BDH Ltd. (Poole, UK). Optiphase HiSafe 2 and Ultima-Flo M scintillant was obtained from PerkinElmer (Milton Keynes, UK). All other chemicals were of analytical grade. 2-Hydroxyimipramine, desmethyliimipramine, sertraline and amlodipine were donated by Pfizer Ltd. (Sandwich, UK). Ondansetron was donated by GSK (Ware, UK). 4-Hydroxypropranolol and N-desisopropylpropranolol were donated by Professor G. Tucker, University of Sheffield, UK. 5-Hydroxypropranolol and 7-hydroxypropranolol were donated by Dr T. Walle, Medical University of South Carolina, USA.

The radiolabelled imipramine and propranolol were checked for radiochemical purity during and after the experimental phase using radio-thin layer chromatography (Raytest RITA linear analyzer (LabLogic, Sheffield, UK) and were found to be sufficiently radiochemically pure (>95 %) to achieve the experimental objectives.
Animals and Treatment. Male Sprague-Dawley rats (240 - 260 g) were obtained from the University of Manchester Biological Services Unit. They were housed 2 - 4 per cage on a bedding of sawdust in rooms maintained at a temperature of 20 ± 2 °C and a humidity of 45 - 55 % with a 12 hr light/dark cycle. They had free access to water and Standard Rat and Mouse Experimental Laboratory Diet (B & K Universal, Hull, UK).

Preparation of hepatocytes and incubations of drug with hepatocytes. Isolated rat hepatocytes were prepared from livers of male rats by collagenase perfusion using a method based on that of Berry and Friend (1969), as detailed by Hayes et al., (1995). [3H]Imipramine or [3H]propranolol was diluted with non-radiolabelled drug in WME to give incubation concentrations of 0.04, 0.2, 0.4, 2, 4, 20, 40, 200 and 400 µM (approximately 70 kBq/ml). Hepatocytes were diluted in WME or WME containing ABT to give incubation concentrations of 0.5, 1, 2, 5 and 10 x 10⁶ cells/ml and a concentration of ABT (where applicable) of 5 mM. Substrate solution (0.25 ml), maintained at 37 °C, was placed in an Eppendorf tube (2 ml capacity) containing a layer of silicone oil (510/50:550 (3:2, density 1.002), 0.1 ml) over a layer of 5 M KOH (0.05 ml). The tube was then placed in an MSE Microcentaur microcentrifuge and the incubation was started by the addition of a portion of cell suspension (0.25 ml) which had been maintained at 37 °C for approximately 15 minutes after preparation. After an incubation period of either 0, 5, 10, 20 or 30 s (for cell concentrations of 2 x 10⁶ cells/ml, and 30 s for other cell concentrations), the incubate was centrifuged at maximum speed for about 20 s. The tube was then placed in powdered dry ice for at least 30 minutes before separation of the tube tip (containing the separated cells) at a position within the oil layer, using a microtube cutter. The tube tip was collected in a plastic
scintillation vial (5 ml capacity) to which was added distilled water (0.5 ml). The remainder of the incubation tube was placed over another Eppendorf tube to collect the supernatant. The incubations described above were repeated with hepatocyte preparations from two other livers in order to incorporate the variability between livers and cell preparations. The extent of the effect of ABT was investigated in similar experiments (with and without ABT pre-treatment) in which the individual drug-related components in the cells were quantified after an incubation period sufficient to reach maximum uptake of radiolabel (30 s). A limited range of low initial concentrations (at which the greatest contribution of metabolism would be expected) was used. The hepatocytes were collected in 0.2 M carbonate buffer (pH 10, 0.05 ml) under the oil. The stability of imipramine and propranolol throughout the incubation, separation in buffer and extraction was assessed in a parallel experiment conducted as described above except without hepatocytes (replaced with WME). Extended incubations of [3H]propranolol (at 0.04 µM in 2 x 10^6 cells/ml) were performed with separation of cells into 0.2 M carbonate buffer (pH 10) (as described above) after 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 s.

To investigate the dependence of uptake on cellular integrity, incubations of imipramine were performed with hepatocytes which had either been freeze-thawed (by placing in a freezer for approximately 30 minutes and thawed by incubating at 37 °C for 30 minutes), pre-treated with ABT (5 mM) and saponin (0.05 %), to give an incubation concentration 1 mM ABT and a saponin concentration of 0.005 %, or pre-treated with ABT (5 mM) and rotenone (0.3 mM), to give an incubation concentration 1 mM ABT and a rotenone concentration of 30 µM.
To investigate the suspectability of the uptake process to competition, incubations of imipramine with hepatocytes were performed with putative competitor drugs to give an incubation concentration of 0.4 µM imipramine and competitor concentrations of either 0.4 or 400 µM; the hepatocytes were pre-treated with ABT (5 mM). Selected inhibitors were subsequently incubated with either imipramine or propranolol (0.4 µM) at concentrations of 0.2, 0.4, 2, 4, 20, 40, 200 and 400 µM.

**Analysis of separated cells.** After digestion of the cells (approximately 24 hours), Optiphase HiSafe 2 scintillant (5 ml) was added to each vial which was capped, shaken and, after a period of several hours, analysed by LSC (using isotope spectrum shape analysis quench correction, over a 5 minute counting period). Duplicate portions (0.025 ml) of the substrate solutions were mixed with scintillant and analysed by liquid scintillation counting (LSC) using a Wallac 1409 counter (PerkinElmer, Milton Keynes, UK).

Cells separated into carbonate buffer (pH 10, 1 ml) were shaken with ethyl acetate (3 ml, for imipramine) or tert-butyl methyl ether (2 ml, for propranolol) using an SMI Multi-tube Vortexer, for approximately 1 min. Each tube was then centrifuged using an MSE Mistral 1000 centrifuge at approximately 1000 rpm for 5 min after which the organic layer was transferred to a separate tube. The extraction was repeated once and the combined organic phase was sampled (by duplicate aliquots of 0.05 ml) then evaporated under a stream of nitrogen (oxygen-free) at approximately 30 °C. The aliquots were mixed with scintillant (Optiphase HiSafe 2, 5 ml) and analysed by LSC. The evaporated samples were stored at about -20 °C and subsequently resuspended in mobile phase (0.2 ml) and centrifuged (Microcentaur, maximum speed) for 5 minutes immediately prior to chromatographic analysis. The aqueous phase remaining after extraction was mixed with scintillant (Optiphase
Hisafe 2, 10 ml) in glass scintillation vials (20 ml capacity) and analysed by LSC. The cell extracts were assayed by high performance liquid chromatography using a Gilson 231 XL Sample Injector and Inertsil ODS(2) column (Gasukuro Kogyo, Japan) reverse phase (ODS) column with isocratic elution of acetonitrile: acetic acid (0.5 M), sodium acetate (0.1 M), triethylamine (0.1 %), pH 3.5 (35:65) (imipramine) or acetonitrile/methanol (1/1): water/acetic acid (56/1), triethylamine (0.1 %), pH 3.5 (30:70) (propranolol) at a flow rate of 1ml/min (maintained by a Waters 510 pump, Waters, Watford, UK). Detection was by UV absorbance (254 nm, Applied Biosystems 759A Absorbance detector (AnaChem, Luton, UK)) (imipramine) or fluorescence (excitation: 310 nm; emission: 380 nm, Hewlett Packard HP 1046 Fluorescence Detector (Agilent, Winnersh, UK)) (propranolol) and by radioactivity (tritium energy range) using flow-through liquid scintillation counting (Packard Radiomatic Flo-One/beta Series A-500 Radio-chromatography Detector (PerkinElmer, Pangbourne, UK)) at an eluant: scintillant ratio of 1:3. The stability of imipramine and propranolol in the extraction procedure was demonstrated by complete extraction (>90 %) of radiolabel from the incubation medium and resolution of parent drug only, by radio-chromatography.

**Cellular protein, cellular volume and adherent water layer volume determination.** The amount of drug in cells was expressed relative to total cellular protein to correct for the differences in the number of cells between preparation batches; the cell protein was assayed using a method based on that of Lowry et al. (1951). In order to evaluate the uptake of drug in terms of cellular concentration, a method for determination of cell volume, based on Miyauchi et al. (1993), was used. Isolated hepatocytes from each preparation were incubated for 30 s with \[^3\text{H}\]water and \[^{14}\text{C}\]sucrose in WME (approximately 10 kBq/ml of each radionuclide). The volume occupied by the radiolabelled water in the cells was determined on the basis that the \[^3\text{H}\]water was assumed to be in equilibrium with the external medium.
and hence the volume was proportional to the initial radiochemical concentration of the $[^3\text{H}]$water. The volume of the cells after separation from the incubation medium by centrifugation through oil was determined after correction for the volume of external adherent water. The volume of the adherent layer was determined by the radiochemical concentration of the $[^1\text{4}\text{C}]$sucrose which was assumed not to have penetrated the cells. The separated cells and aliquots (0.025 ml) of the preparation of radiolabelled drugs in WME were assayed for radioactivity from each radionuclide, simultaneously, by LSC. The total cell volume was calculated from the difference between the ratio of tritium and $^{14}\text{C}$ for separated cells relative to incubation medium. The total cellular volume and total protein was found to increase linearly for the range of cell concentrations used (0.5 - 10 x $10^6$ cells/ml). The total volume was therefore proportional to the cellular protein and the mean volume per mg. protein was $2.2 \pm 0.46 \mu\text{l}$ ($n = 41$). Although the volume of adherent medium was appreciable (95% of cellular volume), the amount of drug it contained was relatively minor due to the high relative affinity of the drugs for the cells.

**Data analysis.** The concentration of drug in the cells ($C_{\text{cell}}$) was calculated using the total amount of drug in cells and the cell volume, for each hepatocyte preparation (Equation 1). The concentration of drug in the medium ($C_{\text{M}}$) was calculated using the total amount of drug in the medium and the total medium volume (total incubation volume minus the total cell volume).

$$C_{\text{cell}} = \frac{A_{\text{cell/ext}} - (C_{\text{M}} \cdot V_{\text{ext}})}{V_{\text{cell}}}$$  \hspace{1cm} (1)
where $A_{\text{cell/ext}} = \text{amount of drug in cells and adherent layer}$; $V_{\text{ext}} = \text{volume of the adherent (external) layer}$; $V_{\text{cell}} = \text{total volume of the cells}$.

The hepatocyte: medium partition coefficient ($K_p$) was calculated based on the ratio of the concentration of drug in the cell to the concentration of drug in the medium as shown in Equation 2.

$$K_p = \frac{C_{\text{cell}}}{C_{\text{M}}}$$ (2)

The accumulation of drug in hepatocytes was quantified in mass action terms on the assumption that intra-cellular binding represented the major mechanism for high $K_p$ values. The concentration of bound drug in the cells was related to the concentration of the unbound drug with a one-site ligand binding model incorporating a linear function for unsaturated binding (Equation 3) using the GraFit (Erithacus Software Ltd.) nonlinear regression modelling program:

$$C_b = \frac{B_{\text{max}} \cdot C_u}{K_d + C_u + B_{\text{NS}} \cdot C_u}$$ (3)

where $C_b = \text{concentration of bound drug}$; $C_u = \text{concentration of unbound drug}$; $B_{\text{max}} = \text{concentration of binding sites (Site 1)}$; $K_d = \text{binding dissociation equilibrium constant}$; $B_{\text{NS}} = \text{non-specific binding constant}$.
The unbound fraction of drug \((f_u)\) in the cells was calculated using \(K_p\) as shown in Equation 4 based on the assumptions that the unbound concentration of drug in the cell is equal to the unbound concentration in the medium and that no active transport occurs.

\[
\text{Intracellular } f_u = \frac{1}{K_p} \tag{4}
\]

Based on the ratio of the cell volume to the incubation volume and the measured hepatocyte:media \(K_p\), the unbound fraction in the whole incubation was calculated using Equation 5.

\[
\text{Incubation } f_u = \frac{1}{1 + K_p \cdot \frac{V_c}{V_{inc}}} \tag{5}
\]

where \(V_c = \text{cell volume and } V_{inc} = \text{incubation volume. Equation 5 was derived from mass balance considerations, again assuming the unbound concentration of drug in the cell is equal to the unbound concentration in the medium and no active transport occurs (Jones et al., 2004).}

The radiochromatographic components extracted from the cells were integrated as proportions of total radioactivity detected for each sample. These proportions were used to convert the total amount of drug-related compounds in the cells to total amounts of the principal components separated, after correction for loss in extraction.

In the studies where drug was co-incubated with competitor, the amount of drug in the cells was expressed relative to when no competitor was present. Where a wide range of competitor
concentrations was used, these values were related to the concentration of the competitor with equation 6 for one-site ligand binding competition (incorporating a linear component for an unsaturated site) using the GraFit program:

\[
B_1 = sB_{\text{max}} + B_{\text{NS}} - \frac{sB_{\text{max}} \cdot C_I}{IC_{50} + C_I}
\]  

where: \(B_1\) = relative proportion of uncompetted binding; \(sB_{\text{max}}\) = maximum proportion of saturable uncompetted binding; \(IC_{50}\) = concentration of competitor reducing binding by 50% (assuming a one site model); \(C_I\) = concentration of competitor; \(B_{\text{NS}}\) = constant for unsaturated binding.

The area under the concentration-time curve for propranolol in the cell, medium and total incubate was calculated using the trapezoidal method and the clearance of propranolol from the medium was calculated from the area under the concentration-time curve.
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Results

Characteristics of uptake. The time course of uptake of radiolabelled imipramine was very rapid (see Figure 1A which expresses the data as the total amount of drug in the cells normalized by the initial drug concentration). The positive intercept at zero incubation time represented a substantial proportion of the total drug and reflected a lag period in the cell separation procedure (estimated to be about 10 s). There was a trend for a decrease in the rate of uptake as incubation concentration increased but at all concentrations a plateau value was achieved within 30 s. Similar time profiles were seen with propranolol (Figure 1B).

The plateau values for cellular uptake, used to calculate $K_p$, were dependent on the initial concentration for both drugs (Figure 2A). The mean maximum values were approximately 360 for imipramine and 280 for propranolol (at <1 µM) and this decreased to a minimum of 110 for imipramine and 70 for propranolol (at >100 µM). The cellular accumulation for both drugs was fitted by nonlinear regression to a two-site model incorporating a high affinity, low capacity site and a low affinity, high capacity site not saturated over the concentration range studied; the maximum concentration accumulated ($B_{\text{max}}$), the dissociation constant ($K_d$) and the constant for the linear accumulation ($B_{\text{NS}}$) are given in Table 1. The $K_p$ at all drug concentrations was independent of the cell concentration used (range 0.5 - 10 x 10^6 cells/ml).

The rate and extent of accumulation of radiolabel was determined in hepatocytes pre-treated with ABT, an irreversible inhibitor of P450, and was found to be similar to those for control cells for both drugs (Table 1).

Uptake of imipramine into disrupted hepatocytes. The $K_p$ of imipramine for hepatocytes pre-treated, either by freeze-thawing or by pre-incubation with the plasma membrane
permeabiliser saponin, was 120 and was not dependent on the initial drug concentration (see Figure 2B for the latter case). The saturable uptake process was therefore effectively eliminated by the structural disruption of the cell membrane. No effect on uptake of imipramine was observed after pre-treatment with the inhibitor of ATP-dependent uptake, rotenone, (data not shown).

**Competition for uptake.** The uptake of imipramine in the presence of a high (400 µM) and low (0.4 µM) concentration of 24 putative competitors is shown in Figure 4. The cellular uptake of imipramine was not affected ($p > 0.05$ by ANOVA) by any of the coincubated drugs at the low concentration. However, at the high competitor concentration, the uptake of imipramine in cells was significantly reduced (by 40 - 70 %, $p < 0.05$ by ANOVA) in the presence of several lipophilic amines (amitriptyline, clomipramine, nortriptyline, trimipramine, amiodipine, sertraline, diltiazem, chlorpromazine, promethazine, verapamil, chloroquine, propranolol, quinine or quinidine). There is thus evidence that the saturable uptake of imipramine is shared by other lipophilic amines. No such evidence of competition was found for the acidic drug warfarin, the non-lipophilic amine serotonin (5-hydroxytryptamine), or the neutral drugs of differing lipophilicity (caffeine, diazepam and nifedipine).

The inhibition of the uptake of imipramine by desmethylimipramine, hydroxyimipramine and propranolol was studied over a wide concentration range (see Figure 4A) and analysed by nonlinear regression using an inhibition model (Table 2). For propranolol, similar inhibition characteristics were observed for competition with imipramine and the pharmacologically inactive enantiomer ($R$)-propranolol (see Fig 4B); the inhibition model parameter values are also given in Table 2.
Extent of metabolism concurrent with uptake. The proportion of metabolites produced was clearly influenced by pre-treatment with ABT and, in the absence of this inhibitor was concentration dependent (see Fig 5 A and B, for imipramine and propranolol, respectively). In addition, the composition of these metabolites was dependent on concentration, presumably due to differences in affinity and capacity between different P450 enzymes responsible for the formation of each metabolite. For imipramine, the predominant metabolite was 2-hydroxyimipramine at the lowest concentrations, but the relative proportion of this decreased with increasing initial imipramine concentration; for propranolol, the predominant metabolites were 4-hydroxypropranolol and 7-hydroxypropranolol and again the relative proportion of these decreased with increasing initial propranolol concentration.

Complete extraction of imipramine-related compounds from incubates by organic solvent was obtained from cells treated with ABT. Following incubation with untreated cells a high proportion of drug-related material (70 % at low conc and 90 % at high) was extracted with the lower recovery values corresponding to the lowest initial concentrations. The residues would be expected to comprise of more polar and therefore relatively unextractable metabolites (eg. glucuronides). For imipramine, the principal components identified, by co-chromatography with reference standards, were imipramine, 2-hydroxyimipramine, desmethylimipramine (consistent with those previously reported by Chiba et al., 1990) and two unknown metabolites, designated M3 and M4. For propranolol, the principal components were propranolol, 4-hydroxypropranolol, 7-hydroxypropranolol, 5-hydroxypropranolol and N-desisopropylpropranolol (consistent with those previously reported by Ishida et al., 1992 and Masubuchi et al., 1993).
Binding of imipramine and propranolol in hepatocytes and within the incubation.

Fraction unbound values were calculated making the assumption that $K_p$ reflects only intracellular binding. The range of $f_u$ within the cells over the concentration range studies corresponded to 0.004 - 0.014 and 0.003 - 0.009 for propranolol and imipramine, respectively. When allowance was made for the relative volume difference for incubation with a hepatocellularity of $2 \times 10^6$ cells/ml (using equation 5) these unbound values increase markedly to 0.42 - 0.73 and 0.30 - 0.56, respectively.

Time-course of propranolol in hepatocytes and clearance. The concentration of propranolol in cells, medium and total incubate (calculated from either the cellular pellet, supernatant or total incubate, respectively) declined in parallel over the time course of 100 s corresponding to 90 % of loss of substrate (Figure 6). The estimation of clearance of propranolol in hepatocytes was based on the changes in concentration of propranolol with time. At an initial drug concentration of 40 nM the propranolol clearance from the medium was $3.1 \pm 0.8$ ml/min/$10^6$ cells (Table 3). In order to allow direct comparison of this extracellular clearance of propranolol with the clearance for total propranolol from the incubation medium (as would be determined in a kinetic assay of metabolite formation) the AUC of total propranolol in the incubate was calculated. This yielded a total incubation clearance of $1.8 \pm 0.4$ ml/min/$10^6$ cells. The AUC of propranolol in cells allowed calculation of an `intracellular` clearance which was very low ($0.04$ ml/min/$10^6$ cells). Although there is a loss in linearity at latter time intervals (see Figure 6) this is unlikely to reflect a change in uptake by the time $>90$ % of drug is eliminated.
Discussion

Investigation of the hepatocellular uptake of drugs using intact cells requires separation of at least three processes: membrane transport, intracellular binding and metabolism. The use of the mechanism based inhibitor, ABT, blocked the latter process to allow a direct determination of $K_p$. This parameter however may reflect intracellular binding and/or transporter activity. The silicon oil procedure allowed rapid separation of cells with minimal changes in drug distribution due to re-equilibration and with the use of radiolabelled drugs a wide concentration range was studied (0.1 - 400 µM). The high $K_p$ values (>100) for both imipramine and propranolol were not surprising for lipophilic bases. However a concentration dependence in the accumulation of imipramine and propranolol in isolated hepatocytes was also demonstrated and in both cases accumulation can be described as a two site process: a high affinity, low capacity process and a low affinity, high capacity process which is not saturable under the experimental conditions used. While there is evidence to indicate that the high affinity, low capacity component may involve membrane transporter(s), these drugs are highly permeable and regarded as Class 1 compounds in the Biopharmaceutical Classification System (Wu and Benet, 2005) and any impact of transporters would not be expected to be substantial. The low affinity, high capacity component is likely to represent solely intracellular binding.

Uptake into intact hepatocytes is rapid for both drugs (with an equilibrium between concentrations within cell and the external media being achieved within 30 s), however as both are high clearance drugs, metabolism is substantial even within this time period. There is considerable evidence for tissue binding of lipophilic basic drugs (Fichtl et al., 1991; Rodgers et al., 2004; Austin et al., 2005). The magnitude of accumulation of imipramine and
propranolol is substantial, with concentrations being two orders of magnitude higher in cells than in the medium. In similar experiments we have observed less accumulation - approximately one order of magnitude - but also a concentration-dependence (e.g. diazepam, phenytoin (Jones et al., 2005), dextromethorphan (Witherow and Houston, 1999); in other cases no accumulation (e.g. caffeine and tolbutamide).

The two component accumulation of imipramine and propranolol into hepatocytes evident in the concentration-dependent uptake profile was also seen in the time profile. The initial uptake which occurred within 10 s (the lag time of the experiment) showed increasing concentration dependence and the subsequent uptake over 30 s was clearly saturable as concentration increased. The saturable component of uptake of imipramine was found to have a similar capacity and affinity (11 and 6.3 µM, respectively) to that of propranolol (66 and 36 µM).

In order to explore the nature of the saturable process and its dependence on cellular functional integrity (including membrane transporters), the uptake of imipramine was examined in cells disrupted both physically and metabolically by freeze-thawing. This level of disruption completely eliminated the saturable process, resolving the non-saturable process. This result also demonstrated that the non-saturable component comprised of a non-metabolically active processes. It was considered that due to the amphiphilic nature of these compounds, the non-saturable uptake occurred by diffusion and subsequent binding to the phospholipid component of membranes, by polar alignment between the phospholipid molecules. This would represent a large capacity site, as would binding to a large range of cellular proteins. The non-saturable uptake process for imipramine and propranolol was an
important contributor to overall uptake; even at low drug concentrations, where saturable uptake was maximal, the contribution was 30 - 40% of total uptake.

Uptake following disruption of a more specific nature was assessed using saponin, a plasma membrane permeabiliser, which provides evidence for the involvement of membrane transporters. It has been observed that some cationic drugs, including propranolol and imipramine, may undergo cellular uptake by active transport (Meijer et al., 1990; Nakamura et al., 1994; van Montfoort et al., 2003; Chandra and Brouwer, 2004) involving members of the OATP and OCT families. It is of interest that the uptake of imipramine was not affected by treatment with rotenone, an inhibitor of ATP-dependent transport (consistent with involvement of SLCO rather than ABCC family of transporter proteins).

The premise that the hepatic uptake of imipramine is representative for a large group of lipophilic amine drugs was tested by competition for saturable uptake with 24 other drugs covering a range of physico-chemical types. All inhibitors of saturable uptake of imipramine were specifically lipophilic amines (including imipramine metabolites). No inhibition was discernable by other drug types, including lipophilic drugs which were not amines, and amines which were not lipophilic. Substantial inhibition was apparently dependent on amphiphilicity: a combination of relatively high lipophilicity (logD >1.5) and relatively high basicity (pKa >8). Non-specific binding of lipophilic compounds is to be expected as a consequence of hydrophobic forces; in addition, a key binding property might be the cationic amine which in this group of amphiphiles is located aliphatically and remote from the hydrophobic (cyclic) moiety. However these competition results do not mean that all of these basic drugs share the same transporter or indeed are substrates. Further detailed studies are required before this conclusion can be drawn.
Regardless of the mechanism(s) responsible for the high $K_p$ values reported, it is important to consider how this phenomenon would impact on determination of intrinsic clearance within a hepatocyte in vitro system. From the $K_p$ values reported, the fraction unbound within the cells would be extremely low ($f_u < 0.01$ for both imipramine and propranolol). These values, obtained using equation 4, represent an extreme case for $K_p$ values (greater than unity) resulting solely from intracellular binding. Within the in vitro system it is necessary to allow for the particular dilution of the cell suspension used and effectively this means calculating the volume ratio for the hepatocytes relative to the incubation volume (equation 5). A commonly used incubation hepatocellularity value would be $1 - 2 \times 10^6$ cells per ml and the corresponding volume ratio for such an incubation would be $0.003 - 0.006$. Thus the equation indicates that $K_p$ values greater than two orders of magnitude are necessary to result in a major change in the fraction unbound in the incubation. Fraction unbound values for the incubation conditions used for imipramine and propranolol (at high concentrations of substrate) are 0.42 and 0.36 respectively. These values correspond well with those reported by Austin et al. (2005) using an equilibrium dialysis procedure.

The implications of the hepatocellular uptake in the determination of clearance were examined by measuring the time-course of propranolol depletion when incubated with hepatocytes. This lipophilic amine was selected in preference to imipramine due to its simpler metabolite kinetics as outlined in Introduction. The clearance of propranolol from the buffer medium (3.1 ml/min/10^6 cells) was greater than the clearance of drug in the total incubate (1.8 ml/min/10^6 cells). Assuming that the concentration of drug in the medium represents the concentration of unbound drug (in the whole incubate), then the ratio of the clearances of total incubate to the medium gives an estimate of the fraction of drug unbound
in the total incubate. This value is approximately 0.6 and is of a similar magnitude to that resulting from non-saturable binding found in hepatocytes. The total apparent binding (non-saturable and saturable uptake) in hepatocytes at equilibrium of total uptake was greater at this low concentration (0.04 µM) of propranolol ($f_u = 0.4$ for total incubate) in the equilibrium partitioning studies. However consideration of the time course of the uptake indicated that the saturable component was only evident in the later time period when most of the drug was eliminated. Therefore, the apparent contribution from only the non-saturable binding component to clearance is consistent with the uptake of drug with respect to time. Whilst these quantitative aspects of uptake are speculative, it is clear that uptake is not rate limiting the clearance of propranolol. In general, for the determination of intrinsic clearance, the relative rates of uptake processes and metabolism should be considered and hence the choice of in vitro systems can be critical. For propranolol a similar predicted clearance is obtained with hepatic microsomes and hepatocytes, both in reasonable agreement with in vivo (Table 3). Recently, Lam and Benet (2004) have demonstrated in rat with digoxin how the complexities of transporters (Oatp 2 and Pgp) can result in hepatocytes but not hepatic microsomes providing realistic prediction of hepatic clearance.

In conclusion, lipophilic amine drugs distribute into the liver primarily by highly favourable (>100-fold) and non-saturating partition with considerable enhancement (2 to 3-fold) by a saturable process at low concentrations. This saturable process may be transporter related. Several lipophilic amines, including propranolol and imipramine have been documented as substrates for OCT and OATP transporters (van Montfoort et al., 2003; Chandra and Brouwer, 2004) yet hepatic uptake does not rate limit the clearance of propranolol. Recently Wu and Benet (2005) have speculated that although many drugs are substrates for transporter
proteins this is of little consequence due to their permeability properties. Propranolol appears to exemplify this notion; for other lipophilic amine drugs, clearance may be dependent on active uptake if the rate of metabolism is slower.
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References


**Legends for Figures**

**Figure 1.** Uptake of imipramine (A) and propranolol (B) into isolated rat hepatocytes, expressed as total drug in the cells after incubation, normalised by initial drug concentration. (○) 0.04; (●) 0.2; (Δ) 0.4; (▲) 2.0; (□) 4.0; (■) 20; (◊) 40; (♦) 200; (∇) 400 μM.

**Figure 2.** Cell-to-medium concentration ratio of imipramine and propranolol in isolated rat hepatocytes. A: The effect of initial concentration, (●) imipramine; (○) propranolol. B: The
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effect of pretreatment with saponin, as a function of imipramine initial concentration, (○) control; (●) saponin-treated.

Data in panel A represent no pre-treatment (separate to dataset for +/- ABT (Table 1)); lines show fit of initial concentration (kinetic parameters in Table 1 derived from unbound concentration).

**Figure 3.** Relative proportion of imipramine in isolated rat hepatocytes following incubation with 24 drugs at 0.4 and 400 µM imipramine concentration.

**Figure 4.** Inhibition of uptake of \[^3\text{H}\]imipramine (A) and (S)-propranolol (B) at 0.4 µM into isolated rat hepatocytes, as a function of competitor concentration. A: (●) desmethyl imipramine; (○) 2-hydroxy imipramine; (grey) propranolol. B: (●) imipramine; (○) (R)-propranolol.

Lines show fit of initial concentration (kinetic parameters in Table 2 derived from unbound concentration).

**Figure 5.** Proportion of total radiolabelled material as metabolites in hepatocytes, with (black) or without (grey) pre-treatment with ABT, after incubation with \[^3\text{H}\]imipramine (A) or propranolol (B) for 30 s, as a function of initial concentration.

**Figure 6.** Concentration of propranolol in hepatocytes, medium and total incubate, as a function of time. (●) cell; (▲) medium; (○) total incubate. Initial propranolol medium concentration was 40 nM.
### TABLE 1

Binding parameter values for imipramine and propranolol in hepatocytes with or without pre-treatment with ABT

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pre-treatment</th>
<th>Binding parameter (± SE)(^a)</th>
<th>(B_{\text{max}})</th>
<th>(K_d)</th>
<th>(B_{\text{NS}})</th>
<th>(K_{p \text{ max}})</th>
<th>(K_{p \text{ min}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipramine</td>
<td>None</td>
<td></td>
<td>11 ± 3.2</td>
<td>6.3 ± 4.6</td>
<td>0.77 ± 0.23</td>
<td>360 ± 120</td>
<td>150 ± 23</td>
</tr>
<tr>
<td></td>
<td>ABT</td>
<td></td>
<td>9.7 ± 3.6</td>
<td>6.2 ± 5.7</td>
<td>1.1 ± 0.31</td>
<td>380 ± 110</td>
<td>160 ± 29</td>
</tr>
<tr>
<td>Propranolol</td>
<td>None</td>
<td></td>
<td>66 ± 39</td>
<td>36 ± 18</td>
<td>0.41 ± 0.11</td>
<td>240 ± 46</td>
<td>69 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>ABT</td>
<td></td>
<td>89 ± 17</td>
<td>62 ± 9.9</td>
<td>0.30 ± 0.14</td>
<td>180 ± 19</td>
<td>63 ± 11</td>
</tr>
</tbody>
</table>

\(^a\) Using Equation 3
### TABLE 2

Binding inhibition parameter values for imipramine and \((S)\)-propranolol in the presence of various competitors

<table>
<thead>
<tr>
<th>Drug</th>
<th>Competitor</th>
<th>Inhibition parameter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(sB_{\text{max}})</th>
<th>(IC_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>Desmethylinipramine</td>
<td>0.43</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-Hydroxyimipramine</td>
<td>0.43</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Propranolol</td>
<td>0.41</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>((S))-Propranolol</td>
<td>Imipramine</td>
<td>0.59</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td></td>
<td>((R))-Propranolol</td>
<td>0.61</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Using Equation 6 and a drug concentration of 0.4 \(\mu M\)

Parameters derived from mean binding data; \(SE <10\%\) mean
TABLE 3

Hepatocyte propranolol clearance, based on cell, medium or total incubate depletion compared to microsomal prediction and observed in vivo clearance.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Hepatocyte clearance</th>
<th>$\text{CL}_{\text{int}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{ml/min/10}^6\text{ cells}^a$</td>
<td>$\text{ml/min/g liver}$</td>
</tr>
<tr>
<td>Cell</td>
<td>0.041 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>3.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Total incubate</td>
<td>1.8 ± 0.4</td>
<td>198</td>
</tr>
<tr>
<td>Microsomal incubate$^b$</td>
<td></td>
<td>193</td>
</tr>
<tr>
<td>In vivo$^c$</td>
<td></td>
<td>245</td>
</tr>
</tbody>
</table>

$^a$Mean of 3 incubations ± SD, 4 nM initial medium concentration

$^b$Hallifax, unpublished data

$^c$Ito and Houston, 2004
Figure 1

**A**

![Graph A](image)

**B**

![Graph B](image)

Incubation time (s)

Radiolabelled material in cells (µmol/10^6 cells/µM)
Figure 2

A

Initial drug concentration ($\mu$M)

Cell-to-medium ratio

0 100 200 300 400 500

0.1 1 10 100

B

Initial imipramine concentration ($\mu$M)

Cell-to-medium ratio

0 100 200 300 400 500

0.01 0.1 1 10 100
Figure 3

Competitor

Nortriptyline
Amitriptyline
Desipramine
Propranolol
Amlodipine
Trimipramine
Hydroxyimipramine
Clomipramine
Sertraline
Quinine
Promethazine
Verapamil
Chloroquine
Diltiazem
Quinidine
Chlorpromazine
Metoprolol
Ondansetron
Diazepam
Atenolol
Warfarin
Caffeine
Serotonin
Nifedipine

Proportion of maximum binding
Figure 4

A

Relative proportion of uncompeted uptake vs. competitor concentration (µM).

B

Relative proportion of uncompeted uptake vs. competitor concentration (µM).
Figure 5

A

Initial imipramine concentration (µM)

Percentage of radiolabel in cells as metabolites

0.04 0.2 0.4 2 4

B

Initial propranolol concentration (µM)

Percentage of radiolabel in cells as metabolites

0.4 4 40 400