Uptake and Intracellular Binding of Lipophilic Amine Drugs by Isolated Rat Hepatocytes and Implications for Prediction of in Vivo Metabolic Clearance

David Hallifax and J. Brian Houston

School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom

Received April 5, 2006; accepted July 27, 2006

ABSTRACT:

The hepatic uptake of imipramine and propranolol was investigated after 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 cytochrome P450). Pretreatment of cells either by freezethawing or with saponin (which permeabilizes 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 nonsaturable component probably represents partition into the phospholipid component of membranes. Propranolol was further investigated to determine the impact of high $K_p$ values on hepatic cellular 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 the nonsaturable 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.

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 pharmaceutical 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).

Although 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 (Houston and Carlile, 1997; Jones et al., 2005). This situation is of concern for human prediction studies since, because of 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 have an 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, because 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 being 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 (Yao and Levy, 2002; Rostami-Hodjegan and Tucker, 2004).

ABBREVIATIONS: ABT, aminobenzotriazole; $CL_{int}$, intrinsic clearance; $f_u$, fraction unbound; $K_p$, cell to medium concentration ratio; WME, Williams’ Medium E.
Lipophilic amine drugs are of particular interest for hepatic uptake, inasmuch as many have been widely reported to extensively partition into this tissue (Fichtl et al., 1991; Austin et al., 2005; Rodgers 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, aminobenzotrazole (ABT) (Ortiz de Montellano and Correia, 1995), which is not hepatotoxic (Shiba and Shimamoto, 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 preincubation with saponin (a plasma membrane permeabilizer (Mick et al., 1988). To characterize the process further, the uptake was also investigated in the presence of 24 potential competitors possessing a range of physicochemical 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 because 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,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).

Materials and Methods

Chemicals. [3H]Imipramine HCl (733 GBq/mmol), (S)-[3H]propranolol HCl (555 GBq/mmol), [3H]water (37.0 MBq/ml), and [14C]sucrose (25.0 GBq/mmol) were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Imipramine (nonradiolabeled), (S)-propranolol, (R)-propranolol, clomipramine, trimipramine, nortriptyline, metoprolol, atenolol, chlorpromazine, promethazine, quinine, quindine, chloroquine, verapamil, diltiazem, diazepam, warfarin, nifedipine, caffeine, serotonin (5-hydroxytryptamine), 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 were obtained from PerkinElmer (Milton Keynes, UK). All other chemicals were of analytical grade. 2-Hydroxyimipramine, desmethylimipramine, sertraline, and amlodipine were donated by Pfizer Ltd. (Sandwich, UK). Ondansetron was donated by GlaxoSmithKline (Ware, UK). 4-Hydroxypropranolol and 4-desisopropylpropranolol were donated by Professor G. Tucker, University of Sheffield (Sheffield, UK). 5-Hydroxypropranolol and 7-hydroxypropranolol were donated by Dr T. Walle, Medical University of South Carolina (Charleston, SC).

The radiolabeled 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 two to four per cage on a bedding of sawdust in rooms maintained at a temperature of 20±2°C and a humidity of 45 to 55% with a 12-h light/dark cycle. The rats had free access to water and Standard Rat and Mouse Experimental Laboratory Diet (B and 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 nonradio labeled 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 × 10^6 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 (VWR, Lutterworth, UK) and the incubation was started by the addition of a portion of cell suspension (0.25 ml) that had been maintained at 37°C for approximately 15 min after preparation. After an incubation period of either 0, 5, 10, 20, or 30 s (for cell concentrations of 2 × 10^6 cells/ml, and 30 s for other cell concentrations), the incubate was centrifuged at maximum speed for approximately 20 s. The tube was then placed in powdered dry ice for at least 30 min 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 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 pretreatment) 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 M) 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 2×10^6 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 that had either been freeze-thawed (by placing in a freezer for approximately 30 min and thawed by incubating at 37°C for 30 min), pretreated with ABT (5 mM) and saponin (0.05%), or pretreated with ABT (5 mM) and rotenone (0.3 mM), to give an incubation concentration of 1 mM ABT and a rotenone concentration of 30 μM.

To investigate the susceptibility 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 competitive concentrations of either 0.4 or 4 μM for ABT (pH 10, 0.05 M) and the hepatocytes were pretreated 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 h), Optiphase HiSafe 2 scintillant (5 ml) was added to each vial, which was capped, shaken, and, after a period of several hours, analyzed by liquid scintillation counting (LSC) (using isotope spectrum shape analysis quench correction, over a 5-min counting period). Duplicate portions (0.025 ml) of the substrate solutions were mixed with scintillant and analyzed by LSC using a Wallac 1409 counter (PerkinElmer).

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 (VWR), for approximately 1 min. Each tube was then centrifuged using an MSE Mistral 1000 centrifuge (Fisher Scientific, Loughborough, UK) 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), and 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 analyzed by LSC. The evaporated samples were stored at approximately −20°C, and subsequently resuspended in mobile phase (0.2 ml).
and centrifuged (Microcentaur, maximum speed) for 5 min immediately before 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 analyzed by LSC. The cell extracts were assayed by high-performance liquid chromatography using a Gilson 231 XL Sample Injector (Anachem, Luton, UK) and an Inertis ODS(2) column (Gasukuro Kogyo, Japan) with isocratic elution of acetonitrile/facetic 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 1 ml/min (maintained by a Waters 510 pump; Waters, Watford, UK). Detection was by UV absorbance (254 nm; Applied Biosystems 759A Absorbance detector; Anachem) (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 radionuclide from the incubation medium and resolution of parent drug only, by radiochromatography.

**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). 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 [3H]water and [14C]sucrose in WME (approximately 10 kBq/ml of each radionuclide). The volume occupied by the radiolabeled water in the cells was determined on the basis that the [3H]water was assumed to be in equilibrium with the external medium and, hence, the volume was proportional to the initial radiochemical concentration of the [3H]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 [14C]sucrose, which was assumed not to have penetrated the cells. The separated cells and aliquots (0.025 ml) of the preparation of radiolabeled drugs in WME were assayed for radioactivity by each radionuclide, simultaneously, by LSC. The total cell volume was calculated from the difference between the ratio of tritium and 14C 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 × 10⁶ cells/ml). The total volume was therefore proportional to the cellular protein, and the mean volume per milligram protein was 2.2 ± 0.46 μl (N = 51). Although the volume of adherent medium was appreciable (95% of cellular volume), the amount of drug it contained was relatively minor because of the high relative affinity of the drugs for the cells.

**Data Analysis.** The concentration of drug in the cells (Ccell) was calculated using the total amount of drug in cells and the cell volume for each hepatocyte preparation (eq. 1). The concentration of drug in the medium (Cmed) was calculated using the total amount of drug in the medium and the total medium volume (total incubation volume minus the total cell volume).

\[
C_{\text{cell}} = \frac{A_{\text{direct}} - (C_{\text{med}} \cdot V_{\text{cell}})}{V_{\text{cell}}}
\]

(1)

where \(A_{\text{direct}}\) = amount of drug in cells and adherent layer; \(V_{\text{cell}}\) = volume of the adherent (external) layer; and \(V_{\text{cell}}\) = 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 eq. 2.

\[
K_p = \frac{C_{\text{cell}}}{C_{\text{med}}} \quad \text{(2)}
\]

The accumulation of drug in hepatocytes was quantified in mass action terms on the assumption that intracellular binding represented the major mechanism for high \(K_p\) values. The concentration of bound drug in the action was related to the concentration of the unbound drug with a one-site ligand binding model incorporating a linear function for unsaturated binding (eq. 3), using the GraFit (Erithacus Software Ltd., Horley, Surrey, UK) nonlinear regression modeling program.

\[
C_s = \frac{B_{\text{max}} \cdot C_s}{K_d + C_s} + B_{\text{NS}} \cdot C_u
\]

(3)

where \(C_s\) = concentration of bound drug; \(C_u\) = concentration of unbound drug; \(B_{\text{max}}\) = concentration of binding sites (site 1); \(K_d\) = binding dissociation equilibrium constant; \(B_{\text{NS}}\) = nonspecific binding constant.

The unbound fraction of drug (\(f_u\)) in the cells was calculated using \(K_p\) as shown in eq. 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.

\[
f_u = \frac{1}{K_p}
\]

(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 eq. 5.

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

(5)

where \(V_c\) = cell volume and \(V_{\text{inc}}\) = 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 in which drug was coincubated 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 eq. 6 for one-site ligand binding competition (incorporating a linear component for an unsaturated site) using the GraFit program.

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

(6)

where \(B_i\) = relative proportion of uncompetet binding; \(sB_{\text{max}}\) = maximum proportion of saturable uncompetet 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 unbound saturation.

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.

**Results**

**Characteristics of Uptake.** The time course of uptake of radiolabeled imipramine was very rapid (see Fig. 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 approximately 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 (Fig. 1B).

The plateau values for cellular uptake, used to calculate \(K_p\), were dependent on the initial concentration for both drugs (Fig. 2A). The mean maximum values were approximately 360 for imipramine and 280 for propranolol (at <1 μM), and this decreased to a minimum of
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$^6$ cells/ml). The rate and extent of accumulation of radiolabel were determined in hepatocytes pretreated 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, pretreated either by freeze-thawing or by preincubation with the plasma membrane permeabilizer saponin, was 120 and was not dependent on the initial drug concentration (see Fig. 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 pretreatment 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 $\mu$M) and low (0.4 $\mu$M) concentration of 24 putative competitors is shown in Fig. 3. The cellular uptake of imipramine was not affected ($p > 0.05$ by analysis of variance) 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 analysis of variance) 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 nonlipophilic 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 Fig. 4A) and analyzed 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 pretreatment 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 because of differences in affinity and capacity between the 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. After incubation with untreated cells, a high proportion of drug-related material (70% at low concentration and 90% at high) was extracted, with the lower recovery values corresponding to the lowest initial concentrations. The residues would be expected to comprise more polar and, therefore, relatively unextractable metabolites (e.g., glucuronides). For imipramine, the principal components, identified by cochromatography 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, 2-hydroxyimipramine, desmethylimipramine (consistent with those previously reported by Chiba et al., 1990), and two unknown metabolites by organic solvent was obtained from cells treated with ABT. After incubation with untreated cells, a high proportion of drug-related material (70% at low concentration and 90% at high) was extracted, with the lower recovery values corresponding to the lowest initial concentrations. The residues would be expected to comprise more polar and, therefore, relatively unextractable metabolites (e.g., glucuronides). For imipramine, the principal components, identified by cochromatography 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 to 0.014 and 0.003 to 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 eq. 5), these unbound values increase markedly to 0.42 to 0.73 and 0.30 to 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 (Fig. 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). 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 Fig. 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 radiolabeled drugs, a wide concentration range was studied (0.04–400 \( \mu M \)). 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 that is not saturable under the experimental conditions used. Although 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 Drug Disposition Classifica-

---

**TABLE 1**

Binding parameter values for imipramine and propranolol in hepatocytes with or without pretreatment with ABT.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pretreatment</th>
<th>( B_{\text{max}} ) (( \mu M ))</th>
<th>( K_d ) (( \mu M ))</th>
<th>( B_{\text{sat}} ) (( \mu M ))</th>
<th>( K_p ) (( \mu M ))</th>
<th>( K_{p_{\text{max}}} ) (( \mu M ))</th>
<th>( K_{p_{\text{min}}} ) (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>None</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>
<td></td>
</tr>
<tr>
<td></td>
<td>ABT</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>
<td></td>
</tr>
<tr>
<td>Propranolol</td>
<td>None</td>
<td>66 ± 39</td>
<td>36 ± 18</td>
<td>0.41 ± 0.11</td>
<td>240 ± 46</td>
<td>69 ± 6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABT</td>
<td>89 ± 17</td>
<td>62 ± 9.9</td>
<td>0.30 ± 0.14</td>
<td>180 ± 19</td>
<td>63 ± 11</td>
<td></td>
</tr>
</tbody>
</table>

\( a \) Using eq. 3.
nent comprised nonmetabolically active processes. It was considered
process. This result also demonstrated that the nonsaturable compo-
uptake of imipramine was examined in cells disrupted both physically
on cellular functional integrity (including membrane transporters), the
/\9262
M).
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>( IC_{50} )</th>
<th>( \mu M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>Desmethylimipramine</td>
<td>0.43</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2-Hydroxyimipramine</td>
<td>0.43</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Propranolol</td>
<td>0.41</td>
<td>64</td>
</tr>
<tr>
<td>(S)-Propranolol</td>
<td>Imipramine</td>
<td>0.59</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>(R)-Propranolol</td>
<td>0.61</td>
<td>150</td>
</tr>
</tbody>
</table>

* Using eq. 6 and a drug concentration of 0.4 \( \mu M \). Parameters were derived from mean binding data; S.E. is <10% of mean.

that, due to the amphiphilic nature of these compounds, the nonsatur-
able 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
nonsaturable uptake process for imipramine and propranolol was an
important contributor to overall uptake; even at low drug concentra-
tions, where saturable uptake was maximal, the contribution was 30 to
40% of total uptake.

Uptake after disruption of a more specific nature was assessed
using saponin, a plasma membrane permeabilizer, which provides
evidence for the involvement of membrane transporters. It has been
observed that some cationic drugs, including propranolol and imipra-
mine, 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 organic anion-transporting
polypeptide and organic cation transporter 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 the
involvement of solute-linked carrier organic anion transporter rather
than the ATP-binding cassette subfamily C 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 physi-
cochemical types. All inhibitors of saturable uptake of imipramine
were specifically lipophilic amines (including imipramine metabo-
lites). No inhibition was discernible by other drug types, including
lipophilic drugs that were not amines, and amines that were not
lipophilic. Substantial inhibition was apparently dependent on am-
phiphilicity: a combination of relatively high lipophilicity (logD
>1.5) and relatively high basicity (\( pK_a >8 \)). Nonspecific binding
of lipophilic compounds is to be expected as a consequence of hydro-
phobic forces; in addition, a key binding property might be the
cationic amine which, in this group of amphiphiles, is located ali-
phatically and remote from the hydrophobic (cyclic) moiety. How-
ever, 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 \( K_a \) values
reported, it is important to consider what impact this phenomenon
would have on determination of intrinsic clearance within a hepato-
cyte in vitro system. From the \( K_a \) values reported, the fraction un-
bound within the cells would be extremely low (\( f_u <0.01 \) for both
imipramine and propranolol). These values, obtained using eq. 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 (eq. 5). A commonly used incubation hepatocellularity value would be 1 to 2 x $10^6$ cells per ml, and the corresponding volume ratio for such an incubation would be 0.003 to 0.006. Thus, the equation indicates that $K_p$ values greater than 2 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 because of its simpler metabolic kinetics as outlined in the 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 nonsaturable binding found in hepatocytes. The total apparent binding (nonsaturable and saturable uptake) in hepatocytes at equilibrium of total uptake was greater at the low concentration (0.04 $\mu$/ml) 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 nonsaturable binding component to clearance is consistent with the uptake of drug with respect to time. Although 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 clearance (Table 3). Recently, Lam and Benet (2004) have demonstrated, in rat with digoxin, how the complexities of transporters (Oatp 2 and P-glycoprotein) 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 favorable (>100-fold) and nonsaturating 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 organic cation transporter and organic anion-transporting polypeptide 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

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Hepatocyte Clearance$^a$</th>
<th>CLint $^c$</th>
<th>ml/min/10^6 cells</th>
<th>ml/min/g liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>0.041 ± 0.009</td>
<td>198</td>
<td>0.009</td>
<td>198</td>
</tr>
<tr>
<td>Medium</td>
<td>3.1 ± 0.8</td>
<td>193</td>
<td>0.009</td>
<td>245</td>
</tr>
<tr>
<td>Total incubate</td>
<td>1.8 ± 0.4</td>
<td>193</td>
<td>0.009</td>
<td>245</td>
</tr>
<tr>
<td>Microsomal incubate$^b$</td>
<td>0.4 ± 0.035</td>
<td>193</td>
<td>0.009</td>
<td>245</td>
</tr>
</tbody>
</table>

$^a$ Mean of three incubations ± S.D.; 4 nM initial medium concentration.

$^b$ D. Hallifax, unpublished data.

$^c$ Ito and Houston (2004).
(2005) have speculated that although many drugs are substrates for transporter proteins, this is of little consequence because of their permeability properties. Propranolol seems to exemplify this notion; for other lipophilic amine drugs, clearance may be dependent on active uptake if the rate of metabolism is slower.

References


Address correspondence to: Professor J. B. Houston, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester M13 9PL, UK. E-mail: brian.houston@manchester.ac.uk