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SATURABLE UPTAKE OF LIPOPHILIC AMINE DRUGS INTO ISOLATED HEPATOCYTES: MECHANISMS AND CONSEQUENCES FOR QUANTITATIVE CLEARANCE PREDICTION

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Running title: Quantitative drug metabolism in isolated hepatocyte systems

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Abbreviations: CL_{int}, intrinsic clearance; K_p, cell to media concentration ratio; $f_{u,inc}$, fraction unbound of total incubation; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; ABT, l-aminobenzotriazole

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Abstract

The hepatic uptake of quinine, fluvoxamine and fluoxetine (0.1 - 10 μM) was investigated with freshly isolated rat hepatocytes. The cell-to-medium concentration ratios (K_p) were concentration-dependent: the mean maximum K_p (at 0.1 μM) were 150 (quinine), 500 (fluvoxamine) and 2000 (fluoxetine). There was also a large capacity site that was not saturable over the concentration range used (possibly partition into the phospholipid component of membranes); representing this site, the mean minimum K_p (at 10 μM) were 30 (quinine), 200 (fluvoxamine) and 500 (fluoxetine). To eliminate concomitant metabolism, cells were pretreated with the irreversible P450 inhibitor, aminobenzotriazole. The saturable uptake was substantially eliminated after exposure to FCCP (ATP inhibitor). The difference between the maximum and minimum K_p for these three amine drugs, as well as for dextromethorphan, propranolol and imipramine, was within a limited range of 3-fold, indicating a common magnitude of saturable uptake. Basic, permeable drugs are expected to be sequestered into lysosomes which actively maintain their low internal pH (~5) using ATP and this process is predictable from the combined effects of pH-driven ion accumulation and unsaturable binding representing partition into membranes. The resultant predicted maximum K_p correlated strongly with the observed maximum K_p. Thus at low substrate concentrations, the fraction of drug unbound in the hepatocyte incubation (critical for assessing drug clearance and drug-drug interaction potential) may be dependent upon saturable as well as unsaturable binding, and for lipophilic, basic drugs this can be readily estimated assuming a common degree of uptake into lysosomes.

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Introduction

The use of metabolite kinetic parameters generated in vitro for the prediction of in vivo drug clearance and drug-drug interaction potential is widespread (McGinnity and Riley, 2001; Obach, 2001; Houston and Galetin, 2003; Rostami-Hodjegan and Tucker, 2004). Expectations have increased beyond that of obtaining information on P450 metabolic reactions to encompass other drug metabolizing enzymes (e.g. UGT, Miners et al., 2006) and transporter proteins (e.g. OATP1B1, Hirano et al., 2004). Thus the traditional use of hepatic microsomes as the routine in vitro system has been challenged (Lam and Benet, 2004) and human cryopreserved hepatocytes are becoming more widely employed. Their utility in predicting human clearance is encouraging (Lau et al., 2002; McGinnity et al., 2004; Hallifax et al., 2005; Brown et al., 2006) and valuable investigations into enzyme-transporter interplay are ongoing (Shitara et al., 2004; Lau et al., 2006; Zamek-Gliszczynski et al., 2006).

Determining the drug concentration available to the enzyme and/or transporter within the in vitro system is a key issue. As accumulation of drugs in hepatocytes may occur via active uptake processes and/or intracellular binding, a concentration difference may exist between hepatocytes and microsomes and whether the drug is a bound or free entity within the cell is of importance. Intracellular binding to sites not involved in the metabolic process may be of little consequence, as the free concentration within the cell will be in equilibrium with the external incubation media concentration (Austin et al., 2005). For microsomal preparations this has been studied extensively and reasonable predictions may be calculated from physiochemical properties (Austin et al., 2002; Hallifax and Houston, 2006a). However for isolated hepatocytes appropriate comprehensive methods have yet to be established.

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We recently reported the characteristics of drug uptake into isolated rat hepatocytes of two lipophilic, basic, prototypic drugs - imipramine and propranolol, (Hallifax and Houston, 2006b). Hepatocellular uptake studied over a wide concentration range was found to be a combination of high capacity unsaturable intracellular binding and a saturable process which was dependent on cell plasma membrane integrity; the latter process was inhibited by 18 other lipophilic amine drugs. The possible role of membrane transporter proteins in the saturable process was discussed yet the high permeability of these drugs and their position in Class 1 in the Biopharmaceutic Drug Disposition Classification System (Wu and Benet, 2005) confounds this explanation. In the present study, the saturable uptake characteristic of this type of drug was investigated further using quinine, fluvoxamine and fluoxetine and the ATP inhibitor FCCP.

Uptake parameters for quinine, fluvoxamine and fluoxetine are used together with previously described data from our laboratory (Witherow and Houston, 1999; Hallifax and Houston, 2006b) on imipramine, propranolol and dextromethorphan to form a set of six drugs with a hepatocellular uptake range of 40-fold. Saturable and nonsaturable uptake processes are explored as a function of physiochemical properties. Complications due to metabolism are avoided by using the non-specific, non cytotoxic P450 inhibitor – aminobenzotriazole (Ortiz de Montellano and Correia, 1995; Shiba and Shimanato, 1999). Putative uptake into lysosomes is estimated for each drug based on the pH effect on ion concentration and the nonsaturable binding. The processes are combined in the form of prediction equations to estimate the total cellular uptake. The overall aims of these investigations were to examine alternative explanations for the substrate concentration dependence of hepatocellular uptake for lipophilic bases in isolated hepatocytes and explore the complexities associated with the

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use of intact cellular systems in assessing drug clearance and drug-drug interaction potential.

Materials and Methods

Chemicals. Fluoxetine hydrochloride, fluvoxamine maleate, quinine hydrochloride, 1-aminobenzotriazole (ABT), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 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). All other chemicals were of analytical grade.

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 Expanded 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). Quinine, fluvoxamine or fluoxetine were diluted with WME to give incubation concentrations of 0.01, 0.02, 0.03, 0.06, 0.1, 0.3, 1, 3 and 10 µM (quinine) or 0.1, 0.15, 0.2, 0.3, 0.5, 1, 1.5, 3, 5 and 10 µM (fluoxetine, fluvoxamine). Hepatocytes were diluted in WME containing ABT to give an incubation concentration of 1 x 10^6 cells/ml and a concentration of ABT of 5 mM. Substrate solution (0.25 ml), maintained at 37 ^{o}C, was placed in an Eppendorf tube (0.5 ml

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capacity) containing a layer of silicone oil (510/50:550 (3:2, density 1.02), 0.05 ml) over a layer of sucrose solution (1 M; 0.025 ml). The tube was then placed in an Eppendorf MiniSpin microcentrifuge and the incubation was started by the addition of a portion of cell suspension (0.1 ml) which had been maintained at 37 ^{o}C for approximately 15 minutes after preparation. After an incubation period of either 10, 30 or 60 s, 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 an Eppendorf tube (1.5 ml capacity) to which was added distilled water (0.1 ml) prior to analysis – below. 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 stability of fluoxetine, fluvoxamine and quinine throughout the incubation and extraction was assessed in a parallel experiment conducted as described above except without hepatocytes (replaced with WME).

To investigate the dependence of uptake on active (ATP-dependent) processes, incubations of the three drugs were performed, as above, with hepatocytes that had been pre-treated with FCCP (in addition to ABT) at an incubation concentration 1 μM (a reduction of cellular ATP by more than 90 % was observed at 2 μM FCCP by Yamazaki et al., 1993).

Analysis of separated cells. Cells separated into sucrose solution (0.05 ml) were mixed (vortex) with methanol (0.1 ml) for approximately 1 minute. Each tube was then centrifuged using an Eppendorf microcentrifuge at approximately 10000 rpm for 5 minutes after which the supernatant was transferred to a separate tube. The extraction was repeated once and the

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combined organic phase was sampled (by duplicate aliquots of 0.05 ml) and analysed by LC-MS/MS.

Each drug, together with dextromethorphan as internal standard, was eluted on a Luna C18(2) 50 x 4.6 mm 3 µm column (Phenomenex, Macclesfield, UK) at 40 °C using either a binary or ternary gradient (comprising acetonitrile, water and formic acid (0.01 M)) maintained at 1 ml/min by a Waters Alliance 2795 HT LC system (Waters, Watford, UK). For each assay, nine calibration standards with a blank were prepared in a matrix identical to the incubation extracts and included levels below and above the expected concentrations. The compounds were detected by multiple reaction monitoring following atmospheric pressure electrospray ionisation using a Micromass Quattro Ultima mass spectrometer and quantified using Micromass QuanLynx 3.5 software. (Waters, Watford, UK). The accuracy of the method was assumed to be adequate as the concentrations were calculated from calibration standards prepared in the same way as the extracts (spike calibration). Values were accepted if the internal standard ratio was greater than a value equal to the calibration regression intercept plus approximately 10 times the estimated standard deviation of the intercept (LLOQ). Repeatability precision was considered adequate if duplicate sample values were within 10 % of each other.

Cellular protein and cellular volume. 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 express the drug in cells in terms of a concentration which could be compared with that in the external medium, a relative cell volume of 4.5 µl/10^6 cells was used; this value

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was previously determined by incubations with radiolabelled water and sucrose from several (n = 41) experiments (Hallifax and Houston, 2006b).

Data analysis The concentration of drug in the cell pellet extract was used to calculate the total amount of drug in cells and hence the concentration of drug in cells (C) using the relative cell volume (see above). The concentration of drug in the external medium (M) was measured directly and the hepatocyte: medium partition coefficient (K_p) was calculated as the ratio C/M. It was assumed that the contribution of drug from the aqueous layer adherent to the separated cells was not significant (at a relative adherent volume of 95 % cell volume and a K_p of 100, the contribution of the adherent medium would be ≤ 1 %).

The concentration dependence of accumulation of drug in hepatocytes was described using a two-site binding model incorporating a saturable site and a linear function for unsaturated binding (eq 1) by nonlinear regression analysis using GraFit (Erithacus Software Ltd, Horley, UK).

$$K_p = K_{p,U1,max} - \frac{K_{p,U1,max} \bullet C}{K_{U1} + C} + K_{p,min} \quad (1)$$

where K_p is cell-to-medium concentration ratio of total uptake; $K_{p,U1,max}$ is maximum K_p for saturable uptake; $K_{p,min}$ is K_p for non-saturable uptake; C is the total incubation concentration; K_{U1} is apparent saturable uptake equilibrium constant. Predicted K_p values for maximum total uptake were calculated as the sum of $K_{p,U1,max}$ and $K_{p,min}$.

Prediction of pH effects For basic drugs, the ratio of ionized to unionized drug is given in eq 2. Moderately basic (pKa ≥8.5) drugs are hence highly ionized at physiological pH.

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$$\frac{C^{+}}{C} = 10^{\text{pKa-pH}} \quad (2)$$

where C^{+} is concentration of ionized drug; C is concentration of unionized drug.

The concentration of drug in hepatocytes will be influenced by the difference in pH between the hepatocyte cytosol (pH = 7.1 – 7.2, Williams and Woodbury, 1971; Nilsson et al., 2004) and the medium (pH = 7.4). The relative concentration of ionized drug molecules (for bases) between the cytosol and the medium is given by eq 3.

$$\frac{C^{+}}{M^{+}} = 10^{\text{pH,med-pH,cell}} \quad (3)$$

where C^{+} is concentration of ionized drug in the hepatocyte cytosol; M^{+} is concentration of ionized drug in the medium.

The K_p (ionized and unionized drug) resulting solely from the difference in pH between cytosol and medium will be (see Appendix 1):

$$K_p = \frac{M_{tot} \bullet (10^{\text{pKa-pH,med}}/1+10^{\text{pKa-pH,med}}) \bullet 10^{\text{pH,med-pH,cyt}} + M_{tot} - [M_{tot} \bullet (10^{\text{pKa-pH,med}}/1+10^{\text{pKa-pH,med}})]}{M_{tot} \bullet (10^{\text{pKa-pH,med}}/1+10^{\text{pKa-pH,med}}) + M_{tot} - [M_{tot} \bullet (10^{\text{pKa-pH,med}}/1+10^{\text{pKa-pH,med}})]} \quad (4)$$

where M_{tot} is total (ionized an unionized) drug concentration in the medium.

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In addition, the concentration of drug in hepatocytes will be partly dependent on the pH difference between the hepatocyte cytosol and the lysosomal space (pH = 5: Myers et al., 1995; Cuervo et al., 1997). Chemical partition into lipophilic media is normally assumed to involve unionized drug; here partition into cell membrane is assumed to involve ionized drug (virtually equivalent to total drug) because of the electrostatic interaction with phopholipids (Austin et al., 1995; Rodgers et al., 2005) in addition to the hydrophobic/lipophilic interaction. The difference in pH between medium and cytosol and between cytosol and intra-lysosomal space as well as intracellular binding can be used to calculate the overall f_u for any given cell concentration (see Appendix 2):

$$f_{u,tot} = \frac{1}{1+\left(\frac{L^+}{C^+}\cdot\frac{C^+}{M^+}\cdot\frac{V_{lys}}{V_{inc}}\right)+\left(\frac{C_{min}}{M}\cdot\frac{V_{cell}}{V_{inc}}\right)+\left(\frac{C_{min}}{M}\cdot\frac{L^+}{C^+}\cdot\frac{C^+}{M^+}\cdot\frac{V_{lys}}{V_{inc}}\right)} \quad (5)$$

where L^+ is concentration of ionized drug in the intralysosomal space; C_{min} is minimum total concentration of drug in the cell; V_{lys} is volume of intralysosomal space (1 %, Alberts et al., 1995); V_{inc} is total incubation; V_{cell} is total intracellular volume.

The overall K_p is related to $f_{u,tot}$ by eq 6

$$f_{u.tot} = \frac{1}{1+K_p \bullet V_{cell}/V_{inc}} \quad (6)$$

Hence -

$$K_p = \frac{1/f_{u,tot} - 1}{V_{cell}/V_{inc}} \quad (7)$$

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Comparison between observed K_p values and those predicted based on intracellular pH differences was made for the three drugs in this study together with dextromethorphan (Witherow and Houston, 1999), imipramine and propranolol (Hallifax and Houston, 2006b).

Results

Characteristics of hepatocellular uptake of fluoxetine, fluvoxamine and quinine

The time course of uptake of fluoxetine, fluvoxamine and quinine appeared to be rapid; the cellular concentration observed after 30 s incubation was close to that at 60 s, indicating attainment of equilibrium. This behaviour is in keeping with previous studies with other lipophilic bases (Hallifax and Houston, 2006b).

The plateau concentration values for cellular uptake used to calculate K_p were dependent on the initial concentration for all three drugs (Fig 1A - C). The cellular accumulation for each drug 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 mean maximum fitted values were 140 for quinine, 580 for fluvoxamine and 2010 for fluoxetine (at <1 μM) and these decreased to a minimum of 37 for quinine, 170 for fluvoxamine and 560 for fluoxetine (at concentrations ≥10 μM) (Table 1). The apparent saturable uptake equilibrium constants were between 1 and 3 μM for quinine, fluvoxamine and fluoxetine (Table 1). The rate and extent of accumulation of drug in the cells was considered not to have been influenced by metabolism as the effect of preincubation of hepatocytes with ABT has been shown to overwhelmingly and irreversibly inhibit cytochrome P450-dependent routes (Hallifax and Houston, 2006b).

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Uptake into hepatocytes treated with ATP inhibitor

The K_p values of fluoxetine, fluvoxamine and quinine in hepatocytes pre-treated with FCCP was also dependent on the initial drug concentration but substantially reduced, at low concentrations, compared to that found in the absence of inhibitor (Fig 1). The inhibited cellular accumulation was fitted by nonlinear regression to a two-site model (as described above) and the mean fitted maximum values were 73 for quinine, 330 for fluvoxamine and 1300 for fluoxetine; these decreased to a minimum of 27 for quinine, 160 for fluvoxamine and 400 for fluoxetine (Table 1).

Concentration of drug in cells due to intracellular differences in pH

Table 2 shows certain physiochemical properties for the three drugs used in this study (quinine, fluvoxamine and fluoxetine) together with three other basic drugs for which concentration-dependent cell K_p values have been reported, namely imipramine, propranolol (Hallifax and Houston, 2006b) and dextromethorphan (Witherow and Houston, 1999). For these drugs the proportion of drug ionized at physiological pH (7.4) will be between 95.2 and 99.8 based on their pKa values. If the hepatocellular cytosolic pH is assumed to be 7.2 (Williams and Woodbury, 1971; Nilsson et al., 2004), the ratio between the unbound ionized drug inside and outside the cell will be approximately 1.6 for all these compounds. This ratio is close to that of total (ionized and unionized) drug because of the degree of ionization (eq 4). If the hepatocellular lysosomal pH is assumed to be 5.0 (Myers et al., 1995; Cuervo et al., 1997), the ratio between the unbound ionized drug inside and outside the lysosomes will be approximately 160 for all six drugs.

For each drug, the minimum K_p (at concentrations ≥10 μM) for hepatocytes is assumed to represent the partition into the cell membranes (Austin et al., 1995; Hallifax and Houston,

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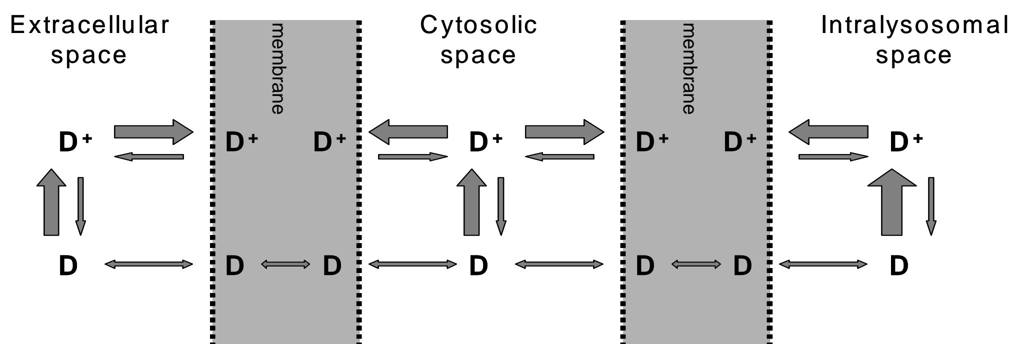
2006b). The ratios of maximal and minimal K_p values for the six bases were between 2.4 and 3.9 (Table 1), indicating a similar magnitude of saturable uptake for these drugs. At low drug concentrations, drug will diffuse into the lysosomes and accumulate due to the lower pH. This process could constitute the major mechanism responsible for $K_{p,max}$, as it would allow high partitioning of ionized drug. The distribution processes for the whole cell are illustrated in Scheme 1. Although ionized drug molecules may become unionized while located in the membrane, the partition of the ionized molecules would predominate, including with the lysosomes. The total maximum uptake into hepatocytes, including (at low drug concentrations) accumulation in lysosomes, can be predicted using eq 5 and 6 (Table 1) using an assumed relative cell volume of 1 % (Alberts et al., 1994); corroboration of this latter approximation is found both in histological analysis (Kumaratilake and Howell, 1989) and by relative protein/lipid content (Zubay, 1993). Predicted maximal K_p values ranged between 55 and 1900 and were in close agreement with those observed (Fig 2A).

Scheme 1. Physicochemical and electrochemical distribution of cationic lipophilic drugs



Prediction of unbound fractions in hepatocyte incubations

Fig 2B shows the poor relationship between $K_{p,min}$ and either log P or log $D_{7.4}$ for the six drugs. Table 3 compares the unbound fractions calculated from the observed K_p values, for two concentrations of cells, and the f_u observed for microsomes (dextromethorphan,

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Witherow and Houston, 1999; quinine, fluoxetine, fluvoxamine, Brown et al., 2006; imipramine, propranolol, Hallifax, unpublished data) at 1 mg microsomal protein/ml. The cell concentrations used were 1 x 10^6 cells/ml and 3.5 x 10^6 cells/ml; the latter was determined as the cell concentration equivalent to 1 mg microsomes/ml based on linear regression of the binding constants in microsomes and hepatocytes on the assumption that $K_{p,min}$ represents the unsaturable partition into membranes. Fig 2C compares the microsomal f_u with the f_u calculated at 3.5 x 10^6 cells/ml using the binding constant from the $K_{p,max}$, and shows that use of microsomes as a surrogate of cells overestimates the in vitro f_u for these drugs. This contrasts with the report of Austin et al., (2005) using a more chemically diverse set of drugs. However, unbound fractions calculated from $K_{p,max}$ and predicted from eq 5 are highly correlated — see Fig 2D. Also shown in Fig 2D is the consequence of ignoring the 3-fold difference between $K_{p,max}$ and $K_{p,min}$, resulting in substantial error in the uptake/binding constants, as indicated above for microsomes.

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Discussion

One aim of this study was to characterize the uptake of quinine, fluvoxamine and fluoxetine selected on the basis of their lipophilic basic nature and their potency as P450 inhibitors. In addition, we investigated the contribution of ATP-dependent processes to uptake using the inhibitor, FCCP. To avoid complications from the simultaneous processes of metabolism, the irreversible P450 inhibitor, ABT, was used (Hallifax and Houston, 2006b). Uptake into intact hepatocytes for quinine, fluvoxamine and fluoxetine was both rapid and substantial in magnitude ($K_{p,max}$ = 143, 577 and 2010 for quinine, fluvoxamine and fluoxetine, respectively). As previously reported for imipramine and propranolol (Hallifax and Houston, 2006b), concentration dependence in the accumulation in isolated hepatocytes for these compounds was demonstrated and 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. The saturable uptake of quinine, fluvoxamine and fluoxetine was substantially, but incompletely, reduced after pre-treatment of hepatocytes with the ATP inhibitor, FCCP, providing evidence of involvement of an active process. Partial inhibition of saturable uptake does not necessarily indicate involvement of an additional, non-active, process; partial inhibition by several ATP inhibitors has been observed in other studies (Dell'Antone, 1988; D'Souza et al., 1987; Van Dyke, 1993). Also, there is evidence to support the specificity of the FCCP cellular effect in reducing ATP (Yamazaki et al., 1993 and 1996). Saturable uptake of quinine, fluvoxamine and fluoxetine appears to be typical of lipophilic amines, but the mechanism of the active process has yet to be unequivocally explained. In our previous study (Hallifax and Houston 2006b), the saturable uptake of imipramine and propranolol was inhibited by a range (n = 16) of lipohpilic amines (including quinine) but was not inhibited by several neutral or acidic drugs.

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Previously, the saturable uptake process was resolved from the non-saturable process using either freeze-thawing or the plasma membrane permeabliser, saponin (Hallifax and Houston, 2006b). The non-saturable component was considered passive and due to the amphiphilic nature (cationic moiety (amine) located in aliphatic part of the molecule, remote from the hydrophobic moiety) of these compounds, a combination of diffusion and subsequent binding to the phospholipid components of membranes, by both electrostatic and lipophilic alignment between the phospholipid molecules. Membrane binding of lipophilic bases has been widely reported (Di Francesco and Bickel, 1977; Fisar et al., 1991; Austin et al., 1995) and it is reasonable to assume that the non-saturable component of uptake of quinine, fluvoxamine and fluoxetine is of this nature. Another lipophilic amine which has been shown to undergo saturable uptake into hepatocytes is dextromethorphan (Witherow and Houston, 1999). When the minimum K_p (membrane partition) for the six drugs is compared with their log P or log $D_{7.4}$, there is no apparent correlation (Fig 2B) indicating that this binding phenomenon is more complex than partition into octanol. For example, dextromethorphan shows a relatively weak partition into cells despite a relatively high log P value; the amine moiety of this drug is located close to the centre of the hydrophobic cyclic part of the molecule in contrast with the other compounds, where the charge is more remote.

Basic, permeable drugs would be expected to accumulate intracellularly due to the difference between the intracellular pH and the extracellular pH, based on the relationship between pH and pKa, regardless of any active uptake or intracellular binding. On this basis, the six lipophilic amines are calculated to equilibrate at a 1.6-fold greater concentration of ionized molecules within hepatocytes. Being moderately basic (pKa = 8.5 - 10), these compounds are almost completely ionized and so the ratio of total drug is also about 1.6-fold greater within the cells (based on a cytosolic pH of 7.2), which represents a minor but significant

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accumulation of unbound drug. In addition to the pH difference between the hepatocyte cytosol and external medium, the more pronounced pH difference between the cytosol and the intra-lysosomal space (pH ~5) is pertinent. There have been numerous reports of the phenomenon of trapping by lysosomes of basic compounds including imipramine and propranolol (Moseley and Van Dyke, 1995; Ishizaki et al., 1996; Siebert et al., 2004). Maintenance of the low lysosomal pH is ATP-dependent (Dell'Antone, 1984, 1988; D'Souza et al., 1987; Moseley and Van Dyke, 1995; Strazzabosco and Boyer, 1996) and so the possibility that the saturable component of uptake represents the lysosomal uptake is supported by the data for uptake in cells pretreated with the ATP inhibitor, FCCP. There are numerous inhibitors of ATP-dependent processes; FCCP, however, has been shown to inhibit lysosomal pH maintenance, specifically (Dell'Antone, 1988). Saturation may be due to pH elevation, at high concentrations of sequestered ions, by exhaustion of ATP-dependent buffering capacity. For example, if the lysosomal buffer capacity, without active maintenance, is about 1 mM, cytosolic concentrations of basic drugs of only 2 - 3 µM would raise the lysosomal pH sufficiently to lower the lysosomal uptake by about 50 %. Hepatocyte cytosolic buffering capacity is in the range 10 - 20 mM (Strazzabosco and Boyer, 1996); the buffering capacity of lysosomes is unknown. Reduction of saturable uptake of chloroquine into rat hepatocytes when exposed to 10 mM NH_4Cl has been demonstrated in a previous investigation into lysosomal uptake, by MacIntyre and Cutler, 1993.

In order to predict the total cell uptake when lysosomal uptake is not saturated, it is necessary to include intra-organellar binding to the lysosomes. Using $K_{p,min}$ to represent partition into membranes, the contribution of partition within the lysosomes to the total cell uptake is calculated to be minor if $K_{p,min}$ is dependent on unionized drug (because the 100-fold greater proportion of ionized drug at the low lysosomal pH). However, if the $K_{p,min}$ is assumed to be

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dependent on total drug, then the intra-lysosomal partition will be proportional to the greatly magnified total intra-lysosomal drug concentration. On this basis, the lysosomal uptake is substantial. The predicted total cellular uptake is in close agreement with that observed for the maximal uptake of the six drugs using a lysosomal pH of 5.0 (Fig 2A**)**. These drugs range in their lipophilicity by two log P units and this correlation therefore emphasizes the dependency of the magnitude of the putative lysosomal uptake on intracellular pH distribution. The assumption that membrane partition depends on ionized molecules is justified for these compounds because the mechanism comprises a lipophilic binding within the membrane together with an electrostatic interaction (Austin et al., 1995 and 2002; Rodgers et al., 2005).

For unsaturable membrane partition, it seems reasonable to assume that binding to microsomes would provide a practical surrogate for the same process in cells. For the six drugs investigated in this study, we have determined the approximate concentration of hepatocytes equivalent to the concentration of microsomes given by 1 mg protein/ml, to be 3.5 x 10^6 cells/ml. However, if this equivalence is used to predict the $f_{u,inc}$ for hepatocytes at low drug concentrations, when uptake involves both the saturable as well as unsaturable components, an overestimate is obtained (Fig 2C). The appropriateness of either microsomes or de-natured hepatocytes (Austin et al., 2005) is therefore questionable for assessment of $f_{u,inc}$ using hepatocytes for this type of drug.

It is important to consider how the characteristic uptake of lipophilic amine drugs impacts on determination of intrinsic clearance and drug-drug interaction potential within the hepatocyte in vitro system. It is reasonable to assume that the concentration of unbound drug at the active site will be equal to the concentration of total drug in the incubation after correcting

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for total binding in the incubation and for the cytosolic pH difference. It should be noted that the effect of the cytosolic/external medium pH differential implies an underprediction of clearance when using non-cellular systems of up to about 50 %, for cationic P450 substrates; although not generally recognized, this effect is pertinent to the extensive use of microsomes for prediction of clearance. Previously, we demonstrated (Hallifax and Houston, 2006b) the implications of this uptake in the determination of clearance by measuring the time-course of propranolol depletion when incubated with hepatocytes. The clearance measurement was only affected by the nonsaturable binding which was rapid compared with the rate of saturable uptake. For this type of assay, it was apparent that the relative rates of uptake and metabolism are an important consideration for the hepatocyte system. However, because of the predominance of metabolic clearance for this particular drug, it was not possible to assess whether the saturable uptake of these drugs involved intracellular accumulation of unbound drug, such as has been observed for other drugs (Lam and Benet, 2004).

Saturability of uptake for these drugs requires that the in vitro f_u is determined over a concentration range. Use of non-saturating uptake values only, such as is implicit in the log P approach recently advocated by Austin et al. (2005), may result in an error in the estimation of the kinetic constants K_M and K_I for this type of drug. The ratio $K_{p,max}/K_{p,min}$, such as provided by this study, may be used to correct the $f_{u,inc}$ due to partition, providing an estimate of the saturating concentration is available (Fig 2D). Among the six lipophilic amines described in this report, the apparent dissociation equilibration constant varied between 1 and 70 μM so it would seem appropriate to apply maximum $f_{u,inc}$ for incubations at greater than 10 μM and minimum $f_{u,inc}$ below 1 μM.

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In conclusion, we have provided further evidence for lipophilic amine drugs distributing into the liver by a combination of highly favourable (>100-fold) and non-saturating partition, with considerable enhancement (3-fold) by a saturable process at low concentrations. This saturable, apparently active, process can be explained by uptake into lysosomes within hepatocytes (rather than by membrane transport), as supported by direct evidence of similar unbound K_I for these drug in microsomes and hepatocytes (Brown HS, Chadwick A and Houston JB, unpublished observations). Cationic amphiphilicity, therefore, appears important both in the saturable and unsaturable uptake of these drugs and the need to correct in vitro parameter values for the appropriate concentration-dependent $f_{u,inc}$ is clear.

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Footnote

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Figure Legends

Fig. 1. Uptake into isolated rat hepatocytes, with (●) or without (○) pre-treatment with FCCP, expressed as cell-to-medium concentration ratio (K_p) as a function of initial concentration of quinine (A), fluvoxamine (B) and fluoxetine (C).

Fig. 2. Prediction of hepatocellular uptake for six lipophilic amine drugs. (A) Correlation of predicted and observed maximal cell-to-medium ratio concentration (K_p); (B) Comparison of unsaturable cell binding ($K_{p,min}$) with log P and log $D_{7.4}$; (C) Comparison of fraction unbound observed in microsomal incubation with that calculated for an equivalent concentration of hepatocytes; (D) Correlation of predicted and observed maximal incubation unbound fraction ($f_{u,inc}$) using either $K_{p,min}$ (○) or $K_{p,max}$ (●).

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TABLE 1

Uptake parameter values (observed and predicted) for six lipophilic amine drugs with or without pre-treatment with FCCP

Substrate	$K_{p,U1,max}$		$K_{p,min}$		K_{U1} (µM)	$K_{p,max}$	$K_{p,min,av}$ [3]	$K_{p,max}$ / $K_{p,min}$	Predicted $K_{p,max}$ [4]
	-FCCP	+FCCP	-FCCP	+FCCP					
Dextromethorphan[1]	35	-	15		70	50	15	3.3	54.9
Quinine	96	46	57	27	1	143	36	3.9	125
Propranolol[2]	171	-	69	-	30	240	69	3.5	239
Imipramine[2]	210	-	150	-	10	360	150	2.4	514
Fluvoxamine	389	181	188	157	1	577	169	3.4	579
Fluoxetine	1280	909	732	396	3	2010	564	3.6	1920

[1] Witherow and Houston, 1999

[2] Hallifax and Houston, 2006

[3] Average $K_{p,min}$ in the absence and presence of FCCP

[4] Using equation 5

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TABLE 2

Physicochemical characteristics and pH-dependent ionization for six lipophilic amine drugs

Drug	pKa	log P	log $D_{7.4}$	% ionisation at pH 7.4	% unionised at pH 7.4	% ionisation at pH 5.0	% unionised at pH 5.0
Dextromethorphan	8.7	4.5	3.2	95.2	4.8	99.980	0.02
Quinine	8.7	3.4	2.1	95.2	4.8	99.980	0.02
Propranolol	9.5	2.3	0.2	99.2	0.8	99.997	0.003
Imipramine	9.5	4.0	1.9	99.2	0.8	99.997	0.003
Fluvoxamine	9.4	3.4	1.4	99.0	1.0	99.996	0.004
Fluoxetine	10.1	4.2	1.5	99.8	0.2	99.999	0.001

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TABLE 3

Unbound fractions in incubations with hepatocytes and microsomes ($f_{u,inc}$)

Drug	$f_{u,inc}$ Hepatocytes (10^6 cells/ml)		Hepatocytes ($3.5^1 \times 10^6$ cells/ml)		Microsomes (1 mg protein/ml)
	at $K_{p,max}$ [2]	at $K_{p,min}$ [3]	at $K_{p,max}$ [2]	at $K_{p,min}$ [3]	
Dextromethorphan	0.80	0.93	0.57	0.82	0.98
Quinine	0.58	0.84	0.32	0.65	0.36
Propranolol	0.45	0.74	0.21	0.49	0.60
Imipramine	0.36	0.57	0.16	0.31	0.45
Fluvoxamine	0.25	0.54	0.10	0.28	0.27
Fluoxetine	0.09	0.26	0.03	0.10	0.09

[1] Ratio $K_{a,msome}$ / $K_{a,cell,app}$

[2] Calculated using maximum K_p

[3] Calculated using minimum K_p

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Fig. 1

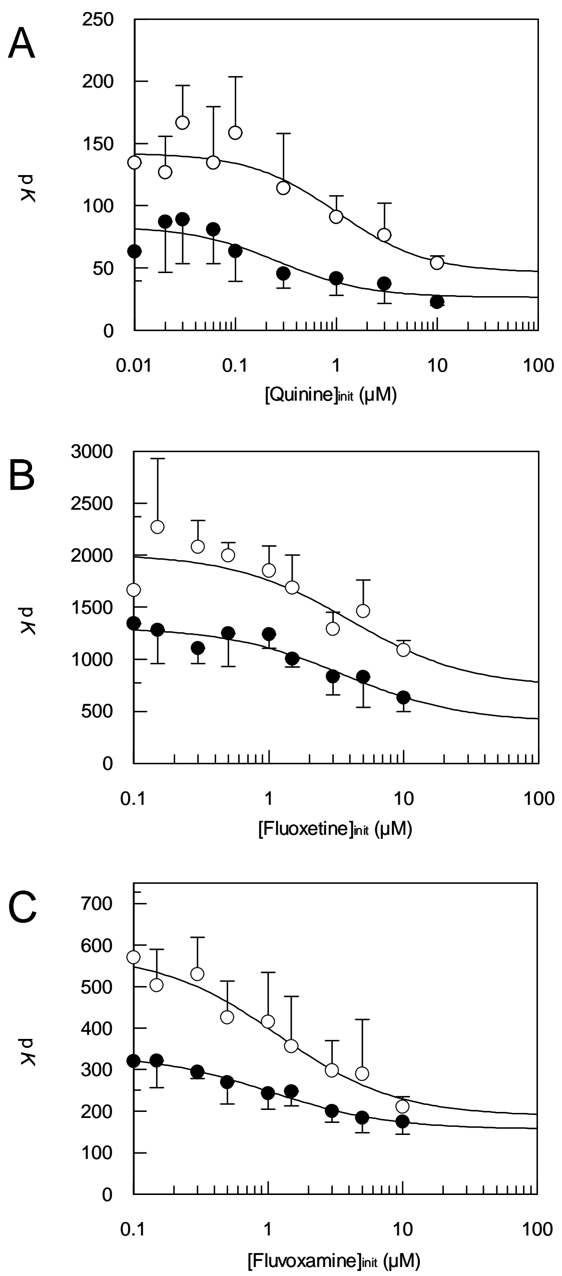

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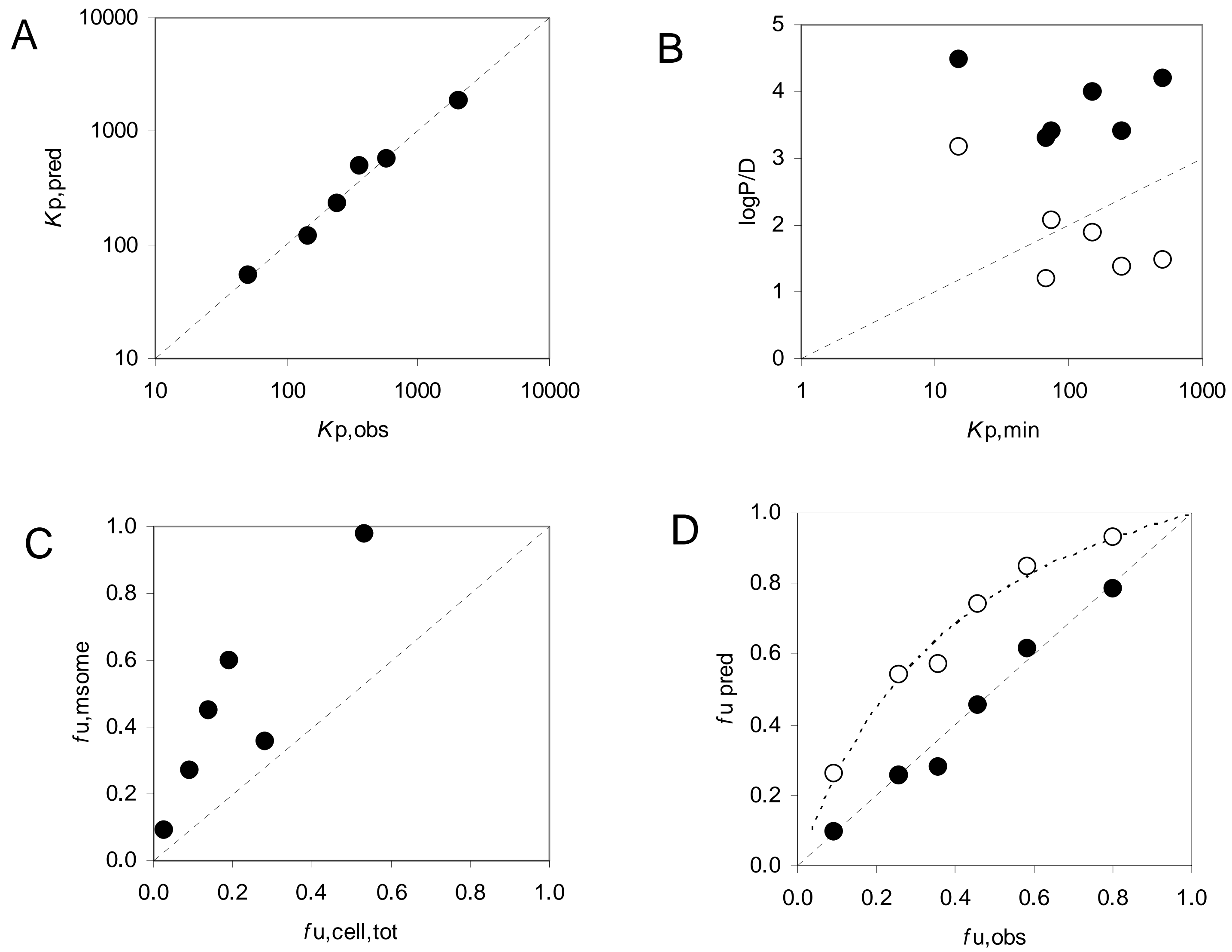
Fig. 2



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Appendix 1

Calculation of K_p due to pH difference between cytosol and external medium (eq 4)

K_p can be written

$$\frac{C + C^+}{M + M^+} \qquad \text{(A1.1)}$$

C^+ can be given by (from eq 3)

$$M^+ \bullet 10^{pH,med-pH,cyt} \qquad \text{(A1.2)}$$

M^+ can be given by (from eq 2)

$$M_{tot} \bullet \frac{M^+}{M^+ + M} = M_{tot} \bullet \frac{10^{pKa-pHmed}}{1 + 10^{pKa-pHmed}} \qquad \text{(A1.3)}$$

where M_{tot} is total (ionized an unionized) drug concentration in the medium.

M can be given by

$$M_{tot} - M^+ \qquad \text{(A1.4)}$$

where M is unionized drug in the medium.

Assuming uptake by diffusion only (at equilibrium)

$$C = M \qquad \text{(A1.5)}$$

where C is unionized drug in the cell.

And, as

$$M_{tot} = M^+ + M, \qquad \text{(A1.6)}$$

the K_p (C_{tot}/M_{tot}) (ionized and unionized drug) resulting solely from the difference in pH between cytosol and medium can be given by:

$$K_p = \frac{M_{tot} \bullet (10^{pKa-pH,med}/1 + 10^{pKa-pH,med}) \bullet 10^{pH,med-pH,cyt} + M_{tot} - [M_{tot} \bullet (10^{pKa-pH,med}/1 + 10^{pKa-pH,med})]}{M_{tot} \bullet (10^{pKa-pH,med}/1 + 10^{pKa-pH,med}) + M_{tot} - [M_{tot} \bullet (10^{pKa-pH,med}/1 + 10^{pKa-pH,med})]}$$

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Appendix 2

Calculation of total incubation fu at maximal uptake (eq 5)

The fraction of drug unbound in an in vitro incubation is dependent on the binding (partition) equilibrium constant and the concentration (or relative volume) of the binding compartment in an incubation system (Witherow and Houston, 1999),

$$f_{\mathrm{u,tot}} = \frac{1}{1 + K_{\mathrm{p}} \bullet \frac{V_{\mathrm{cell}}}{V_{\mathrm{inc}}}} \qquad \text{(A2.1)}$$

where V_{cell} is the total incubation cell volume; V_{inc} is the total incubation volume,

This eq can be extended to cover a number of binding components (i) when their respective volumes (V_{i}) are known.

$$f_{\mathrm{u,tot}} = \frac{1}{1 + \sum K_{\mathrm{p},i} \bullet \frac{V_{\mathrm{i}}}{V_{\mathrm{inc}}}} \qquad \text{(A2.2)}$$

The f_{u} for total cell uptake of the drugs in this study was calculated by using specific ratios and relative volumes for four distinct components:

1) Ionised drug concentration ratio across the cell membrane

$$\frac{\mathrm{C}^{+}}{\mathrm{M}^{+}} \bullet \frac{V_{\mathrm{cell}}}{V_{\mathrm{inc}}} \qquad \text{(A2.3)}$$

where C^{+} is concentration of ionized drug in the cell; M^{+} is concentration of ionized drug in the medium

2) Ionised drug concentration ratio across the lysosomal membrane

$$\frac{\mathrm{L}^{+}}{\mathrm{C}^{+}} \bullet \frac{\mathrm{C}^{+}}{\mathrm{M}^{+}} \bullet \frac{V_{\mathrm{lys}}}{V_{\mathrm{inc}}} \qquad \text{(A2.4)}$$

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where L^+ is concentration of ionized drug in the lysosomes; V_{lys} is the total lysosomal volume (calculated from reported proportion (1 %) of cell volume (Alberts et al., 1995))

3) Lipophilic partition into whole cell (non-saturable)

$$\frac{C_{min}}{M} \bullet \frac{V_{cell}}{V_{inc}} \qquad \text{(A2.5)}$$

where C_{min} is minimum concentration of total drug in cells (at saturating drug concentration); M is the concentration of total drug in the medium

4) Lipophilic partition into lysosome

$$\frac{C_{min}}{M} \bullet \frac{L^+}{C^+} \bullet \frac{C^+}{M^+} \bullet \frac{V_{lys}}{V_{inc}} \qquad \text{(A2.6)}$$

As component A3 has a very minor contribution to f_u (approx. 1 %), this can be omitted and A4, A5 and A6 combined resulting in eq 5:

$$f_{u,tot} = \frac{1}{1 + \left(\frac{L^+}{C^+} \cdot \frac{C^+}{M^+} \cdot \frac{V_{lys}}{V_{inc}}\right) + \left(\frac{C_{min}}{M} \cdot \frac{V_{cell}}{V_{inc}}\right) + \left(\frac{C_{min}}{M} \cdot \frac{L^+}{C^+} \cdot \frac{C^+}{M^+} \cdot \frac{V_{lys}}{V_{inc}}\right)}$$

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