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Ion-trapping, microsomal binding and unbound drug distribution in the hepatic retention of basic drugs[φ]

Gerhard A Siebert, Daniel Y Hung, Ping Chang and Michael S Roberts

Department of Medicine, University of Queensland, Princess Alexandra Hospital, Woolloongabba, Queensland, Australia

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Running title: Hepatic disposition of basic drugs

Corresponding author:

Professor Michael S Roberts

Department of Medicine, University of Queensland,

Princess Alexandra Hospital, Woolloongabba Qld 4102

Australia

Phone +61 7 3240 2546

Email mroberts@soms.uq.edu.au

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List of Abbreviations

CL_{int}	intrinsic elimination clearance	K_v	equilibrium amount ratio characterising the vesicular ion-trapping sites (ion-trapping parameter)
CV^2	normalized variance	k_{vc}	rate constant for transport from acidic vesicles into cytosol
DMO	dimethyloxazolidine-2,4-dione	*MP*	microsomal protein
E	hepatic extraction ratio	*MTT*	mean transit time
f_{uB}	drug fraction unbound in perfusate	pK_a	the negative logarithm of the ionisation constant
$f_{u,MP}$	drug fraction unbound in microsomal protein	*PS*	permeability-surface area product
K_b	equilibrium amount ratio characterising the intracellular binding sites	*Q*	blood flow
k_{cv}	rate constant for transport from cytosol into acidic vesicles	*RBC*	red blood cell
k_e	elimination rate constant	V_B	extracellular reference space
k_{in}	influx rate constant	V_C	cellular water volume
Km	Michaelis-Menten constant	V_{max}	maximum velocity
k_{out}	efflux rate constant		

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Abstract

This study investigated the relative contribution of ion-trapping, microsomal binding and distribution of unbound drug as determinants in the hepatic retention of basic drugs in the isolated perfused rat liver. The ionophore monensin was used to abolish the vesicular proton gradient and thus allow an estimation of ion-trapping by acidic hepatic vesicles of cationic drugs. In vitro microsomal studies were used to independently estimate microsomal binding and metabolism. Hepatic vesicular ion-trapping, intrinsic elimination clearance, permeability-surface area product and intracellular binding were derived using a physiologically based pharmacokinetic model. Modelling showed that the ion-trapping was significantly lower after monensin treatment for atenolol and propranolol, but not for antipyrine. However, no changes induced by monensin treatment were observed in intrinsic clearance, permeability or binding for the three model drugs. Monensin did not affect binding or metabolic activity *in vitro* for the drugs. The observed ion-trapping was similar to theoretical values estimated using the pHs and fractional volumes of the acidic vesicles and the pK_as of drugs. Lipophilicity and pK_a determined hepatic drug retention: a drug with low pK_a and low lipophilicity (e.g. antipyrine) distributes as unbound drug, a drug with high pK_a and low lipophilicity (e.g. atenolol) by ion-trapping and a drug with a high pK_a and high lipophilicity (e.g. propranolol) is retained by ion-trapping and intracellular binding. In conclusion, monensin inhibits the ion-trapping of high pK_a basic drugs leading to a reduction in hepatic retention but with no effect on hepatic drug extraction.

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Basic lipophilic compounds are characterized by a high volume of distribution as a result of extensive tissue uptake. The main mechanisms of such a distribution pattern are non-specific binding to membrane phospholipids (Bickel and Steele, 1974; Francesco and Bickel, 1977; Romer and Bickel, 1979), binding to microsomal protein (Hung et al., 2002) and the sequestration of the compounds into acidic vesicular compartments such as lysosomes or mitochondria (Daniel et al., 1995). A potential consequence of an apparent irreversible sequestration of basic drugs into acidic vesicles is a potentially reduced drug bioavailability (de Duve et al., 1974; Ohkuma and Poole, 1978) or drug interactions (Daniel and Wojcikowski, 1999b; Nebbia et al., 1999). Lysosomal trapping of basic lipophilic drugs has also been demonstrated to be an important determinant of disposition for desipramine and chloroquine and psychotropic compounds such as the piperidine and piperazine-type neuroleptics (Daniel et al., 2001). The lysosomotropic properties of basic drugs are particularly important determining drug disposition and pharmacokinetics in lysosome-rich organs such as lungs, kidneys or the liver.

Specific studies determining the relative contribution of ion-trapping and microsomal binding to the hepatic retention of drugs or relating the relative uptake to the physico-chemical properties of drugs do not appear to have been undertaken so far and are the focus of the present study. Fluorescence microscopy, acridine orange staining and other studies using primary hepatocyte cultures have shown that H^+ ionophores, such as the carboxylic antibiotic monensin, abolish the pH gradient in the approximately 170 acidic vesicles in hepatocytes (Lake et al., 1987). Such studies have not been carried out in the isolated perfused rat liver which not only retains intact cellular integrity and fully functional lysosomes but allows the effects of drug structure on hepatocyte wall permeability, hepatic binding, sequestration and metabolism to be described (Hung et al., 2001; Hung et al., 2002). Monensin infusions should allow the acidic vesicle pH gradient (and thus the ability for ion trapping of basic drugs) to be

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experimentally abolished without affecting other permeation, binding and clearance processes.

To evaluate the importance of physico-chemical drug characteristics such as lipophilicity and pK_a on ion-trapping and microsomal binding and thus drug retention, we compared two drugs with similar pK_a but different lipophilicity (apparent log octanol-water partition coefficient, log $P_{app,}$ atenolol and propranolol) and two drugs with a similar lipophilicity but different pK_a (atenolol and antipyrine). The study follows up on the recently reported structure-hepatic disposition relationships of cationic drugs in normal and diseased rat liver (Hung et al., 2001; Hung et al., 2002). Pharmacokinetic parameters such as hepatocellular influx, efflux, binding and elimination for the three drugs were determined and kinetic parameters were derived from an improved two-phase physiologically based organ pharmacokinetic model (Weiss and Roberts, 1996; Hung et al., 2001; Hung et al., 2003) and the relative contribution of ion-trapping, intracellular binding and unbound drug to hepatic drug retention was calculated.

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Material and Methods

Chemicals

Atenolol (4-[2-hydroxy-3-[(1-methylethyl)amino[propoxy]benzeneacetamide), antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3*H*-pyraol-3-one), Monensin sodium (2-[5-ethyltetrahydro-5-[tetrahydro-3-methyl-5-[tetrahydro-6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-2H-pyran-2-yl]-2-furyl]-2-furyl]-9-hydroxy-β-methoxy-α,γ,2,8-tetramethyl-1,6-dioxaspiro[4,5]decane-7-butyric sodium salt), propranolol (1-[(1-methylethyl)amino]-3-(1-naphtalenyloxy)-2-propanol) all were obtained from Sigma Chemical Co., St Louis, MO. [U-^{14}C]DMO (dimethyloxazolidine-2,4-dione), ^{14}C-sucrose and ^{3}H-water were purchased from Amersham, Buckinghamshire, UK.

In situ perfusion of the isolated rat liver

The experimental protocol was approved by the University of Queensland Animal Ethics Committee. Perfusion of the isolated rat liver used in this study was performed as described elsewhere (Cheung et al., 1996). Briefly, male Wistar rats, weighing 200-250 g were anaesthetised using an intraperitoneal injection of xylazine/ketamine (10/80 mg kg^{-1}). The laparatomised rats were heparinized with 200 units heparin injected into the inferior vena cava. The bile duct and the portal vein were cannulated (PE-10, Clay Adams, Franklin Lakes, NJ) and using an intravenous 16-gauge catheter, respectively. The liver was then perfused with MOPS [3-(N-morpholino-)propanesulfonic acid]-buffer containing 2% BSA and 15% washed canine red blood cells, adjusted to pH 7.40 and oxygenated via a silastic tubing lungwith 100% pure oxygen. A non-circulating peristaltic pump was used as perfusion system. Once perfusion was established the animals were sacrificed by thoracotomy and the inferior vena cava was cannulated for sample collection. The animals were placed in a

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temperature-controlled environment at 37°C. Assessment of liver viability was by macroscopic appearance, measurement of bile flow, oxygen consumption and portal resistance pressure (Cheung et al., 1996).

Perfusions were adjusted to a flow rate of 15 ml/min and given a 10-min stabilising period before the injection of the first bolus. Each bolus consisted of 50 µl of buffer containing a given concentration of radiolabelled compound (^{14}C-sucrose, ^{3}H-water, ^{14}C-DMO) or cationic drug, in the presence and absence of 0.5 mM monensin). The injection was timed to coincide with the start of a computer-controlled fraction collector (samples were collected over 4 min: 20 x 1 s, 5 x 4 s, 5 x 10 s, 4 x 30 s).

The collected samples were centrifuged and 75 µl aliquots of the supernatant containing [^{3}H-water, ^{14}C-sucrose, ^{14}C-antipyrine or ^{14}C-DMO were taken for scintillation counting (MINAXI beta TRI-CARB 4000 series liquid scintillation counter, Packard Instruments Co., USA). The remainder was vortexed for HPLC analysis and the atenolol and propranolol outflow concentrations were determined (Hung et al., 2001).

In vitro binding of cationic drugs to a microsomal protein preparation

To assess the effect of monensin on hepatic drug binding and metabolism an in vitro study with a microsomal protein preparation in the presence and absence of monensin was carried out, using buffer containing 0.35 mg/mL MP from normal livers. The unbound fraction of cationic drug in each buffer solution was estimated using an ultra-filtration method. A known concentration of the cationic drug stock solution was added to 500 µL of each buffer solution to make final concentration of 0.05 µM and placed in a centrifugal filter device (Microcon YM-30, 30,000 MWCO, Millipore Corp., Bedford, MA) and then centrifuged at 3000 x g for 10 min. The ultra-filtrate (in triplicate) was assayed by HPLC. The fraction of drug unbound

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in the microsomal protein preparation (f_{uMP}) was determined as the ratio of the free concentration to total concentration of solute.

The impact of monensin on hepatic drug metabolism was assessed in these studies using intrinsic elimination clearance CL_{int} in the presence and absence of monensin estimated by the formula $CL_{\text{int}} = \frac{V_{\max}}{K_m + C}$ where V_{max} is the maximum velocity, K_m is the Michaelis-Menten constant and C the concentration of the drug. The respective K_m values were taken from the literature: antipyrine (Roberts and Rowland, 1986), atenolol (Bagwell et al., 1989), propranolol (Ishida et al., 1992). V_{max} was determined by drug incubation with the microsomal protein preparation at 37°C and sampling at time points 0, 5, 10 and 20 min. The concentrations of drugs were analyzed by high performance liquid chromatography (Hung et al., 2002). Data were fitted to the formula and CL_{int} estimated using Scientist (Micromath Scientist, Salt Lake City, UT) (Hung et al., 2001; Hung et al., 2002). The perfused liver intrinsic clearance was based on the assumption that 70 % of the harvested microsomal protein was metabolically active and an average amount of 50 mg microsomal protein was obtained per g liver (Roberts and Rowland, 1986).

Analytical procedure

The high performance liquid chromatography method employed in this study has been described and validated previously (Hung et al., 2001).

Modelling and data fitting of the outflow concentration-time profiles of extracellular and cellular references

Calculation of intracellular pH (pH_i)

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Changes in intracellular pH were calculated according to the method of Le Couteur et al (Le Couteur et al., 1993).

Data analysis

The two-phase physiologically based organ pharmacokinetic model describing inter-sinusoidal mixing (Roberts et al., 1988), transfer across the hepatocyte membrane, and the intracellular distribution and elimination kinetics (Weiss and Roberts, 1996; Weiss et al., 1997) has been further developed to account for both the ion-trapping effect (K_v, characterizing the acidic vesicular ion-trapping sites) and intracellular binding (K_b, characterizing the total of intracellular binding) for the three model cationic. Briefly, as shown in Fig. 1 the model assumes drug transfer across the cytoplasmic mambrane with influx and efflux rate constants k_{in} and k_{out}, respectively, recognising that solute concentrations change in space and time in both phases. The stochastic approach represents the transit of a molecule through the organ as a series of sojourns in one of the two regions described by density functions. The density of cellular residence times $\hat{f}_y(s)$ describes the hepatocellular distribution and elimination kinetics.

The retention time distribution $f_y(t)$ of a drug after a single excursion in the cellular space for the resulting two-compartment cell model was obtained by standard methods in the Laplace domain, $\hat{f}_y(s) = L^{-1}[f_y(t)]$, as described earlier (Weiss, 1999; Weiss et al., 2000).

$$\hat{f}_y(s) = \frac{(s + k_{vc})k_{in}}{s^2(k_{in}/k_{out})(1+K_b) + s((k_{in}/k_{out})(k_{vc} + K_b k_{vc} + k_e + k_{cv}) + k_{in}) + (k_{in}/k_{out})k_e k_{vc} + k_{in}k_{vc}} \quad \textbf{(1)}$$

where the influx rate constant $k_{in} = f_{uB}PS/V_B$, is the permeation clearance per extracellular volume V_B , PS is the permeability-surface product, f_{uB} is the unbound fraction of solute in the perfusate, k_{cv} and k_{vc} represent the rate constant for transport from cytosol into the acidic

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compartment (lysosomes and mitochondria) or from acidic vesicles into cytosol, respectively, determining the equilibrium amount ratio $K_v = k_{cv}/k_{vc}$ characterising ion-trapping. K_b is the equilibrium amount ratio characterizing the intracellular binding sites (microsomal and non-specific binding) and the elimination rate constant defined as $k_e = CL_{int} /V_C$ is the intrinsic elimination clearance normalised per cellular volume V_C (Pang et al., 1995). On comparison of the CL_{int} obtained in microsomal metabolism studies with the one determined when K_b was assigned as a slow distribution process it was found that this resulted in an unrealistically high clearance rate. Therefore it was assumed that K_b is characterized by an instantaneous distribution process (eliminating one fitting parameter at the same time).

The hepatic transit time density function $\hat{f}(s)$ of solutes can be derived in terms of the extracellular transit time density of a non-permeating reference molecule (sucrose) $\hat{f}_B(s)$ (see Equation 2), and the density function of successive sojourn times $\hat{f}_y(s)$ of the drug molecules into the cellular space

$$\hat{f}(s) = \hat{f}_B[s + k_{in}(1 - \hat{f}_y(s))] \qquad \textbf{(2)}$$

The fractional outflow versus time data were fitted in the time domain using a numerical inverse Laplace transformation of the appropriate transit time density function using the non-linear regression program SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT). Data were analysed by a sequential procedure: First, the fractional outflow curve $C_{sucr}(t)$ of the extracellular marker [U-^{14}C]sucrose was fitted by Equation (3), whereby $\hat{f}_{cath}(s)$ accounts for the catheter and $\hat{f}_B(s)$ includes the large vessel transit time

$$C_{sucr}(t) = \frac{Dose}{Q} L^{-1}\left\{ \hat{f}_{cath}(s)\hat{f}_B(s) \right\} \qquad \textbf{(3)}$$

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and the transit time density (*TTD*) of the non-permeating indicator is given by

$$\hat{f}_B(s) = p\hat{f}_1(s) + (1-p)\hat{f}_2(s) \tag{4}$$

with

$$\hat{f}_i(s) = \exp\left\{\frac{1}{CV_i^2} - \left[\frac{MTT_i}{CV_i^2/2}\left(s + \frac{1}{2MTT_i CV_i^2}\right)\right]^{1/2}\right\} (i=1,2) \tag{5}$$

Equation (5) is the Laplace transform of the inverse Gaussian density function with mean MTT_i and relative dispersion CV_i^2. Equations (3) - (5) have been shown to adequately describe the *TTD* of vascular markers in the perfused rat liver (Weiss et al., 1997).

Catheter transit time density was calculated by an independent experiment fitting Equations (4) and (5) to the catheter system outflow profile. The four parameters describing $\hat{f}_{cath}(s)$ were then fixed while fitting the liver outflow data.

The mean transit time of the extracellular reference, $MTT_B = \int_0^\infty t f_B(t)dt$, is given by

$$MTT_B = pMTT_1 + (1-p)MTT_2 \tag{6}$$

$V_B = MTT_B Q(1\text{-}hematocrit)$, is the sum of the sinusoidal plasma space volume accessible to sucrose, and the Disse space, $V_B = V_{Plasma} + V_{Disse}$. $Q(1\text{-}hematocrit)$ denotes the plasma flow rate]. Sucrose does not distribute into erythrocytes, therefore this extracellular space value has to be corrected for hematocrit (Varin and Huet, 1985). Second, utilising this information the outflow concentration data of the permeating drugs, $C(t)$, were analysed, i.e. the parameters MTT_i, CV_i^2 (i = 1, 2), and p of the individual fits of [U-^{14}C]sucrose data were substituted as fixed parameters in $\hat{f}_B(s)$ of the model (Equation 2)

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$$C(t) = \frac{Dose}{Q} L^{-1}\left\{ \hat{f}_{cath}(s)\,\hat{f}(s) \right\} \quad \textbf{(7)}$$

and the parameters k_{in}, k_{out}, K_v, K_b and k_e were estimated.

In this study a uniform $K_v = 0.25$ was assumed in monensin treatment (the K_v value was estimated from the volume ratios of the subcellular compartments to the remaining cytoplasmic fraction: 0.21 for mitochondria plus lysosomes and 0.79 for cytosol (Rhoades and Pflanzer, 1996), and used as a fixed parameter in data fitting to obtain k_{in}, k_e, k_{out} and K_b. The parameters k_{in}, k_{out}, k_e, and K_b were then fixed to estimate K_v for the data in the not monensin-treated groups.

The relative contribution of ion-trapping, intracellular binding and distribution of unbound drug for hepatic sequestration of the three model drugs is therefore given by:

$$\frac{K_v}{1+K_v+K_b}, \quad \frac{K_b}{1+K_v+K_b} \text{ and } \frac{1}{1+K_v+K_b}, \text{ respectively.} \quad \textbf{(8)}$$

The cellular distribution volume of water was estimated by fitting the [^{3}H]water outflow data with Equation (7) using the density function for water $\hat{f}_W(s)$ instead of $\hat{f}(s)$. The latter differ only with regard to the respective tissue retention time densities $\hat{f}_y(s)$ assuming no cytoplasmic binding of water and $k_{out,w} = k_{in,w}v_{c,w}$ where $v_{c,w}$ denotes the normalised cellular water volume Equation (1) for well-mixed intracellular distribution reduces (Weiss et al., 2000)

$$\hat{f}_{y,w}(s) = \frac{k_{in,w}/v_{c,w}}{k_{in,w}/v_{c,w} + s} \quad \textbf{(9)}$$

and is substituted in Equations (2) and (7).

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Non-parametric estimates of hepatic availability, mean transit time and normalized variance were determined from the outflow concentration versus time profiles for the reference from Equations (10) - (13) using the trapezoidal method with exponential tail approximation.

$$F = \frac{Q \, . \, AUC}{D} \qquad \textbf{(10)}$$

$AUC = \int_0^\infty C(t)dt$ is the area under the solute concentration versus time curve, Q is the perfusate flow rate and D is the drug dose administered (expressed in molar equivalents).

$$\mathrm{MTT} = \frac{\int_0^\infty tC(t)dt}{AUC} \qquad \textbf{(11)}$$

$$CV^2 = \frac{\sigma^2}{MTT^2} \qquad \textbf{(12)}$$

where:

$$\sigma^2 = \frac{\int_0^\infty t^2 \; C(t) \; dt}{\int_0^\infty C(t) \; dt} - MTT^2 \qquad \textbf{(13)}$$

Statistical analysis

All data are presented as mean ± SD unless otherwise stated. Statistical analysis was performed using Tukey *post-hoc* or Student's *t* test where appropriate. A $p < 0.05$ was taken as significant.

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Results

Fig. 2 shows typical logarithms of measured and predicted (fitted data) time-outflow fraction profiles before and after monensin treatment for the three model cationic drugs antipyrine, atenolol and propranolol. The peak outflow concentrations were in the order atenolol < antipyrine < propranolol and are inversely related to drug lipophilicity as defined by their individual log P_{app} (atenolol 0.14, antipyrine 0.33, propranolol 3.10). Monensin treatment did not affect the outflow concentration-time profile of antipyrine (with the lowest pK_a and a low log P_{app}) but changed the outflow profiles of atenolol and propranolol (Fig. 2). The effect of monensin on the outflow profile of propranolol is dramatic (Fig. 2) and in the presence of monensin the profile assumes a shape similar to antipyrine (Fig. 2). The outflow profiles of antipyrine (as a positive control that does not show ion-trapping) remained unchanged and were virtually superimposed before and after treatment (Fig. 2).

Table 1 shows the non-parametric moments parameters hepatic extraction, mean transit time and normalized variance for the three model drugs. The mean transit time of propranolol showed treatment-induced significant differences ($p < 0.01$). No other differences for comparison of controls and monensin treatment groups were observed ($p > 0.05$, Table 1).

The model-derived kinetic parameters K_v, K_b, CL_{int} and PS for hepatic drug disposition of the three model drugs are summarized in Table 2. The vesicular ion-trapping constant K_v was significantly lower after monensin treatment for atenolol and propranolol (K_v control/K_v treatment: 30- and 35-fold, respectively, $p < 0.001$), but not for antipyrine. However, no changes induced by monensin treatment were observed for the intracellular binding constant, the intrinsic elimination clearance or the permeability-surface area product values for the three model drugs (Table 2).

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A comparison of the predicted and observed model-derived ion-trapping parameter K_v values are presented in Table 3. The theoretical and observed values were in good agreement. Atenolol and propranolol both showed considerable ion-trapping (high K_v values), whereas the corresponding value for antipyrine was relatively small (Table 3).

The results of an *in vitro* binding, the calculated microsomal association constant and *in vitro* intrinsic elimination clearance for the three drugs in a microsomal protein preparation are shown in Table 4. Monensin treatment had no effect on binding behaviour or metabolic drug elimination. No differences before and after treatment were found to exist for the three model drugs (Table 4). It is also apparent that the *in vitro* microsomal estimates of K_b (Table 4) compare favourably with those obtained from modelling the perfused liver data (Table 2). The microsomal CL_{int} values obtained *in vitro* are also in general good agreement with the model-derived CL_{int} values in the *in situ* isolated perfused liver study (Tables 2 and 4).

The relative contribution of ion-trapping, intracellular (microsomal and unspecific) binding and the distribution of unbound drug to the hepatic retention of the three drugs are summarized in Fig. 3. Ion-trapping is the most important determinant of hepatic drug retention for atenolol and the least important contributor for antipyrine, whereas ion-trapping and intracellular binding are equally important for hepatic drug retention for propranolol (Fig. 3). The distribution of unbound drug is the least important determinant for propranolol and the most important for antipyrine (Fig. 3).

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Discussion

The study presented here was undertaken to define the relative importance of intracellular binding, vesicular (lysosomal and mitochondrial) drug sequestration and distribution of unbound drug as determinants of hepatic solute retention and also to investigate the relative importance of pK_a lipophilicity for this process. Monensin was used to determine the relative contribution of vesicular ion-trapping in the hepatic retention of three model cationic drugs, atenolol, antipyrine and propranolol. *In vitro* microsomal studies produced independent estimates of microsomal binding and intrinsic clearance that showed that monensin treatment does not affect these parameters and therefore can be used to estimate the contribution of vesicular ion-trapping to hepatic drug retention. The difference in K_b calculated from *in vitro* microsomal data (Table 4) and the model-derived values from the perfused liver (Table 2) suggests that microsomal binding accounts for most of hepatic binding, as has been previously suggested for the normal rat and human livers (McLure et al., 2000; Hung et al., 2002). A comparison of CL_{int} values obtained in the *in vitro* study (Table 4) and results from the *in situ* liver perfusion (Table 2) shows the reliability and suitability of the model used in the study.

In this investigation, the intact, viable perfused *in situ* rat liver was used to study sequestration of basic drugs in acidic organelles of the liver. Using monensin as an inhibitor of vesicular sequestration, the processes governing the pharmacokinetic events in the disposition of these basic drugs were quantified. This is, as far as we are aware, the first attempt to determine the differential contribution of ion-trapping, microsomal binding and distribution of unbound solute to the hepatic retention of basic drugs with differing physico-chemical properties in the isolated perfused rat liver.

Previous studies with ionophores have used cultured hepatocytes (Lake et al., 1987; Myers et al., 1995) or liver slices (Daniel et al., 1995; Daniel and Wojcikowski, 1999a). Lake et al.

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1987 used monensin to characterize the number of acidic compartments in intact hepatocytes (Lake et al., 1987; Myers et al., 1995) and Myers et al. (Lake et al., 1987; Myers et al., 1995) applied flow cytometry to make dynamic measurements of the lysosomal pH in living hepatocytes. The studies of Daniel et al. have been focussed on the contribution of two factors, non-specific drug binding to membrane phospholipids and ion-trapping of cationic drugs in tissue slices (Daniel et al., 1995; Daniel and Wojcikowski, 1999a) and the contribution of lysosomal trapping to the total tissue uptake of neuroleptics (Daniel et al., 1995; Daniel and Wojcikowski, 1999a). The extent of uptake of drugs into tissue slices was found to be tissue-specific, and the contribution of the two uptake mechanisms to be strongly drug-dependent (the contribution of ion-trapping to the total drug uptake of perazine in the liver was given as 40%).

The model used in our investigation differs from previous studies in that a perfused liver, in which the vascular hepatic architecture remains intact and the liver is oxygenated by red blood cells, was used to ensure optimal hepatic vitality and drug extraction in the perfused organ in situ. In our present study characterized both hepatic extraction and retention, including the respective relative contribution of ion-trapping, but also those of microsomal protein binding (the major hepatic factor contributing to drug binding, Hung et al., 2002) and of unbound drug uptake into the liver. Results thus obtained are of considerable clinical relevance especially for drugs that show high first pass hepatic extraction and are targeted to the liver, such as the statins (Garcia et al., 2003) or cytostatic drugs.

Analysis of hepatic extraction, mean transit time and normalized variance by moments showed that there were no differences in these parameters for the three drugs before and after monensin treatment (Table 1) with the exception of propranolol that showed a decrease in mean transit time from 119 sec to 79.5 sec *post*-treatment. This change is also evident in Fig. 2, where the change in outflow profile and especially the different tail sections of the curves

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are indicative of this change. Together with monensin not affecting in vitro microsomal binding and intrinsic clearance, it is evident that the major impact of monensin is on ion-trapping of basic drugs in hepatic acidic vesicles, as has been shown previously by histology (Lake et al., 1987).

According to the hepatic transport model used in this work (Fig. 1), hepatic extraction is affected by metabolism, permeability and perfusate flow, whereas the mean transit time is defined not only by these parameters but also by ion-trapping (K_v) and microsomal binding (K_b). Confirmation of the relationships is provided by an independent assessment of the effects of monensin on hepatic extraction and mean transit time (Table 1), relative to controls. Further, the in vitro study suggests that the drug fraction unbound by microsomal protein (surrogate of K_b) remains unchanged by monensin treatment (Table 4). Changes in mean transit time following monensin treatment are most evident for propranolol using a model-independent approach (Table 1). The change in K_v (Table 2) is consistent with this change in mean transit time due to ion-trapping.

The comparison of kinetic parameters before and after monensin treatment showed no differences for intracellular binding, intrinsic elimination clearance or permeability (Table 2). Differences between control and treatment groups were observed for the vesicular ion-trapping of atenolol and propranolol, (with their relatively high pK_a) but not for antipyrine (Table 2). The post-treatment ion-trapping was estimated from the volume ratios of the subcellular compartments to the remaining cytoplasmic fraction and fixed at 0.25. This calculated value is very close to the ion-trapping found for antipyrine in the control group. As ion-trapping is defined by the relative permeation rates into and out of the acidic vesicles it becomes obvious that this parameter is a potent marker of vesicular ion-trapping.

Ion-trapping may be estimated from considerations of the pHs and fractional volumes of the various cellular components, assuming that distribution is instantaneous and the resulting

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steady state ratios are reflect in the observed ratios from dynamic (non-steady state) studies. When the unbound drug concentration is assumed to be identical in both the intracellular and perfusate compartments, the intracellular to perfusate concentration ratio for a drug with a given pK_a is given by $\frac{1+10^{pKa-pHi}}{1+10^{pKa-pHp}}$ (Goldstein et al., 1974), where pH_i is the intracellular pH and pH_p is the perfusate pH (Table 3). A comparison of the predicted ion-trapping and the experimental ratios obtained by data fitting showed good similarity (Table 3). It is recognised that the predicted ion-trapping are only an approximation as a range pHs have been reported for intracellular pH (7.19 to 7.29) (Le Couteur et al., 1993; Burns et al., 1999; Pietri et al., 2001), mitochondria pH (6.7 to 7.0) (Soboll et al., 1980), and lysosomal pH (4 to 5) (MacIntyre and Cutler, 1988; Myers et al., 1995; Proost et al., 1997) as well as differing lysosomal fractional volumes (0.68% to 1%) (Rhoades and Pflanzer, 1996).

The relative contributions of ion-trapping, microsomal binding and distribution of unbound drug to the hepatic sequestration of the three model drugs is summarized in Fig. 3. It is apparent that neither pK_a nor lipophilicity are sole determinants of drug distribution. Whereas the retention of the polar drug atenolol with its high pK_a is mainly determined by ion-trapping, and intracellular binding and distribution of unbound drug contribute only in a minor way, the retention of the neutral drug antipyrine is mainly decided by the distribution of intracellular unbound drug. The combination of high lipophilicity and high pK_a Retention of propranolol results in intracellular binding and ion-trapping determining its retention in equal parts (Fig. 3).

It has been suggested that lysosomes account for as much as 10 % of the distribution of the basic drugs imipramine, biperiden and chlorpromazine in the rat liver (Ishizaki et al., 1996). This effect is likely to be reduced by the neutralisation of the lysosomal and mitochondrial pH at the higher therapeutic plasma concentration of the drugs. Furthermore it has been shown

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that external propranolol concentrations of 10^{-6}M, 10^{-5}M and 10^{-4}M increased the intra lysosomal pH in highly purified rat liver, from pH 5.5 to approximate pHs of 5.6, 5.75 and 6.1, respectively (Ishizaki et al., 2000). Given that the uptake of imipramine into purified lysosomes shows a lack of dependence on either temperature or counter-transport (Ishizaki et al., 2000), it is probable that a passive ion-trapping mechanism is responsible for the uptake of cationic drugs into lysosomes. The effect of neutralisation of pH on uptake into mitochondria is less marked because their pH is only an order of magnitude different to physiological pH whereas the intra-lysosomal differs by several orders of magnitude. Ishizaki et al (Ishizaki et al., 1996) also showed that weak bases suppressed uptake of bases into lysosomes more than into mitochondria.

Could abolishing of the ion-trapping effect be justified therapeutically? Monensin is routinely used in the cattle and poultry industry as feed additive, and lysosomal inhibitors have already been proposed as possible potent antimalarial therapeutics (Gumila et al., 1997) and also been considered as anticancer drugs (Singh et al., 1999; Park et al., 2002). However, monensin has been characterised by a narrow safety margin and may cause lethal toxicoses, especially when co-administered with other drugs (Nebbia et al., 1999). Further, accidental poisoning in animals (Nebbia et al., 1999) and an isolated case of lethal monensin ingestion in a human patient suggest that large doses of lysosomal inhibitors must be considered potentially dangerous for humans (Caldeira et al., 2001). Nevertheless the possibility to use lysosomal inhibitors to manipulate hepatic (or pulmonary) drug extraction to achieve a higher systemic availability for drugs that show a pronounced first pass effect is of interest.

In conclusion, the relative contributions of ion-trapping, intracellular binding and distribution of unbound drug of the basic drugs atenolol, antipyrine and propranolol and the importance of pK_a and lipophilicity for total hepato-cellular retention were determined. Ion-trapping is the dominant determinant of intracellular distribution of a polar drug with high pK_a and low

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lipophilicity (i.e. atenolol) but for a drug with a comparable high pK_a and high lipophilicity (i.e. propranolol) intracellular binding and ion-trapping are equally important for hepatic drug retention. The intracellular distribution of a drug with low pK_a and low lipophilicity (i.e. antipyrine) is characterized mainly by the distribution of intracellular unbound drug. The methodology employed in this study may become a powerful and important tool in assessing and predicting differential intracellular drug distribution and retention pharmacokinetics.

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Table 1 Non-parametric moments for model cationic drugs (mean ± SD, n = 6)

Drug			Hepatic extraction ratio		Mean transit time (*sec*)		Normalized variance	
	log P_{app}	pK_a	Control	Treatment	Control	Treatment	Control	Treatment
Atenolol	0.14	9.60	0.26 ± 0.10	0.16 ± 0.02	30.9 ± 2.76	29.1 ± 2.88	1.44 ± 0.14	1.52 ± 0.39
Antipyrine	0.33	1.45	0.20 ± 0.03	0.17 ± 0.09	67.5 ± 10.1	55.8 ± 7.34	0.48 ± 0.12	0.62 ± 0.10
Propranolol	3.10	9.45	0.95 ± 0.03	0.92 ± 0.05	119 ± 20.3	79.5 ± 15.3**	1.07 ± 0.25	0.86 ± 0.39

log P_{app}: log octanol/water partition coefficient; pK_a: negative logarithm of the ionisation constant. There were significant differences between controls and treatment groups for the mean transit time of propranolol (**p < 0.01). No other differences for the non-parametric moment parameters were found between control and treatment groups (p > 0.05).

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Table 2 Kinetic parameters derived from the two-phase stochastic model fitting for model cationic drugs (mean ± SD, n = 6).

Drug			K_v			K_b			CL_{int} (mL min^{-1} g^{-1} liver)			PS (mL min^{-1} g^{-1} liver)		
	log P_{app}	pK_a	Control	Treatment	p	Control	Treatment	p	Control	Treatment	p	Control	Treatment	p
Atenolol	0.14	9.60	7.37 ± 1.89	0.25	***	0.81 ± 0.45	0.81 ± 0.45	-	1.68 ± 1.73	1.70 ± 1.73	-	3.01 ± 1.54	3.14 ± 1.66	-
Antipyrine	0.33	1.45	0.24 ± 0.06	0.25	-	0.51 ± 0.14	0.51 ± 0.14	-	0.47 ± 0.41	0.49 ± 0.43	-	26.2 ± 7.87	27.4 ± 7.87	-
Propranolol	3.10	9.45	8.79 ± 1.79	0.25	***	8.65 ± 1.13	8.65 ± 1.13	-	11.8 ± 2.44	12.1 ± 2.31	-	35.9 ± 8.64	37.29 ± 9.07	-

K_v (= k_{on}/k_{off}): the equilibrium amount ratio characterising the slowly accessible pool for ion-trapping; K_b: the rapidly equilibrating intracellular binding sites (microsomal and non-specific binding); PS: permeability-surface area product; CL_{int}: intrinsic elimination clearance. There were significant differences for comparison of control and treatment groups for atenolol and propranolol ($p < 0.001$) but not for antipyrine.

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Table 3 Comparison of predicted and observed, model-derived K_v (equilibrium amount ratio characterising the vesicular ion-trapping) values for the three model drugs.

Drug	pK_a	[a]Lysosomal to intracellular concentration ratio	[a]Mitochondrial to intracellular concentration ratio	[b]Predicted K_v	[c]Observed model-derived K_v
Atenolol	9.60	738	3.97	8.17	7.37 ± 1.89
Antipyrine	1.45	1.00	1.00	0.21	0.24 ± 0.06
Propranolol	9.45	736	3.96	8.16	8.97 ± 1.79

[a]Vesicular to cytosolic concentration ratio = $\frac{1+10^{pKa-pHv}}{1+10^{pKa-pHi}}$ (Myers et al., 1995), where pHi ≈ 7.27 is the assumed cytosolic pH (Le Couteur et al., 1993), pHv ≈ 4.4 is the assumed lysosomal pH (Daniel et al., 2001) and pHv ≈ 6.67 is the assumed mitochondrial pH in the fasted state (Pietri et al., 2001). [b]Given that of lysosomal and mitochondrial to the cytosolic fraction is 1 and 20% (Rhoades and Pflanzer, 1996), the overall unbound drug vesicles/intracellular distribution ratio (v:i) for the three model drugs can be estimated from the individual organelle volume fraction and concentration ratio above using the equation: $K_v = f_{lys}$ x l:i + f_{mito} x m:i. [c]Data fitting results using the physiological two-phase organ pharmacokinetic model.

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Table 4 In vitro binding and intrinsic elimination clearance for the model cationic drugs in a microsomal protein preparation (mean ± SD, n = 4).

Drug			Drug fraction unbound by microsomal protein		Microsomal K_b (mL^{-1} mg)		Microsomal CL_{int} (mL min^{-1} g^{-1}liver)	
	log P_{app}	pK_a	Control	Treatment	Control	Treatment	Control	Treatment
Atenolol	0.14	9.60	0.91 ± 0.06	0.91 ± 0.07	0.43 ± 0.09	0.46 ± 0.09	1.08 ± 0.35	1.11 ± 0.24
Antipyrine	0.33	1.45	1.00 ± 0.09	0.98 ± 0.07	0.61 ± 0.04	0.60 ± 0.06	0.75 ± 0.28	0.68 ± 0.13
Propranolol	3.10	9.45	0.49 ± 0.05	0.47 ± 0.02	5.93 ± 0.71	5.07 ± 0.89	9.44 ± 1.14	9.46 ± 0.73

log P_{app}: log octanol/water partition coefficient; pK_a: negative logarithm of the ionisation constant; microsomal K_b: calculated association constant for the microsomal protein preparation; microsomal CL_{int}: intrinsic elimination clearance for the microsomal protein preparation. No significant differences before and after treatment were found to exist for drug fraction unbound by microsomal protein, calculated microsomal association constant and internal elimination clearance for the three drugs ($p > 0.05$).

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Figure legends

Figure 1: Schematic overview of hepatocellular drug transport and intracellular drug distribution, including ion-trapping, intracellular binding and drug metabolism. D: drug; DH^+: protonated drug; f_{uB}: fraction of drug unbound in the perfusate; k_{in}: influx rate constant; k_{out}: efflux rate constant; k_e: elimination rate constant; K_b: equilibrium amount ratio characterising the intracellular binding sites (microsomal and non-specific binding sites); k_{cv} and k_{vc}: rate constant for transport from cytosol into acidic vesicles (lysosomes and mitochondria) or from acidic vesicles into cytosol, respectively, determining the equilibrium amount ratio K_v ($K_v = k_{VC}/k_{CV}$) that characterises ion-trapping; V_B: extracellular volume (vascular + Disse space).

Figure 2: Outflow profile fractions (log scale) for antipyrine, atenolol and propranolol in controls and following treatment. Empty symbols represent controls and solid symbols represent monensin treatment. Dashed and solid lines stand for fitted data in control and treatments, respectively.

Figure 3: Comparison of the relative contribution (%) of vesicular ion-trapping (black bars), intracellular binding (lighter bars) and the fraction of unbound drug (white bars) to hepatic disposition of the three model drugs.

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Figure 1

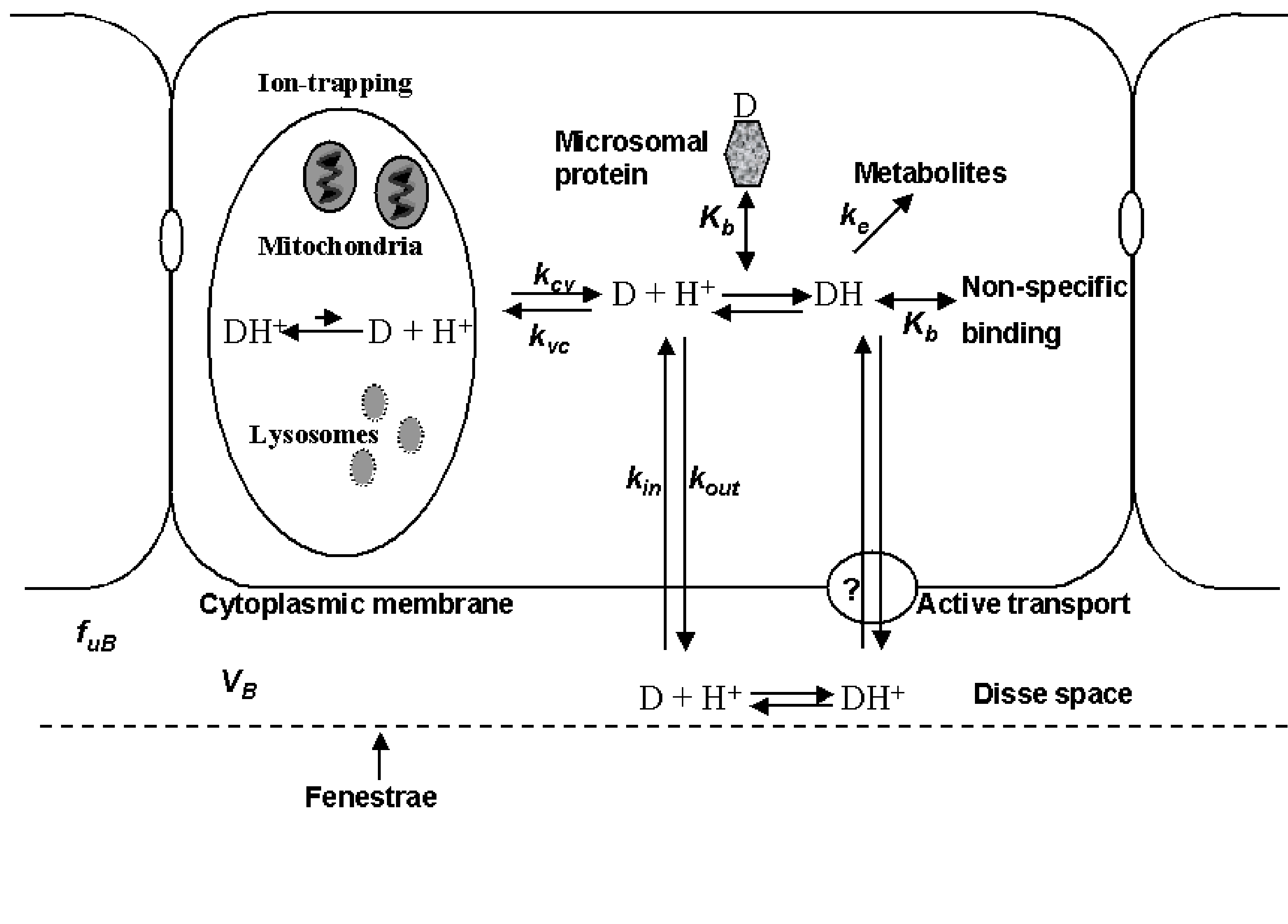

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Figure 2

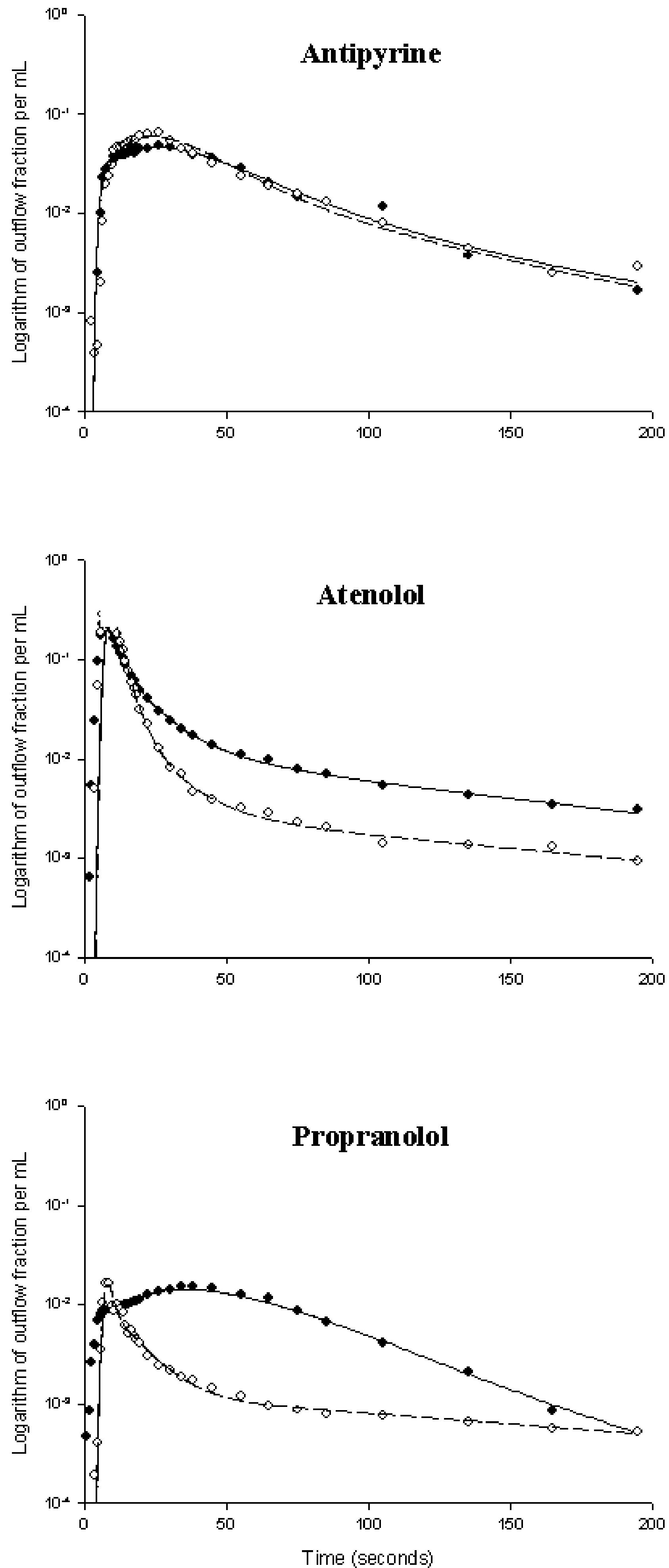

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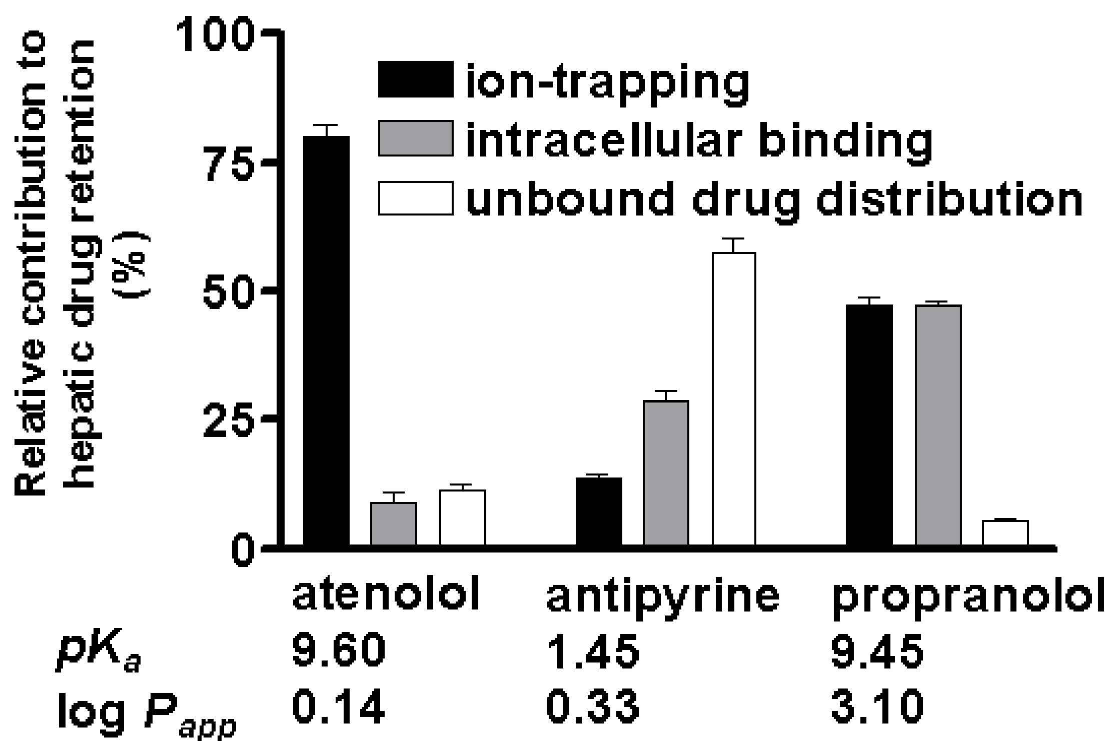
Figure 3



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