Hepatic pharmacokinetics of cationic drugs in a high-fat emulsion induced rat model of non-alcoholic steatohepatitis

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CL_{int}, intrinsic elimination clearance; CV^2, normalized variance; CYP, cytochrome P450; E, hepatic extraction ratio; FI, fibrosis index; HDL, high density lipoprotein; IPRL, in situ perfused rat liver; K_v, apparent distribution ratio; LDL, low density lipoprotein; logP_{app}, apparent partition coefficient; MID, multiple indicator dilution; MTT, Mean transit time; NASH, non-alcoholic steatohepatitis; PS, permeability-surface area product; SI, steatosis index; TG, triglyceride; vLDL, very low density lipoprotein.
Abstract

The hepatic pharmacokinetics of five selected cationic drugs (propranolol, labetalol, metoprolol, antipyrine and atenolol) was studied in the liver from control rats and from those with high-fat emulsion-induced non-alcoholic steatohepatitis (NASH). Studies were undertaken using an in situ perfused rat liver (IPRL) and multiple indicator dilution (MID), with outflow data being analysed with a physiologically-based organ pharmacokinetic model. Hepatic extraction was significantly lower in the NASH model with lipophilicity (log $P_{app}$) being the main solute structural determinant of the observed differences in intrinsic elimination clearance ($CL_{int}$) and permeability-surface area product ($PS$) with $pK_a$ defining the extent of sequestration in the liver ($K_v$). The main pathophysiological determinants were liver fibrosis, leading to a decreased $PS$, liver fat causing an increase in $K_v$, and an increase in both total liver cytochrome P450 (CYP) concentration and CYP isoform expression for Cyp3a2 and Cyp2d2, causing an increase $CL_{int}$ in NASH rat livers compared to control livers.

Changes in hepatic pharmacokinetics ($PS$, $K_v$, $CL_{int}$ and $E$) due to NASH were related to the physicochemical properties of drugs (log $P_{app}$ or $pK_a$) and hepatic histopathological changes (FI, SI, and CYP concentration) by stepwise regression analysis. It thus appears that in NASH, counteracting mechanisms to facilitate hepatic removal are created in NASH induced CYP expression whereas NASH induced fibrosis and steatohepatitis inhibit hepatic extraction by decreasing hepatocyte permeability through fibrosis and hepatic sequestration.
Introduction

Non-alcoholic steatohepatitis (NASH) is a fatty inflammation of the liver that arises without excessive alcohol use and is characterized histologically by steatosis, necroinflammation and fibrosis (Sheth et al., 1997). The fibrosis is characterized by collagen deposition in the sinusoids and space of Disse which can lead to defenestration and sinusoidal portal hypertension (Ishak et al., 1991; Sheth et al., 1997). Whilst the histopathological characteristics of NASH are well described, exactly how this syndrome influences drug disposition in the liver is unclear.

The effects of other liver disease and aging on hepatic drug disposition have been recognized for a long time (Williams and Mamelok, 1980; Le Couteur et al., 2005). Our previous research in rats has shown that carbon tetrachloride induced fibrosis and cirrhosis can significantly alter hepatic pharmacokinetics (Hung et al., 2002a; Hung et al., 2002b). In these studies, changes in hepatic pharmacokinetics were related to alteration in hepatocellular morphology and biochemistry and the nature of the drug. Hepatocyte permeability, intrinsic metabolic clearance, ion-trapping and microsomal binding were all changed in diseased livers (Siebert et al., 2004). An altered hepatic CYP concentration was also seen in adjuvant-induced systemic inflammation (Hung et al., 2006).

There is limited work published on how NASH may affect hepatic pharmacokinetics. Publications have focused on potential changes in hepatic CYP, showing, for instance, that liver CYP2E1 and CYP4A levels were increased in NASH patients (Niemela et al., 2000; Chtioui et al., 2007). However, Weltman et al. (1998) reported lower liver CYP3A levels in NASH. Lickteig et al. (Lickteig et al., 2007) showed increases in liver transporter mRNA and protein levels as well as a decreased
biliary excretion of acetaminophen conjugates in rats fed a methionine- and choline-deficient (MCD) diet for 8 weeks. Pharmacokinetic studies in NASH patients and the NASH rat model have shown a significant decrease in the hepatic extraction ratio ($E$) (Siepmann et al., 1998; Hung et al., 2005; Schrieber et al., 2008). However, the actual effects of steatosis, necroinflammation and damaged micro-circulation in the NASH liver on the individual hepatic pharmacokinetic processes have not, as yet, been well addressed.

The aim of this work was to characterise the hepatic pharmacokinetics of cationic drugs in NASH and relate the changes in individual pharmacokinetic processes, such as membrane permeability, metabolic clearance and sequestration to the pathophysiology caused by NASH and the nature of drug studied. The hepatic disposition of five selected cationic drugs, including propranolol, labetalol, metoprolol, antipyrine and atenolol, was studied both in healthy (control) rats and in a high-fat emulsion-induced rat model of NASH. The high fat diet-induced NASH model has been reported to replicate the key pathological features of human NASH and the typical diagnostic aspects of human NASH, such as obesity, abnormal aminotransferase, hyperlipidemia, hyperinsulinemia, hyperglycemia and insulin resistance (Zou et al., 2006). In order to appropriately characterise the pharmacokinetic processes without recirculation effects, studies were undertaken in a single pass isolated perfused rat liver (IPRL) in which the drug and multiple indicators were co-administered as a bolus and their outflow profiles used to define the hepatic pharmacokinetics (commonly described as multiple indicator dilution (MID)) (Roberts and Anissimov, 1999; Hung et al., 2001). The IPRL-coupled MID method has been previously applied to studies of cationic drug disposition in control and other diseased livers (Hung et al., 2001; Hung et al., 2002a). The perfusate
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outflow data was analysed by a two-phase physiologically based organ pharmacokinetic model that recognised the presence of vascular dispersion, transfer across the hepatocyte membrane, intracellular distribution and metabolic clearance (Weiss et al., 1997). The extent of fibrosis, steatohepatitis and CYP levels were determined in both control and diseased rat livers and used to interrelate changes in hepatic pharmacokinetics with NASH induced changes in hepatocellular histopathology. The results should contribute to a better understanding of whether and how fat deposition, collagen deposition and alteration in CYP expression will affect the hepatic pharmacokinetics of cationic drugs.
Materials and methods

Chemicals. Propranolol, labetalol, metoprolol, antipyrine and atenolol were all obtained from Sigma Aldrich (St. Louis, MO). Physicochemical properties of these five cationic drugs are summarized in Table 1. [14C]sucrose and [3H]water were purchased from New England Nuclear (Boston, MA). All other chemicals used in this research were obtained from Sigma Aldrich (St. Louis, MO).

Rat model of NASH. The rat model of NASH was induced in rats using the high-fat emulsion method following a procedure outlined previously (Zou et al., 2006). Briefly, male Wistar rats (approximately 250 g) were assigned to two groups: control group and NASH group. All rats were fed with standard rat chow and water. In addition, they were allowed free access to saccharose in water (18%). The NASH group was gavaged with the high-fat emulsion (10 mL/kg), which contains corn oil, saccharose and cholesterol et al., once a day for 6 weeks (Zou et al., 2006). The control group was gavaged with saline instead of the high-fat emulsion. Body weight, food intake and water intake were monitored daily for rats in both groups for the entire duration of the experiment. After 6 weeks, the animals were sacrificed for IPRL studies and histopathological examination. All animal studies were carried out according to protocols approved by the University of Queensland Animal Ethics Committee.

Liver biochemistry determination. Blood lipid and liver function tests of the control and disease rats were performed at the Pathology Laboratory of the Princess Alexandra Hospital (Brisbane, Australia). Serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), triglyceride (TG), high
density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (vLDL), total bilirubin and albumin were measured on a Synchron LX®20 Clinical System (Beckman Coulter, Fullerton, CA).

**IPRL.** The IPRL preparation used in this study was prepared as described previously (Cheung et al., 1996; Hung et al., 2001). Wistar rats were anesthetized by intraperitoneal injection of xylazine 10 mg/kg (Bayer Australia) and ketamine-hydrochloride 80 mg/kg (Parnell Laboratories Australia). Following laparotomy, the bile duct was cannulated with PE-10 tubing (Becton Dickinson and Company, Sparks, MD). The animal was heparinized by a bolus injection of 200 units of heparin sodium (David Bull Laboratories Australia) via the inferior vena cava. The portal vein was cannulated with a 16-gauge intravenous catheter. Via this cannula, the liver was then perfused with MOPS buffer which contained 2% (w/v) BSA and 15% (v/v) prewashed canine RBCs (Veterinary Specialist Services Pty Ltd, Brisbane, Australia) at pH 7.4 (Cheung et al., 1996). The perfusion was conducted at 15 mL/min in each liver to reflect *in vivo* microcirculation. The perfusion medium was pumped with a peristaltic pump (Cole-Palmer, Vernon Hills, HL) and oxygenated using a silastic tubing artificial lung ventilated with 100% pure oxygen (BOC Gases Australia, Brisbane, QLD). After the perfusion commenced the animal was sacrificed by thoracotomy and the thoracic inferior vena cava was cannulated within a PE-240 tubing (Becton Dickinson and Company, Sparks, MD). The animal was placed in a temperature-controlled cabinet with the temperature set at 37 °C. Liver viability was evaluated by oxygen consumption, portal vein pressure, bile flow and macroscopic appearance as previously described by Cheung et al. (1996).
Bolus injection study. After a 10 minute perfusion stabilization period, the MID study was performed by bolus injection into the liver of aliquots (50 μL) of perfusion medium which contained [14C]sucrose (1.5×10^5 dpm) and [3H]water (3×10^5 dpm). The outlet samples were collected via a fraction collector at short intervals over 4 minutes. Aliquots (50 μL) of cationic drugs (8 mM propranolol, 6 mM labetalol, 3 mM metoprolol, 4 mM antipyrine, 4 mM atenolol) were separately bolus injected into the perfused liver. In each liver, the order of injection was randomized and there was no repeat of the same injection in the same liver. A wash out and stabilization period of about 10 minutes was applied after every injection. The total perfusion time for each liver was less than 2 hours. All outflow samples were centrifuged and aliquots (100 μL) of supernatant were taken for analysis.

Analytical procedure. The MID samples (which contain [14C]sucrose and [3H]water) were taken for scintillation counting of dpm using a MINAXI beta TRICARB 4000 series liquid scintillation counter (Packard Instruments, Meriden, CT). The outflow samples of cationic drugs were determined with established HPLC methods which have been described and validated previously (Hung et al., 2001).

Histopathology examination and quantitation of histology index. Three to five slices of tissue were selected randomly from each liver and fixed in 10% neutral buffered formalin and then embedded in paraffin. Sections of 5 μm thickness were prepared and stained with hematoxylin-eosin (H&E), Masson’s Trichrome and Sirius Red to determine the degree of steatosis or inflammation, respectively. The light microscopy digital images were acquired with a ScanScope digital slide scanner (Aperio Technologies, Vista, CA) at the Pathology Laboratory of the Queensland
Institute of Medical Research. The collagen and fat deposition were expressed as FI and SI, respectively, which were quantified with computer-assisted image analysis (Image Scope version 10, Aperio Technologies, Vista, CA) of the stained sections. For each rat, the area of stained fibrotic tissue and fat deposition in five randomly selected fields was measured on the binary image. The FI was quantified as the total area of fibrosis divided by the total area of the section, as previously described (O’Brien et al., 2000). The steatosis index was quantified as the total area of fat deposition divided by the total area of the selected section, as previously described (Marsman et al., 2004).

**Determination of liver CYP450 concentration and fat weight.** Rat livers were harvested from the sacrificed rat and perfused through the portal vein with a mixed solution of calcium and magnesium-free Hank’s balanced salt solution (5 mM EDTA and 10 mM HEPES) at 15 mL/min for 5 minutes to remove protein and blood from the sinusoid bed. The liver was then snap frozen in liquid nitrogen and stored at -80 °C until required for analysis. Liver tissue (1 g) was then thawed and homogenized in 2.5 mL of ice-cold 0.25 M sucrose containing 50 mM Tris-HCl buffer (pH 7.4) using a tissue homogenizer. To separate the liver microsome fraction, gradient centrifugation was performed on the liver homogenates as previously described (Hung et al., 2002b). The CYP concentration in the microsome fraction was then determined by the dithionite-reduced difference spectrum of carbon monoxide-bubbled samples using the molar extinction difference at peak position (about 450 nm), as previously described (Matsubara et al., 1976). As an independent measure of fat deposition, the total fat weight in the rat liver was determined by the Folch method (Folch et al., 1957).
RNA extraction, cDNA synthesis and Real-Time RT-PCR. The RNA was extracted from liver tissue using TRIzol reagent (Invitrogen, Mt. Waverley, Australia). The concentration of RNA per sample was measured by spectrophotometry. DNA within the RNA sample was removed by DNase treatment. Then, first-strand cDNA was synthesised from 1 g total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Mt. Waverley, Australia) and oligo (dT(15)), according to the manufacturer’s instructions.

Real-time RT-PCR was performed to measure gene expression of CYP enzymes and a control housekeeping gene (Gapdh). Primers were designed using Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) and the gene sequences for each forward and reverse primer used for the PCR are listed in Table 2. A Corbett Rotor Gene 3000 (QIAGEN, Doncaster, VIC, Australia) was used for real-time RT-PCR amplification. The reactions were performed in a total volume of 20 μL, with 400 nM forward primer, 400 nM reverse primer, 12.5 ng cDNA, and 10 μL QuantiTect SYBR Green PCR master mix (QIAGEN, Clifton Hill, Australia). Semi-quantitation of gene expression was performed using the Standard Curve method and gene expression was normalized with the housekeeping gene expression as external standard. The result for each sample was expressed as a percentage when compared with gene expression in the liver from control rats.

Modelling and data fitting of the outflow concentration-time profiles. Modelling and data fitting were performed by using the dispersion model coupled two-phase physiologically based organ pharmacokinetic model, as previously described (Hung et al., 2001). This dispersion model coupled two-phase physiologically based organ
pharmacokinetic model describes liver sinusoidal mixing (Roberts and Rowland, 1986; Roberts et al., 1988), transfer across the hepatocyte membrane, intracellular distribution and elimination kinetics (Weiss et al., 1997; Hung et al., 2001; Hung et al., 2002a). Briefly, the stochastic approach represents the transit of a molecule through the organ as a series of sojourns in one of two regions described by density functions, as shown in Fig. 1. The apparent distribution ratio between cellular and extracellular space ($K_v$) for the unbound solute is defined by $k_{in}/k_{out}$. The cellular binding processes were assumed to be one rapid and one slow dissociation process, respectively. The rapid binding process was simplified to an instantaneous equilibration process characterized by $K_R = k_{on,R}/k_{off,R}$, as the dissociation rate constant of the rapid process was extremely high. The slow binding process contains the intracellular binding rate constant ($k_{on}$) and intracellular unbinding rate constant ($k_{off}$), respectively. The elimination rate constant $k_e = CL_{int}/V_c$ is the intrinsic elimination clearance per cellular water volume ($V_c$). The density of cellular residence times $\hat{f}_y(s)$ describes the hepatocellular distribution and elimination kinetics. The sojourn time distribution $f_y(t)$ of a molecule after a single excursion in the cellular space for the resulting two-compartment cell model can be obtained by standard methods in the Laplace domain, $f_y(t) = L^{-1}[\hat{f}_y(s)]$, as previously described (Weiss, 1999; Weiss et al., 2000):

$$\hat{f}_y(s) = \frac{k_{out}(s+k_{off})}{s^2(1+K_R)+s(k_{off}+K_Rk_{off}+k_e+k_{on}+k_{out})+k_{off}(k_e+k_{out})}$$  \hspace{1cm} (1)

The hepatic transit time density function $\hat{f}(s)$ of solutes can be evaluated in terms of the extracellular transit time density of a nonpermeating reference molecule (in this study, sucrose) $\hat{f}_B(s)$, see equation 2 and the density function of successive sojourn times $\hat{f}_y(s)$ of the drug molecules in the cellular space:

$$\hat{f}(s) = \hat{f}_B[s + \hat{k}_in\left(1 - \hat{f}_y(s)\right)]$$  \hspace{1cm} (2)
The fractional outflow versus time data were fitted in the time domain by applying a numerical inverse Laplace transformation of the appropriate transit time density function using the nonlinear regression program SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT).

**Estimation of noncompartmental pharmacokinetic parameters.**

Noncompartmental estimates of hepatic pharmacokinetic parameters including hepatic availability and mean transit time were determined from the outflow concentration-time profiles for reference from equation 3 to 6 using the trapezoidal method (extrapolated to infinity) with exponential tail approximation:

\[ F = \frac{Q \cdot AUC}{D} \]  

\[ AUC = \int_0^\infty C(t) dt \]  

\[ MTT = \frac{\int_0^\infty t \cdot C(t) dt}{AUC} \]

**Statistical analysis.** All data are presented as mean ± standard deviation unless otherwise stated. Statistical analysis was performed with two-way analysis of variance, student’s t-test and regression analysis (where appropriate). Stepwise regression analysis was performed with SPSS 14.1 for Windows (SPSS Inc, Chicago, IL) and p < 0.05 was taken as significant. Linear regression equations have only been considered when \( r^2 > 0.5 \). Correlation analysis between two parameters was performed with SPSS 14.1 for windows (SPSS Inc, Chicago, IL) to assess the Pearson’s product-moment correlation coefficient and p < 0.05 was taken as a significant correlation.
Results

Table 3 shows a comparison of various physiological changes found in normal rats and in those fed with a high-fat emulsion to induce NASH. NASH rats had a significantly higher final body weight, liver weight, and liver fat weight than the control rats and are comparable to those previously reported (Zou et al., 2006). The NASH rats had impaired bile excretion, with their bile flow being significantly lower than the control group. Hepatic oxygen consumption in the NASH group was also lower than in the control group. The NASH rat livers had significantly decreased vascular sucrose space as shown in Table 3. However, there was no significant change with the cellular water space in the NASH rat livers. Furthermore, also shown in Table 3 are that the serum biochemistry from NASH rats was abnormal compared to control rats. All biochemistry parameters, other than TG, are significantly higher, indicating that NASH is associated with substantial impairment in liver function.

Representative liver sections of Masson’s Trichrome stained liver slides from control and NASH rats are shown in Fig. 2. Sections of control livers show typical architecture under light microscopy, such as the cords of hepatocytes (one cell wide) radiating out from each hepatic venule towards the portal tract. The NASH liver sections show substantial fat deposition, ballooning hepatocytes and inflammatory damage which are accepted as diagnostic features of NASH (Matteoni et al., 1999). Collagen deposition was also found within the sinusoids of the NASH model rats. The FI and SI estimated by computer-assisted image analysis for control and NASH rats are listed in Table 4. The NASH model rats show significantly higher FI and SI than control rats. The total CYP concentrations in NASH liver are higher than that in control livers (Table 4).
Typical outflow perfusate concentration – time profiles for each cationic drug in control and NASH rats are shown in Fig. 3. Each profile is characterised by a rapid throughput to yield a peak within seconds of administration followed by a fast and then a slower decline in concentration over time. Table 5 shows noncompartmental pharmacokinetic parameters for the cationic drugs derived from these profiles in control and NASH rat livers as well as some physicochemical properties of the drugs. The extraction ratios, $E$, had significantly decreased in NASH livers for all cationic drugs other than propranolol, and was higher for the more lipophilic drugs, as defined by an increased $\log P_{app}$. The mean transit times ($MTT$) of all five cationic drugs were longer in NASH livers relative to the control liver.

Also shown for each profile in Fig. 3 are the nonlinear regression lines obtained using a heterogeneous (barrier-limited and space-distributed) transit time model and a data weighting of $1/(y_{obs})^2$. The pharmacokinetic parameters derived from the model fitting for each cationic drug are summarized in Table 6. The NASH livers had significantly lower $PS$ for each cationic drug compared to the control livers. In contrast, cationic drugs had a significantly higher $CL_{int}$ and $K_v$ in NASH livers than in control livers. Larger $PS$ and $CL_{int}$ values were observed for the more lipophilic cationic drugs, as defined by $\log P_{app}$, whereas the $K_v$ values were related to their $pK_a$ values (Fig. 4). Associated with the increased $CL_{int}$ of cationic drugs was an increase in total CYP concentration. Stepwise regression analysis, used to examine the potential relationships between the pharmacokinetic parameters ($\log PS$, $\log K_v$ and $\log CL_{int}$), the cationic drug physicochemical properties ($\log P_{app}$ and $pK_a$) and the histopathological results (FI, SI and CYP concentration), yielded the following relationships:

1) $\log PS=0.721-0.186*\log FI+0.211*\log P_{app} (r^2=0.762, n=30, p<0.001)$;
2) $\log K_v = 0.382 + 0.163 \cdot \log SI + 0.066 \cdot pK_a \ (r^2 = 0.928, \ n = 30, \ p < 0.001)$;

3) $\log CL_{int} = -4.220 + 1.757 \cdot \log CYP + 0.453 \cdot \log P_{app} \ (r^2 = 0.819, \ n = 30, \ p < 0.001)$;

4) $\log E = -1.460 + 0.230 \cdot \log CYP - 0.123 \cdot \log FI + 0.365 \cdot \log P_{app} \ (r^2 = 0.867, \ n = 30, \ p < 0.001)$;

A comparison of the observed and predicted pharmacokinetic parameters derived from the stepwise regression analysis are shown in Fig. 5. The correlations suggest that SI, a measure of liver steatosis levels, is a predictor for cationic drug trapping in the hepatocytes and that their $CL_{int}$ is related to the CYP levels. However, it needs to be emphasised that labetalol is metabolized by glucuronoyltransferase and the reduced labetalol clearance might due to the decreased expression of glucuronyltransferase.

An examination of the mRNA expression of $Cyps$ ($Cyp3a2$, $Cyp2d2$ and $Cyp2e1$) in NASH rat livers showed that the mRNA expression of $Cyp3a2$, $Cyp2d2$ and $Cyp2e1$ was significantly increased in NASH rat liver compared to the control rat liver (Fig. 6).
Discussion

In this work, we studied the hepatic pharmacokinetics of cationic drugs in rat livers that had NASH induced by a high-fat emulsion diet. This high fat diet-induced NASH model has the same key pathological features as reported for other rat NASH models (Koteish and Mae Diehl, 2002). The presence of substantial fat deposition, inflammatory damage and collagen deposition in NASH livers (Fig. 2) are typical features of a NASH syndrome in rat livers (Ishak et al., 1991; Sheth et al., 1997). Significantly increased serum levels of HDL, LDL, and vLDL (Table 3) are most likely caused by the long term high fat diet. The NASH rat livers were significantly larger than that in normal rats, arising mainly from an increase in fat content, which, in turn, is reflected by significantly increased serum lipid levels (Table 3), and this is consistent with previous research (Zou et al., 2006). Since the liver is larger in NASH rats, the perfusion rate per gram of liver is lower in the NASH rats compared with the control rats (Table 3). This might cause decreased vascular sucrose space in the NASH rat livers (Table 3), as recognized in previous work (Cheung et al., 1996). However, the cellular water space in the NASH rat livers was no affected (Table 3).

The hepatic extraction, $E$ for four cationic drugs was significantly decreased in NASH rat livers compared to control rat livers with the exception of other than for propranolol (Table 5). This finding is consistent with the results reported for the extraction of other compounds in human and rat NASH livers. For instance, silymarin has a significantly increased AUC in human NASH (Schrieber et al., 2008) whereas propiverine has a small but in significant change (Siepmann et al., 1998). We have shown that palmitate hepatic extraction $E$ is lower in NASH rat livers (Hung et al., 2005). The minimal effects of NASH on propiverine and on propranolol may reflect
the dependence of $E$ for these highly hepatic extracted drugs on hepatic blood flow, which has been shown to determine the hepatic extraction of compounds with a high $\text{CL}_{\text{int}}$ (Pang and Rowland, 1977).

In this work, we sought to dissect the mechanisms by which NASH was associated with altered cationic solute pharmacokinetics. Both linear regression and stepwise regression analysis showed that solute lipophilicity, $\log P_{\text{app}}$, is a key predictor for $\text{CL}_{\text{int}}$ and $PS$ whereas solute $pK_a$ defines ion-trapping in the cells as defined by $K_v$. These findings are consistent with the relationships we have reported previously for other liver conditions (Hung et al., 2001; Hung et al., 2002a). In addition, we showed that the observed decrease in $PS$ for NASH rat livers could be related to a significantly increased FI in the livers, as we have also shown for other liver conditions (Hung et al., 2002a; Hung et al., 2002b). We had previously used FI as a predictor for $PS$ control, fibrotic, and cirrhotic livers (Hung et al., 2002a). In the present analysis, we found that the logarithm of FI was a better predictor of $PS$ ($r^2 = 0.762$) than FI alone ($r^2 = 0.613$) in the stepwise regression analyses. Consistent with $PS$ and $\text{CL}_{\text{int}}$ being defined as predictors of $E$ for low and moderately hepatic extracted drugs (Pang and Rowland, 1977; Roberts and Rowland, 1986; Roberts and Anissimov, 1999; Hung et al., 2002a), $E$ can be expressed in terms of their determinants, the lipophilicity of the drugs, $\log P_{\text{app}}$, and changes in FI and CYP concentration with much of the variability in the data being explained by these parameters ($r^2 = 0.876$) (Fig. 5). The predicted $E$ values acquired from this regression showed a good correlation with the observed $E$ values. The changes in overall total CYP concentration in the NASH rat livers was reflected by an increase in expression of the individual hepatic CYP isoforms $\text{Cyp3a2}$ and $\text{Cyp2d2}$ (Fig. 6). These results are consistent with other studies which have shown a significantly increased expression
of Cyp2e1, Cyp1a1 in NASH rat models (Weltman et al., 1996; Zou et al., 2006; Fisher et al., 2008) and in CYP2E1 and CYP4A in NASH patient livers (Niemela et al., 2000; Chtioui et al., 2007; Prompila et al., 2008). In this work, we showed that the logarithm of CYP was a good predictor for logCLint ($r^2 = 0.819$) (Fig. 5).

The MTTs for all five cationic drugs were increased in the NASH rat livers compared with the control rat livers (Table 5) which is consistent with the NASH livers being significantly larger (Table 3) and consistent with our earlier finding of a higher MTT of palmitate in NASH rat livers (Hung et al., 2005). The increase in liver size is likely to be due to a combination of increased fat, collagen and CYP containing organelles in the NASH livers. SI is a surrogate measurement for fat deposition in the hepatocyte during NASH and can be used to explain the increase in cationic drug partitioning, as defined by $K_v$ within NASH livers (Fig. 5).

Clinical studies indicate that NASH patients have greater hepatic fibrosis and increased hepatic CYP expression (Niemela et al., 2000; Chtioui et al., 2007; Prompila et al., 2008). However, the relationships between hepatic clearance and changes in liver morphology and biochemistry in NASH have not previously been studied. The present work suggests that the $E$ of drugs with lower $E$ is likely to be severely reduced in NASH patients due to collagen deposition even through there is a balancing increase in CLint due to enhanced CYP expression. Therefore, a reduction in dosing may be required for patients with NASH who have been given hepatically cleared drugs. However, the measurement of collagen deposition normally entails a liver biopsy, which can be painful and has a significant morbidity. An alternative measure of collagen deposition is noninvasive elastography (Wang et al., 2009). The stiffness of the liver measured with this method correlates well with the liver fibrosis level. The fibrosis score is another non-invasive estimate of liver fibrosis which can
give a semi-quantitative evaluation of the fibrosis level in patients through analysis of routinely measured and readily available clinical and laboratory data (Wong et al., 2008). The observed relationship between fibrosis index and $E$, as reported in this work, provides another possibility, namely, the use of hepatic drug extraction data as a measure of the extent of fibrosis in NASH patients. The difficulty in using drugs for such a purpose is that a decrease in drug extraction could also arise from alterations in hepatic blood flow, other clearance mechanisms and altered hepatic metabolism.

The IPRL model provides controlled and reproducible conditions under which the disposition of various drugs in liver diseases can be studied. It also allows the effects of other organs and recirculation from those organs to be avoided, as well as the ability to study toxic drugs or drugs in excess of their usual therapeutic concentrations. NASH in human livers appears to be similar to NASH in rat liver (Zou et al., 2006) and therefore the results obtained here should be applicable to patients with NASH. But, it does have a limitation in that it does not simulate all in vivo conditions and especially events occurring in man. For instance, NASH may also cause significantly increased serum levels of cholesterol and lipoproteins (Table 3). These in turn can affect the binding of lipophilic drugs in the blood and hence their elimination in the liver (Yeganeh and McLachlan, 2002). Other drugs, such as some of the $\beta$-blockers studied here, can induce haemodynamic effects resulting in a change in hepatic blood flow (Sabba et al., 2001). The function of the human liver also differs from that of the rat liver in its morphology (e.g. bile from rat livers does not drain into a gall bladder) and CYP expression. Furthermore, a limitation in the rat liver disease model used here is that it does not precisely represent conditions in humans. NASH in humans also contains heterogeneity in terms of both morphology and function, which the liver disease model here lacks.
In summary, this study has shown that the hepatic pharmacokinetics of cationic drugs are significantly changed when NASH has been induced by a high-fat diet. We showed that the individual hepatic pharmacokinetic parameters (PS, $K_v$, $\text{CL}_{\text{int}}$, and $E$) could be related to the physicochemical properties of cationic drugs ($\log P_{\text{app}}$ or $pK_a$) and liver histopathological changes (FI, SI, and CYP concentration) in the NASH livers. This work adds to our overall knowledge on how liver diseases and aging affects hepatic drug disposition, as also studied by other groups (Le Couteur et al., 2005; Fisher et al., 2009; Makino et al., 2009).
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Authorship Contribution

Participated in research design: PL, LMF, DC, MSR

Conducted experiments: PL, CAT, QZ

Contributed new reagents or analytic tools: TAR, LMF

Performed data analysis: PL, MSR

Wrote or contributed to the writing of the manuscript: PL, TAR, QZ, DC, MSR
References


glucoside, is absorbed more easily than other quercetin glycosides or aglycone after oral administration in rats. *Biol Pharm Bull* **32**:2034-2040.


Footnotes

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Legends for Figures

Fig. 1. Schematic Overview of the two compartment convection dispersion model applied to cationic drugs disposition in the liver. $k_{in}$, influx rat constant; $k_{out}$, efflux rat constant; $k_{in}/k_{out}$ defines the apparent distribution ratio between cellular and extracellular space ($K_v$) for the unbound solute; $k_e$ elimination rat constant; $K_R$, equilibrium amount ratio charactering the fast binding process; $k_{on}$, $k_{off}$, rate constant for drug transport from cytosol into slow binding site which determines the equilibrium amount ratio $K_S$ ($K_S=k_{on}/k_{off}$) that characterized the slow binding process.

Fig. 2. Representative liver sections from control and non-alcoholic steatohepatitis (NASH) rats. (A) control (X200). (B) NASH (X200).

Fig. 3. Representative outflow profile for the sucrose and cationic drugs in control (⚫) and non-alcoholic steatohepatitis (NASH) (△) rats.

Fig. 4. Relationship between the physicochemical properties and the hepatic pharmacokinetic parameters for the cationic drugs in control and non-alcoholic steatohepatitis (NASH) rats. A. The logarithm of octanol/water partition coefficient ($\log P_{app}$) for the cationic drugs and the logarithm of permeability-surface area product ($\log PS$). B. The logarithm of octanol/water partition coefficient ($\log P_{app}$) for the cationic drugs and logarithm of intrinsic elimination clearance ($\log CL_{int}$). C. The $pK_a$ for the cationic drugs and logarithm of apparent distribution ratio ($\log K_v$). The line represents the linear regression line. The data are represented as the mean ± S.D.
Fig. 5. Comparison of observed and predicted pharmacokinetic parameters of cationic drugs in control (●) and non-alcoholic steatohepatitis (NASH) (▲) rat liver.

Fig. 6. mRNA expression of Cyp3a2, Cyp2d2 and Cyp2e1 in control and NASH rat liver. The expression levels were normalized using housekeeping gene (GAPDH) expression as an external standard and expressed as a percentage when compared with average gene expression in control rat liver ± S.D.
Table 1. Physicochemical properties of selected cationic drugs

<table>
<thead>
<tr>
<th>Cationic drugs</th>
<th>Molecular weight</th>
<th>logP&lt;sub&gt;app&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>f&lt;sub&gt;uB&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Metabolism</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>259.34</td>
<td>3.10</td>
<td>0.69</td>
<td>9.45</td>
<td></td>
<td>CYP2D2</td>
</tr>
<tr>
<td>Labetalol</td>
<td>328.41</td>
<td>2.69</td>
<td>0.57</td>
<td>7.4</td>
<td>glucuronosyl transferase</td>
<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td>267.36</td>
<td>1.79</td>
<td>0.76</td>
<td>9.17</td>
<td></td>
<td>CYP2D2/3A2</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>188.23</td>
<td>0.33</td>
<td>1.00</td>
<td>1.45</td>
<td></td>
<td>CYP3A2</td>
</tr>
<tr>
<td>Atenolol</td>
<td>266.34</td>
<td>0.14</td>
<td>0.47</td>
<td>9.6</td>
<td></td>
<td>CYP2D2</td>
</tr>
</tbody>
</table>

<sup>a</sup> log octanol/water partition coefficient at pH 7.4 values (Hung et al., 2001).

<sup>b</sup> fraction unbound in perfusate acquired with microfiltration method.

<sup>c</sup> negative logarithm of the ionization constant.
Table 2. Primer sequence for real-time RT-PCR analysis of mRNA expression

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Accession number</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A2</td>
<td>NM_153312</td>
<td>CTGACAGACAAGCAGGGATG</td>
<td>TGGGTTCAAGTCGGTAGAG</td>
</tr>
<tr>
<td>CYP2D2</td>
<td>NM_012730</td>
<td>TGAGGACCCTTTTTCTCAACAG</td>
<td>AGAATTGGGATTCGTTTCAG</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>NM_031543</td>
<td>TGGGGAAACAGGGTAATGAG</td>
<td>CTGGCCCTTGGTGCTTTTGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>GATGGTGAAAGGTCGGTGTG</td>
<td>ATGAAGGGGTCGGTATGG</td>
</tr>
</tbody>
</table>
Table 3. Comparison of liver perfusion and biochemistry parameters between control and NASH rats (mean ± S.D., n=6)

<table>
<thead>
<tr>
<th>Perfusion parameters</th>
<th>Animal model</th>
<th>Control rats</th>
<th>NASH rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final rat body weight (g)</td>
<td></td>
<td>355 ± 22</td>
<td>425 ± 36**</td>
</tr>
<tr>
<td>Liver wet weight (g)</td>
<td></td>
<td>10.36 ± 1.31</td>
<td>13.84 ± 0.67**</td>
</tr>
<tr>
<td>Liver fat weight (g)</td>
<td></td>
<td>0.33 ± 0.10</td>
<td>2.08 ± 0.40**</td>
</tr>
<tr>
<td>Perfusion rate (mL·min⁻¹·g liver⁻¹)</td>
<td></td>
<td>1.47 ± 0.19</td>
<td>1.09 ± 0.06**</td>
</tr>
<tr>
<td>Perfusion pressure (cmH₂O)</td>
<td></td>
<td>26.00 ± 2.21</td>
<td>28.67 ± 3.56</td>
</tr>
<tr>
<td>Bile flow (μL·min⁻¹·g liver⁻¹)</td>
<td></td>
<td>0.83 ± 0.13</td>
<td>0.34 ± 0.12**</td>
</tr>
<tr>
<td>O₂ consumption (μmol·min⁻¹·g liver⁻¹)</td>
<td></td>
<td>2.32 ± 0.37</td>
<td>1.49 ± 0.25*</td>
</tr>
<tr>
<td>Cellular water space (μL·g liver⁻¹)</td>
<td></td>
<td>528 ± 52</td>
<td>508 ± 100</td>
</tr>
<tr>
<td>Vascular sucrose space (μL·g liver⁻¹)</td>
<td></td>
<td>300 ± 28</td>
<td>262 ± 32*</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td></td>
<td>81.2 ± 11.4</td>
<td>131.7 ± 29.6**</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td></td>
<td>119.0 ± 7.8</td>
<td>134.1 ± 17.1**</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td></td>
<td>28.8 ± 3.0</td>
<td>39.0 ± 3.0*</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td></td>
<td>0.88 ± 0.18</td>
<td>1.70 ± 0.47*</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td></td>
<td>0.59 ± 0.25</td>
<td>0.78 ± 0.32</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td></td>
<td>0.91 ± 0.12</td>
<td>0.59 ± 0.12**</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td></td>
<td>&lt;0.1</td>
<td>1.1 ± 0.6**</td>
</tr>
<tr>
<td>vLDL (mmol/L)</td>
<td></td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.2*</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01.
Table 4. Comparison of fibrosis index, steatosis index and liver CYP450 concentration between control and NASH rat liver (mean ±S.D., n=6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control rats</th>
<th>NASH rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrosis index (%)</td>
<td>0.42 ± 0.11</td>
<td>7.32 ± 1.40**</td>
</tr>
<tr>
<td>Steatosis index (%)</td>
<td>0.73 ± 0.22</td>
<td>7.23 ± 1.17**</td>
</tr>
<tr>
<td>CYP450 (nmol/g liver)</td>
<td>110 ± 8</td>
<td>135 ± 10 **</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01.
Table 5. Nonparametric estimates of cationic drug pharmacokinetic parameters in perfused rat livers (mean ± S.D., n=6)

<table>
<thead>
<tr>
<th>Cationic drugs</th>
<th>E</th>
<th>MTT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NASH</td>
</tr>
<tr>
<td>Propranolol</td>
<td>0.98 ± 0.02</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td>Labetalol</td>
<td>0.86 ± 0.02</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>0.84 ± 0.05</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>0.16 ± 0.03</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Atenolol</td>
<td>0.16 ± 0.03</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01.
Table 6. Kinetic parameters derived from two-phase stochastic model fitting for cationic drugs in control and NASH model rat livers

(mean ± S.D., n=6)

<table>
<thead>
<tr>
<th>Cationic drugs</th>
<th>PS (mL·min⁻¹·g liver)</th>
<th>$K_v$ (mL·min⁻¹·g liver)</th>
<th>CL$_{int}$ (mL·min⁻¹·g liver)</th>
<th>$K_S$ (mL·min⁻¹·g liver)</th>
<th>$K_R$ (mL·min⁻¹·g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.28 ± 2.43</td>
<td>10.76 ± 1.40</td>
<td>16.31 ± 1.25$^*$</td>
<td>11.31 ± 1.64$^*$</td>
<td>13.59 ± 2.04</td>
</tr>
<tr>
<td>NASH</td>
<td>25.94 ± 2.12$^{**}$</td>
<td>7.50 ± 1.26</td>
<td>13.59 ± 1.64$^*$</td>
<td>5.35 ± 1.45$^{**}$</td>
<td>1.96 ± 0.85</td>
</tr>
<tr>
<td>Control</td>
<td>16.22 ± 1.46</td>
<td>6.45 ± 1.13</td>
<td>10.94 ± 1.02$^{**}$</td>
<td>5.72 ± 1.63$^{**}$</td>
<td>6.78 ± 1.45</td>
</tr>
<tr>
<td>NASH</td>
<td>8.97 ± 1.13$^{**}$</td>
<td>3.34 ± 0.69</td>
<td>11.81 ± 2.62$^{**}$</td>
<td>1.08 ± 0.27</td>
<td>1.94 ± 0.80$^*$</td>
</tr>
<tr>
<td>Control</td>
<td>13.43 ± 1.24</td>
<td>9.64 ± 1.07</td>
<td>11.97 ± 0.76$^{**}$</td>
<td>1.80 ± 0.56$^*$</td>
<td>2.27 ± 0.71</td>
</tr>
<tr>
<td>NASH</td>
<td>7.59 ± 1.40$^{**}$</td>
<td>1.13 ± 0.20</td>
<td>1.78 ± 0.38</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>10.01 ± 0.90</td>
<td>6.97 ± 0.96$^{**}$</td>
<td>2.67 ± 0.22</td>
<td>0.14 ± 0.04</td>
<td>0.24 ± 0.04$^{**}$</td>
</tr>
<tr>
<td>NASH</td>
<td>2.60 ± 0.45$^{**}$</td>
<td>4.50 ± 0.57$^{**}$</td>
<td>0.24 ± 0.04$^{**}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>5.91 ± 0.67</td>
<td>9.90 ± 0.92</td>
<td>12.51 ± 1.09$^{**}$</td>
<td>0.65 ± 0.18</td>
<td>11.85 ± 1.59</td>
</tr>
<tr>
<td>NASH</td>
<td>2.60 ± 0.45$^{**}$</td>
<td>9.30 ± 2.16</td>
<td>9.30 ± 2.16</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^*$P<0.05, $^{**}$P<0.01.
Figure 1

The diagram illustrates the metabolic processes within a hepatocyte, focusing on the movement of metabolites between the cytoplasmic compartment (VC) and other cellular organelles. The key parameters and fluxes include:

- $K_S$: Affinity constant for substrate binding.
- $K_R$: Affinity constant for a different binding site.
- $f_{AB}$: Blood flow rate.
- $V_B$: Blood volume.
- $k_{on}$: On-rate constant for substrate binding.
- $k_{off}$: Off-rate constant for substrate dissociation.
- $k_e$: Rate constant for enzyme catalysis.
- $k_{in}$: Influx rate.
- $k_{out}$: Outflux rate.

The Disse space and blood flow (Q) are also depicted, indicating the exchange between the cellular and extracellular environments.
Figure 3

Sucrose

Propranolol

Labetalol

Metoprolol

Antipyrine

Atenolol
Figure 4(A)

The graph shows the relationship between logarithmic liver perfusion rate (logPS) and logarithmic apparent permeability (logP<sub>app</sub>). Two equations are given:

For control:

\[ \log PS_{\text{Control}} = 0.199 \times \log P_{\text{app}} + 0.816 \]

\[ r^2 = 0.826 \]

For NASH:

\[ \log PS_{\text{NASH}} = 0.222 \times \log P_{\text{app}} + 0.543 \]

\[ r^2 = 0.706 \]

The graph includes data points for both control and NASH conditions, indicating a positive correlation between logPS and logP<sub>app</sub>.
Figure 4(B)

\[
\log C_{\text{int}}^{\text{NASH}} = 0.395\times \log P_{\text{app}} - 0.286 \\
\quad r^2 = 0.919
\]

\[
\log C_{\text{int}}^{\text{Control}} = 0.452\times \log P_{\text{app}} - 0.647 \\
\quad r^2 = 0.824
\]
Figure 4(C)

\[ \log K_{v,\text{NASH}} = 0.060 \times pK_a + 0.572 \]
\[ r^2 = 0.942 \]

\[ \log K_{v,\text{Control}} = 0.073 \times pK_a + 0.312 \]
\[ r^2 = 0.988 \]
Figure 5

\[ \log PS = 0.721 \times \log Fl + 0.211 \times \log P_{app} \]  
\( r^2 = 0.762, n=30, p<0.001 \)

\[ \log K_s = 0.382 + 0.163 \times \log SI + 0.066 \times pK_s \]  
\( r^2 = 0.928, n=30, p<0.001 \)

\[ \log Cl_{int} = -4.220 + 1.757 \times \log CYP + 0.453 \times \log P_{app} \]  
\( r^2 = 0.819, n=30, p<0.001 \)

\[ \log E = -1.460 + 0.230 \times \log CYP - 0.123 \times \log Fl + 0.365 \times \log P_{app} \]  
\( r^2 = 0.867, n=30, p<0.001 \)
Figure 6

![Bar graph showing normalized mRNA expression for Cyp3a2, Cyp2d2, and Cyp2e1 in Control and NASH groups.](image)

*P<0.05, †P<0.01