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The Impact of Hepatic Uptake on the Pharmacokinetics of Organic Anions

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Abbreviations: $CL_{int,met}$, unbound metabolic intrinsic clearance; CL_{inc}, clearance from the incubation; $CL_{int,L}$, liver unbound intrinsic clearance; fu_{inc}, fraction unbound in the incubation; LC-MS/MS, liquid chromatography/tandem mass spectrometry; PPB, plasma protein binding; B:P, blood to plasma ratio; $CL_{int,efflux}$, unbound sinusoidal

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efflux intrinsic clearance; $CL_{int,uptake}$, unbound sinusoidal uptake intrinsic clearance; $CL_{int,pass}$, unbound passive diffusion intrinsic clearance; k_{mem}, proportionality constant between amount in membrane and concentration in medium; Vss, steady-state volume of distribution; CL_{med}, clearance from the medium; fu_{cell}, unbound fraction of drug within the cells; $f_{med,ss}$, fraction in the medium at steady-state; T, tissue; I, interstitial fluid; LV, liver vascular; L,mem, liver cell membrane; LI, liver interstitial; L,cell, liver cellular; $CL_{int,L,pass}$, liver unbound passive diffusion intrinsic clearance; $CL_{int,L,uptake}$, liver unbound sinusoidal uptake intrinsic clearance; $CL_{int,L,efflux}$, liver unbound sinusoidal efflux intrinsic clearance; $k_{L,mem}$, proportionality constant between free blood concentration and the amount of drug in the cell membranes of the liver; MRT, mean residence time.

Abstract

The disposition of 7 marketed and 2 AstraZeneca acid (organic anion) compounds with a range of Vss and clearance have been profiled in rat and dog. PK parameters along with liver and muscle tissue levels were collected and their contribution to total Vss were calculated. The physiologically based prediction of Vss correlated (all predictions within 2-fold) with the Vss obtained from plasma PK analysis. The Vss of the acid drugs with atypically high values could be explained by significant sequestering of compound to the liver. A 'media loss' in vitro hepatocyte assay that monitors loss of compound from the incubation media along with PBPK modelling was assessed for its ability to accurately predict the impact of hepatic uptake on both clearance and Vss. This methodology significantly improved the prediction of metabolic in vivo clearance compared to standard hepatocyte scaling approaches that do not take into account hepatic uptake. Predictions of Vss from the “media loss” assay also correlate with the measured values from plasma PK analysis. However, hepatic uptake will have little overall impact on half-life, due to the concomitant impact on both Cl and Vss, as long as hepatic extraction is not high. The methodology described here is particularly useful when there is no allometric relationship between species as a result of inter-species differences in liver uptake. In this situation, the potential use of human hepatocytes combined with PBPK modelling avoids the question of which species PK is most predictive to man.

Introduction

Within the drug discovery environment the ability to accurately predict human in vivo pharmacokinetics leading to the successful progression of drug candidates is one of the most challenging aspects of drug metabolism and pharmacokinetics (PK). Of particular importance is the ability to be able to predict the clearance (CL) and volume of distribution (Vd) of a candidate drug, which when combined can be used to predict human half-life ($T_{1/2}$). Therefore, inclusion of all factors that influence CL and Vd are of paramount importance in the accurate prediction of the human PK of a candidate drug.

For many drugs the major route of clearance is via metabolism within the liver. Prediction of hepatic clearance from a variety of in vitro systems has formed a cornerstone of the design and selection of small molecule drug candidates within the pharmaceutical industry for more than a decade. For compounds whose clearance is predominantly via hepatic metabolism the approaches taken have been relatively successful, (Riley et al, 2005, Ito and Houston, 2004, Soars et al, 2002) although not without their failures (Soars et al, 2007). The realization that hepatic transporters may add additional layers of complexity has added to the challenge in terms of both the modelling required (Sirianni and Pang, 1997, Liu and Pang, 2005) and the in vitro assays utilized (Soars et al, 2007). This has allowed the opportunity to reassess compounds for which metabolic clearance was poorly predicted via more conventional methods.

The PK parameter commonly used to characterize distribution of a drug is the volume of distribution at steady-state (Vss). The Vss represents the ratio of the amount of drug in the body to the plasma concentration at steady-state. The human Vss of a candidate drug is commonly predicted using an allometric scaling approach. This is

generally regarded as a successful method in most cases, whether using one species, usually rat (Caldwell et al 2004), or two or more species (Mahmood et al, 1999). Successful allometric scaling of Vss to man assumes similar tissue binding between species. Therefore any distribution differences between species due to mechanistic dissimilarity may lead to poor predictions to man. Physiological based PK (PBPK) models have been developed and used in the last 30 years for the prediction of Vss of drugs and chemicals. PBPK models strive to be mechanistic by mathematically transcribing anatomical, physiological, physical, and chemical descriptions of the phenomena involved in the complex ADME processes (Kawai et al, 1994, Charnick et al, 1995). A large degree of residual simplification and empiricism is still present in PKPB models, but they have an extended domain of applicability compared to that of empirical allometric methods.

Recently, there have been several publications highlighting the impact of hepatic uptake on the Vss of a drug (Paine et al, 2008, Grover and Benet, 2009) and the potential impact of drug-drug interactions. The Vss of acid drugs (which are typically characterised by low Vss (0.1-0.25 L/Kg) due to their poor tissue distribution and high plasma protein binding) are particularly sensitive to transporter effects. Paine et al, 2008 have shown that the liver levels for the acid drug atorvastatin are many times higher than that of the blood levels at steady-state in a rat bile duct canulated study. The high concentration of atorvastatin in an organ that has a significant contribution to body weight led to a Vss many times higher than a typical acid drug. This Vss value along with the liver levels could be quantitatively predicted by a PBPK model that uses in vitro hepatocyte uptake data in combination with various physiological parameters. Therefore, inhibition and induction of hepatic uptake transporters may

lead to changes in Vss and species differences in the transporters may lead to poor predictions of PK in man.

The aim of this work was to investigate 7 marketed acid drugs and 2 AstraZeneca acid compounds and determine their PK in both rat and dog bile duct cannulated studies. Bile duct cannulated studies were used to assess the contribution of biliary and renal clearance to the overall clearance value. Tissue (blood, liver, muscle) distribution for the compounds was investigated in both rat and dog and a simple PBPK model used to assess tissue contribution to Vss. The 9 compounds were assessed in an in vitro hepatocyte assay for their cellular disposition and data fitted to a 2-compartmental model (Paine et al, 2008) in order to determine in vitro clearance values for active/passive transport and metabolic clearance in both rat and dog. These parameters were then used in conjunction with a 7-compartmental PBPK model to predict the impact of hepatic uptake on PK parameters. Results were compared between the in vivo measurements and the predicted PK parameters.

Materials and Methods

Chemicals

All chemicals and reagents used were of the highest available grade. Telmisartan was sourced from Sequoia Research Products Ltd. (Oxford, UK). Ramatroban and the AstraZeneca compounds were synthesised in house by AstraZeneca. S-ibuprofen, indomethacin, losartan, tolmetin and sulfisoxazole and all other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK). Hepatocyte suspension buffer consisted of 2.34 g Na HEPES, 0.4 g D-fructose, 2 g bovine serum albumin (BSA), 1 L powder-equivalent of Dulbecco's modified Eagle's medium (Sigma, Gillingham, UK) diluted in 1 L of water and adjusted to pH 7.4 with 1 M HCl.

Preparation of hepatocytes

Rat

Rat hepatocytes were isolated from male Sprague-Dawley rats using the two-step in situ collagenase perfusion method of Seglen (1976) described in detail in Soars et. al. (2007). Cells were resuspended in suspension buffer without BSA and an estimation of hepatocyte yield and viability was obtained using the trypan blue exclusion method. Only cells with a viability of >80% were used.

Dog

Dog hepatocytes were isolated in house from male beagle dogs approximately 1 year old. The isolation procedure was based on the two-step in situ collagenase perfusion method described in more detail by McGinnity et al. (2004). Hepatocytes were resuspended for use in suspension buffer. Estimation of yield and viability was assessed using the trypan blue exclusion method and only batches with a viability >80% were used.

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Determination of intrinsic clearance (CL_{inc}) in hepatocytes using the standard incubation method (cells + media assay)

The rate of turnover in hepatocyte suspensions (CL_{inc}) was estimated using a procedure that sampled the whole incubation. 10 µl of DMSO stocks of ibuprofen indomethacin, losartan, ramatroban, sulfisoxazole, telmisartan, tolmetin, AZ1 AZ2 (100 µM) were added to 490 µl aliquots of hepatocyte buffer (protein-free) and warmed to 37°C. Hepatocytes were diluted to 2 x 10^6 cells/mL in protein-free hepatocyte buffer and warmed to 37°C. At time zero 0.5 mL of cells was added to the appropriate substrate solution, mixed and placed in a shaking water bath (37°C and 80 oscillations/min). 40 µl aliquots were removed at 5, 15, 30, 45, 60, 75 and 90 minutes and quenched with 80 µl of ice-cold methanol. Samples were mixed and then stored at -20°C while awaiting analysis and to enhance protein precipitation. Prior to analysis samples were spun at 2000 x g for 20 minutes at 4°C to pellet protein and the supernatant transferred to a 96-well agilent plate for LC-MS/MS analysis. Parent peak area was plotted against time and CL_{inc} estimated by multiplying the incubation volume by the elimination rate constant. Each compound was incubated on at least three separate occasions.

Determination of loss from media (CL_{med}) using hepatocytes (”media loss” assay)

CL_{med} values were determined essentially as described above except that 2 mL incubations were prepared. Aliquots (80 µl) were removed at 0, 0.5, 1, 2, 4, 6 15, 30, 45, 60, 75 and 90 min and placed into centrifuge tubes. These aliquots were immediately centrifuged at 7000 g for 30 s using a MSE MicroCentaur® centrifuge (Fisher Scientific, Loughborough, UK) and 40 µl of the supernatant was pipetted into 80 µl of ice-cold methanol. Samples were then frozen for 1 h at –20 °C, and

centrifuged at 2000 g for 20 min at 4°C. The supernatants were removed and analysed as described below. Each compound was incubated on at least three separate occasions and the CL_{med} was estimated as described in data analysis section.

Data was fitted to the 2-compartment model shown in Figure 1 and in vitro parameters calculated using ModelMaker 3 (ModelKinetix, The Magdalen Centre, Oxford Science Park, Oxford, OX4 4GA, UK).

Determination of plasma protein binding (PPB)

Plasma protein binding was measured using equilibrium dialysis at least twice for each compound. Ibuprofen, indomethacin, losartan, ramatroban, sulfisoxazole, telmisartan, tolmetin, AZ1 and AZ2 (final concentration 10 μM) were spiked into rat or dog plasma and placed on one side of a dialysis cell; the other side contained only buffer. The compounds were dialysed through a 50kD membrane in a Dianorm rotating unit (Diachema, Switzerland) for 18 hours at 37°C. Aliquots from the buffer and dialysate side of the membrane were then quenched in methanol and analysed via LC-MS/MS as described below. The extent of plasma protein binding (fu_p) was calculated by dividing the concentration of compound in the absence of plasma (buffer side) by the concentration of compound in the presence of plasma (dialysate side)

Determination of fraction unbound in the incubation (fu_{inc})

Unbound fraction in hepatocytes (1×10^6 cells/mL) was measured at least twice for ibuprofen, indomethacin, losartan, ramatroban, sulfisoxazole, telmisartan, tolmetin, AZ1 and AZ2 at 1 μM by equilibrium dialysis over a 3 hour period at 37°C as has previously been described by Austin et al., 2005.

Determination of fraction unbound in liver tissue (fu_L)

Unbound fraction in 25% liver homogenate was measured at least twice for ibuprofen, indomethacin, losartan, ramatroban, sulfisoxazole, telmisartan, tolmetin, AZ1 and AZ2 at 1 μM by equilibrium dialysis at 37°C for 18 hours. Free fraction in whole liver was calculated by correcting for the homogenate dilution using the equation outlined and validated by Austin et al. (2002).

$$fu_2 = \frac{1}{\frac{C_2}{C_1}\left(\frac{1-fu_1}{fu_1}\right)+1}$$

where fu_2 in this case is the free fraction in whole liver, fu_1 is the measured free fraction in 25% homogenate and C_1 and C_2 are the concentrations of the homogenate i.e. 25 and 100% respectively.

Determination of blood-plasma ratio (B:P)

Ibuprofen, indomethacin, losartan, ramatroban, sulfisoxazole, telmisartan, tolmetin, AZ1 and AZ2 at 100 x final concentration were spiked into aliquots of fresh rat or dog blood and plasma (0.5 mL) and incubated for 15 minutes at 37ºC in a shaking waterbath followed by centrifugation in a MSE MicroCentaur® centrifuge (Fisher Scientific, Loughborough, UK) at 11,000 rpm for 4 minutes. Aliquots of plasma from both the blood and plasma incubations were quenched with methanol and stored at –20ºC for at least one hour. Prior to analysis, samples were centrifuged at 2000 × g for 20 minutes and the supernatant transferred into HPLC vials for LC-MS/MS analysis. Blood-plasma ratio was calculated by dividing the peak area from directly spiked plasma by the peak area from plasma isolated from spiked blood.

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Determination of k_{mem}

k_{mem} for each compound was determined by measuring the instantaneous binding of compound to the cell by adding compound to freshly prepared hepatocytes (1 µM, 1 million cells/ml), immediately sampling followed by centrifugation (7000 g), taking the supernatant and comparing the compound levels with the total incubation concentration using LC-MS/MS analysis. This was done 5 times for each compound.

In vivo studies

All in vivo work was subject to internal ethical review and conducted in accordance with Home Office requirements under the Animals Scientific Procedures Act (1986). Healthy virus antibody-free male Sprague Dawley rats were obtained from Charles River (Margate, UK). They were housed in a light-controlled room, kept at a temperature of 19°C ± 2°C and 55% ± 10% humidity. They received a Teklad 2021 diet (Harlan) and had access to water ad libitum. Healthy male beagles (bred in-house) were housed in pairs in a controlled environment (temperature 18°C ± 2°C and humidity 55% ± 10%). They were fed with a SDS D3 (E) Dog Maintenance diet and had access to water ad libitum. They were fully vaccinated and wormed, and had daily access to an external play area for exercise and socialisation. After at least 4 weeks of acclimatisation, a chronic bile duct cannulation was performed on two dogs following the technique described by Kissinger and Garver (1998). Dogs were allowed to recover from surgery for a minimum of one month.

Pharmacokinetics studies in the Rat

After at least 1 week of acclimatisation rats (250-350g) were surgically prepared under isoflurane anaesthesia. The bile duct was cannulated and cannulae were also

implanted into the jugular vein (dosing cannula) and carotid artery (blood sampling cannula). Dose solutions were administered via the intravenous (jugular vein) cannula. Doses administered were calculated by the weight difference of the dosing syringe before and after administration. Serial plasma samples (200 - 300 uL) were taken from the intra-arterial (carotid artery) cannula. Approximately 200ul of blood was drawn through the cannula prior to taking a sampling aliquot to ensure circulating blood was sampled through the cannula. Serial blood samples were taken over a 7 hour time-course. Blood was centrifuged at 1110 × g for 10 minutes at 4°C. Plasma was transferred into plain polypropylene tubes, each prepared in advance to contain 1.5 µl of concentrated phosphoric acid to stabilize any potential acyl glucuronide metabolites. Samples were then immediately frozen upright on dry ice and stored at -20°C. Bile and urine samples were treated with phosphoric acid, frozen and stored as described previously. Livers and sartorius muscle tissue were taken at termination for all compounds.

Pharmacokinetics studies in the Dog

Test compounds were dissolved in bicarbonate buffer (1 mg/mL) containing 10% ethanol to a final concentration of 1 mg/mL. The dose was administered to the dogs (n=2) via a 30-minute infusion in the cephalic vein. Approximately 2.5 mL of blood was collected on EDTA via the jugular vein 0, 15, 30, 60, 120, 180, 300, 420, 720 and 1440 minutes after the beginning of the infusion. Bile was collected over 24 hours. Dogs were kept in metabolism cages over 24 hours for urine collection onto dry-ice. Plasma, bile and urine samples were prepared within 30 minutes of collection according to the following procedure. Blood was centrifuged at 1110 × g for 10 minutes at 4°C. Plasma was transferred into plain polypropylene tubes, each

prepared in advance to contain a modicum of concentrated phosphoric acid to stabilize any potential acyl glucuronide metabolite. Samples were then immediately frozen upright on dry ice and stored at -20°C. Bile and urine samples were treated with phosphoric acid, frozen and stored as described previously. Where terminal studies in the dog were performed they were carried out as above but no samples were collected during the study except liver and sartorius muscle tissue which were taken at termination. Due to a shortage of availability of terminal dogs it was only possible to monitor tissue levels of three compounds; telmisartan, losartan and ibuprofen.

Tissue Measurements

Tissues were homogenised and all samples were analysed as described below. Tissue concentrations were measured and their contribution to total Vss using volume of distribution of tissue $= V_t \times K_p \times (1-E)$, where V_t = volume of tissue, K_p = plasma tissue partition coefficient and E = the tissue extraction ratio. Other pharmacokinetic parameters were calculated using noncompartmental analysis in WinNonlin (v. 3.2, Pharsight Corporation, Mountain View, CA, USA).
Metabolic clearance was calculated by subtracting any renal or biliary clearance from the total clearance.

In vivo sample preparation

Plasma was dispensed into 50 µl aliquots and 150 µl methanol added (containing internal standard) and mixed. Bile and urine were diluted in water prior to analysis. Liver and muscle were weighed, diluted with water and homogenised. Homogenate was dispensed into 50 µl aliquots in triplicate and processed as per plasma.

Appropriate standard curves and quality control (QC samples) prepared in the equivalent blank tissue were used for each analysis.

Sample analysis

Samples were analysed by LC-MS/MS with an HP1100 HPLC system (Hewlett Packard) linked to a Quattro Ultima mass spectrometer (Micromass, Waters, Milford, MA, USA) in negative or positive electrospray ionization mode with data analysis on Quanlynx software (v. 4.0, Micromass). Cone voltage and collision energy were optimised for each compound. In these analyses, chromatographic separation was achieved using a Waters Symmetry C8 3.5 µm (2.1 x 30 mm) column using 10 µl of each sample. The mobile phase consisted of an aqueous phase of water with 0.1 % (v/v) formic acid and an organic phase of methanol with 0.1 % (v/v) formic acid. Samples were quantified using appropriate calibration curves and quality controls. Where plasma, liver and muscle levels were fitted to the 7-compartment model (Figure 2) the software Berkeley Madonna version 8.3.18, University of California, CA. was used to estimate in vivo parameters.

Data Analysis

In vitro

A hepatocyte incubation model that has been previously described by Paine et al, 2008 was used and includes medium, cellular and cell membrane compartments (Figure 1). Following the addition of a test compound, clearance from the incubation (CL_{inc}) is obtained from standard procedures by sampling from the hepatocyte / medium suspension. Clearance from the incubation can be estimated from the slope of the natural log (concentration in incubation)-time plot. However, CL_{inc} has limited

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applications when uptake occurs as the free concentration of drug is different between the medium and hepatocyte. A more appropriate in vitro clearance term is the overall clearance from the medium (CL_{med}) and can be expressed as follows:

$$CL_{med} = CL_{\text{int},met}\frac{fu_{cell}C_{cell}}{fu_{med}C_{med}} = CL_{\text{int},met}\Psi \qquad Equation\ 1$$

Where the subscripts cell and med refer to the cell (excluding the membrane) and medium compartments, respectively. C is the total drug concentration and fu is the free fraction of the corresponding compartment. CL_{med} is the clearance from the medium and $CL_{int,met}$ the unbound metabolic intrinsic clearance from the cell. Albumin was not present in the cell suspension, therefore fu_{med} was assumed equal to 1. Therefore, Ψ represents the ratio of the free concentration inside the cell to the free concentration in the medium at steady-state.

The amount of drug bound to the cell membrane (X_{mem}) is directly proportional to the concentration of drug in the medium.

$$X_{mem} = k_{mem}C_{med} \qquad Equation\ 2$$

Where k_{mem} is the proportionality constant between amount in membrane and free concentration in medium and has units of volume. In this model, the initial concentration in the medium is given by:

$$C_{med(t=0)} = \frac{Dose}{V_{med} + k_{mem}} \qquad Equation\ 3$$

Where dose is total amount of drug added to the suspension, V_{med} is the volume of the medium and $C_{med(t=0)}$ is the medium concentration at time zero. Hence, k_{mem} can

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be estimated by sampling the medium as soon as possible after addition of test compound.

CL_{med} can also be expressed in terms of passive, uptake, efflux and metabolic intrinsic clearances.

$$CL_{med} = CL_{\text{int},met}\frac{CL_{\text{int},pass} + CL_{\text{int},uptake}}{CL_{\text{int},pass} + CL_{\text{int},efflux} + CL_{\text{int},met}} \qquad Equation\ 4$$

Intrinsic clearances in equation 4 were obtained from fitting the model in Figure 1 to the concentration of drug in medium with time and the assumption that efflux was negligible. CL_{med} was then estimated for the test compounds by substituting the intrinsic terms into Equation 4.

Hence from equations 1 and 4 the ratio of the free concentration inside the cell to the free concentration in the medium (Ψ) can be expressed as follows:

$$\Psi = \frac{CL_{\text{int},pass} + CL_{\text{int},uptake}}{CL_{\text{int},pass} + CL_{\text{int},efflux} + CL_{\text{int},met}} \qquad Equation\ 5$$

When V_{med} approximates to the volume of the incubation (V_{inc}), CL_{med} also equals the incubational clearance (CL_{inc}) divided by the fraction in the medium at steady-state ($f_{med,ss}$) as below:

$$CL_{med} = \frac{CL_{inc}}{f_{med,ss}} \qquad Equation\ 6$$

When no uptake occurs $f_{med,ss}$ is equal to the fraction unbound in the incubation (fu_{inc}).

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In vivo

A 7-compartment physiological model (Figure 2) as described by Paine et al, 2008 illustrates sinusoidal bi-directional passive permeation and active uptake/efflux between the liver vascular and cellular compartments and is described by the in vivo intrinsic clearances; $CL_{int,L,pass}$, $CL_{int,L,uptake}$ and $CL_{int,L,efflux}$. Drug is eliminated from the cellular compartment with in vivo intrinsic clearance ($CL_{int,L}$). The model has been designed to be analogous to the in vitro model shown in Figure 1.

Predicting in vivo from in vitro

Equations for blood clearance and V_{ss} for the above 7-compartment physiological model (Figure 2) have been described by Paine et al, 2008. These equations were used to predict the clearance and V_{ss} of the test compounds in rat and dog. Assumptions on physiological volumes, blood flows and the fraction unbound in body tissues are also described in Paine et al, 2008.

The in vitro parameters $CL_{int,pass}$, $CL_{int,uptake}$, $CL_{int,met}$ and k_{mem} were scaled to their in vivo analogues $CL_{int,L,pass}$, $CL_{int,L,uptake}$, $CL_{int,L}$ and $k_{L,mem}$ using standard rat and dog biological scaling factors (where $k_{L,mem}$ is the proportionality constant between free blood concentration and the amount of drug in the cell membranes of the liver). However, using standard biological scaling factors to scale in vitro $CL_{int,met}$ generated in isolated human hepatocytes suggests a systematic under-prediction of in vivo $CL_{int,L}$ (Riley et al., 2005; Ito and Houston, 2005). A similar systematic bias of approximately 5-fold has been observed in the rat data in this laboratory (Grime and Riley, 2006) and can be found in the rat $CL_{int,met}$ data presented by some academic laboratories (Naritomi et al., 2001) but not others (Ito and Houston, 2004). Moreover, little is known when scaling $CL_{int,uptake}$ in isolated hepatocytes to $CL_{int,L,uptake}$ in the

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intact liver and this work allows the opportunity to investigate any discrepancies. Therefore, the plasma and liver levels of compounds that showed significant uptake in hepatocytes ($f_{med,ss} < 0.5 \times fu_{inc}$) were fitted to the 7-compartmental model as shown in Figure 2 and values for $CL_{int,L,pass}$, $CL_{int,L,uptake}$, and $CL_{int,L}$ were determined. These values were compared to the scaled in vitro parameters using standard biological scaling factors and correction factors were estimated based upon any discrepancies. These correction factors were then applied to all 9 compounds and predictions made for both Vss and clearance.

Results

Physical Properties

The physical properties of the 9 compounds are shown in Table 1. Overall the range of physical properties for the compounds is typical of small molecule acid drugs. For example, the molecular weights range from 200 to 500, acid pKas range from 3 to 5 and lipophilicity ranges from a $logD_{7.4}$ of -1 to 2. AZ1 and AZ2 in most regards have very similar properties to the marketed drugs, albeit being the most acidic.

In vivo

The major PK parameters in rat and dog are shown in Table 2. In most cases metabolic clearance is the main clearance mechanism; with the exception of sulfisoxazole in the dog where renal is the major component. It was noticeable that the PK parameters changed very little between biliary cannulated and non biliary cannulated animals (data not shown). In the rat, five of the compounds have Vss values outside the typical acid range (0.1-0.25 L/Kg), losartan, telmisartan, AZ1, AZ2 and ramatroban. For the most part the Vss values in the dog fall within the expected range for acids, with the exception of ramatroban (Vss of 2.2 L/Kg) and to some extent telmisartan (Vss of 0.7, 0.4 L/Kg). The contribution of the different tissues to the total Vss is shown in Table 3 and Figure 3. The Vss of the acid drugs outside the normal range in the rat are explained by the large contribution by the liver. Those compounds with higher Vss in the rat have high concentrations in the liver. The liver levels measured in the dog were low and this was consistent with the Vss of these compounds. The physiologically based prediction of Vss correlates very well with the measured values (all predictions within 2-fold, Figure 4).

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In Vitro

Hepatocyte assay “media loss” plots for each of the compounds in rat and dog are shown in Figure 5 (rat) and Figure 6 (dog). Indomethacin, losartan, ramatraban, AZ1 and AZ2 all show significantly bi-phasic disappearance from the media in rat hepatocytes (Figure 5). In dog, losartan, AZ1 and to a much lesser extent ramatraban and sulphisoxazole show bi-phasic disappearance from the media (Figure 6). The data was fitted using the 2-compartment model in Figure 1 to obtain in vitro parameters. The measured in vitro parameters are shown in Table 4. These indicate that based on $f_{med,ss}$ and fu_{inc} measurements the compounds that are most actively transported in hepatocytes are losartan, telmisartan, AZ1, AZ2 and ramatroban in the rat. Table 5 shows the in vitro and in vivo values of the individual intrinsic clearance parameters for these compounds in the rat. Also in table 5, the parameter Ψ, which represents the ratio of the free concentration inside the hepatocytes relative to the concentration in the media, is >1 for all 5 compounds. In general, there was little active transport observed in dog hepatocytes and therefore dog data was not used for comparing intrinsic clearances between the in vitro and in vivo cases. With this limited data set it appears that the passive correction factor from in vitro to in vivo is close to unity suggesting that the value can be simply scaled based upon hepatocellularity. The correction factor for metabolic intrinsic clearance is two-fold and is consistent with known literature differences between measured and scaled metabolic clearance from hepatocytes (see analysis section). However, the correction factor for hepatic uptake is over six times that of the scaled value using hepatocellularity alone. These mean correction factors have been applied to the in vitro intrinsic clearances of all the compounds to predict the pharmacokinetics.

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Prediction of in vivo parameters

In general the “media loss” assay improves the prediction of clearance in the rat when compared to the standard ”cells and media” assay (AFE 2.4 and 4.2, respectively) (Table 6 and Figure 7 & 8). Moreover, in contrast to the standard assay the prediction of losartan, ramatroban, telmisartan and AZ1 are predicted within 2-fold using the “media loss” assay. Inclusion of uptake tends to have less impact on the predictions of clearance in the dog, which are generally well predicted by both assays, with the exception of sulfisoxazole – this maybe due to the difficulty of measuring the low turnover of the compound coupled with relatively high free levels in plasma. However, there is some evidence of a bias towards an over prediction in the correlation with the “media loss” assay (Figure 8), especially with dog data. This may be a reflection of the correction factors applied being generated from rat data. Predictions of Vss from the “media loss” assay correlate well with the measured values (Figure 9). Most predictions are within 2-fold with the exception of telmisartan in the rat and indomethacin in the dog, which are within 4-fold.

Discussion

The results show that when an acid compound is actively sequestered into the liver both clearance and Vss can be affected. Moreover, scaling methods such as allometry may fail due to species differences in the sequestering of drug within the liver and therefore predictions to man may be inaccurate. For instance, telmisartan and losartan have much larger Vss in rat compared to dog even though they have higher PPB in rat. This can be explained by the greater liver sequestration of drug in rat compared to dog. Therefore, an in vitro tool to predict the level of hepatic uptake in an individual species is required in order to understand discrepancies in species PK parameters and ultimately more accurately predict to man.

The in vivo metabolic clearance of compounds that undergo insignificant hepatic uptake are well predicted using the standard homogeneous sampling (cells + media) methodology (Riley et al., 2005; Ito and Houston, 2005). Whether a compound undergoes active uptake or not this methodology should always be used for an initial understanding of hepatic metabolic turnover. If the in vivo metabolic clearance is well predicted using the standard cells + media assay then this should be the method of choice as it has far fewer variables than the in vitro “media loss” assay coupled with PBPK modelling. However, when an under-prediction of clearance occurs due to significant hepatic uptake then the “media loss” assay coupled with PBPK modelling has been shown in this work to be successful at predicting the PK of a series of acid compounds with a range of active hepatic uptake in both rat and dog. The overall compound disposition is not related to the magnitude of active uptake alone, but in its interplay with other factors such as passive transport and plasma binding. For example, Paine et al, 2008 showed that in the rat indomethacin has a large active uptake component but due to high passive hepatic transport and high plasma binding the impact on distribution and clearance is low and behaves like a typical acid

compound (Metabolic clearance 0.3 mL/min/Kg, Vss 0.1 L/Kg). Furthermore, our data has shown that, in rat, ramatroban has a much lower passive component and lower plasma binding than indomethacin resulting in a significant impact on clearance and Vss (Metabolic clearance 14 mL/min/Kg, Vss 1.3 L/Kg). The data also suggests that uptake will have little overall impact on half-life, due to the concomitant impact on both Cl and Vss, as long as hepatic extraction is not high. It does, however, have the potential to raise free levels of substrate compounds in the liver which may lead to adverse drug reactions.

Various laboratories have published on the discrepancy between scaled hepatocyte data using hepatocelluarity factors and measured metabolic clearance and in most cases an under-prediction of metabolic clearance is observed. We have observed a similar phenomenon for metabolic clearance but no discrepancy for the passive component. However, an in vitro/in vivo mean correction factor over hepatocellularity of around six is observed for the uptake component and suggests that there is a lower transporter capacity in the isolated hepatocyte relative to the in vivo case. Going forward, further refinement of the uptake correction factors would be desirable and this should be possible as the number of data sets increase. Moreover, this would enable a more sophisticated statistical approach to model the discrepancy between isolated hepatocytes and the in vivo situation (e.g. regression analysis). Importantly, the results presented herein suggest that this methodology could be applied to predicting the impact of hepatic uptake on human Vss as well as clearance using human hepatocytes. This is particularly useful when there is no allometric relationship between rat and dog as a result of inter species differences in liver uptake. In this situation, the use of human hepatocytes combined with PBPK modelling avoids the question of which species PK is most predictive to man.

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Authorship Contributions

Participated in research design: Paine and Gardiner.

Conducted experiments: Paine and Gardiner.

Performed data analysis: Paine and Gardiner.

Wrote or contributed to the writing of the manuscript: Paine and Gardiner.

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Legends for Figures

Figure 1: Compartmental model describing hepatocyte incubation

Figure 2: Seven-compartmental model describing sinusoidal bidirectional passive permeation and active uptake/efflux in the liver

Figure 3: Contribution from tissues to Total Vss

Figure 4: Correlation of a physiologically predicted Vss with Vss calculated from plasma (the solid line represents the line of unity, dotted lines signify 2-fold errors between the predicted and observed values, statistics were calculated using log data)

Figure 5: Typical media loss assay plots for each compound in rat and associated fits to a 2-compartment model (Figure 1)

Figure 6: Typical media loss assay plots for each compound in dog and associated fits to a 2-compartment model (Figure 1)

Figure 7: Correlation of predicted clearance using the cells + media assay and metabolic clearance calculated from plasma (the solid line represents the line of unity, dotted lines signify 2-fold errors between the predicted and observed values, statistics were calculated using log data) (△ dog, ▲ rat)

Figure 8: Correlation of predicted clearance using the media loss assay and metabolic clearance calculated from plasma (the solid line represents the line of

unity, dotted lines signify 2-fold errors between the predicted and observed values, statistics were calculated using log data) (△ dog, ▲ rat)

Figure 9: Correlation of predicted Vss using the media loss assay and Vss calculated from plasma (the solid line represents the line of unity, dotted lines signify 2-fold errors between the predicted and observed values, statistics were calculated using log data) (△ dog, ▲ rat)

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Table 1: Physical properties

Compound	MW	pKa	$LogD_{7.4}$	TPSA	Rotatable bonds	H bond donor	H bond acceptor
Ibuprofen	206	4.4	0.98	41	4	1	2
Indomethacin	358	4.0	1.00	69	5	1	5
Losartan	422	4.2	0.83	87	8	2	7
Ramatroban	416	4.6	0.99	94	6	2	6
Sulfisoxazole	267	4.8	-0.26	103	2	3	6
Telmisartan	515	3.8	2.01	62	7	1	6
Tolmetin	257	4.2	-1.02	60	4	1	4
AZ1	421	3.0	0.91	87	6	1	5
AZ2	439	3.0	0.92	87	6	1	5

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Table 2: Observed *in vivo* parameters in bile duct cannulated rats and dogs

Compound	*Species*	Clearance (mL/min/Kg)				Vss (L/Kg)
		Total	**Metabolic**	**Biliary**	**Renal**	
Ibuprofen	Rat	2.4, 1.5	2.4, 1.5	0, 0	0.002, 0.001	0.2, 0.2
Indomethacin	Rat	0.2, 0.3	0.2, 0.3	0.0009, 0.0007	0.0001, 0.0001	0.1, 0.1
Losartan	Rat	4.3, 5.0	4.1, 4.4	0.2, 0.6	0.0004, 0.0005	2.0, 1.2
Ramatroban	Rat	17, 14	15, 13	1.7, 0.6	0.001, 0.002	1.3, 1.3
Sulfisoxazole	Rat	0.1, 0.1	0.1, 0.1	0.001, 0.001	0.009, 0.009	0.2, 0.2
Telmisartan	Rat	6.0, 7.5	5.8, 7.3	0.2, 0.2	0, 0	2.4, 2.7
Tolmetin	Rat	1.5 ± 0.4	1.5 ± 0.4	0, 0	0.024 ± 0.006	0.11 ± 0.03
AZ1	Rat	6.0 ± 2.6	5.6 ± 2.4	0.4 ± 0.2	0.006 ± 0.003	0.9 ± 0.2
AZ2	Rat	8.0 ± 2.3	6.1 ± 1.8	1.6 ± 0.5	0.3 ± 0.1	0.6 ± 0.3
Ibuprofen	Dog	0.5, 0.7	0.5, 0.7	0.0004, 0.0007	0.0005, 0.0002	0.08, 0.07
Indomethacin	Dog	4.0, 2.3	3.8, 2.1	0.3, 0.2	0.0004, 0.0003	0.09, 0.05
Losartan	Dog	27, 44	24, 34	0.3, 6.6	2.8, 3.2	0.4, 0.3
Ramatroban	Dog	9.8 ± 4.8	7.6 ± 3.4	1.1 ± 1.2	1.1 ± 1.0	2.2 ± 1.4
Sulfisoxazole	Dog	1.6 ± 0.8	0.2 ± 0.2	0.0005 ± 0.0009	1.4 ± 0.9	0.3 ± 0.1
Telmisartan	Dog	6.1, 4.0	4.1, 2.3	1.9, 1.7	0.002, 0.002	0.7, 0.4
Tolmetin	Dog	0.4, 0.8	0.3, 0.7	0.004, 0.009	0.009, 0.1	0.1, 0.4
AZ1	Dog	1.9, 1.0	1.5, 0.8	0.1, 0.2	0.3, 0.1	0.2, 0.4
AZ2	Dog	1.2, 2.4	0.9, 2.0	0.3, 0.3	0.05, 0.07	0.4, 0.3

All data represents individual animals or mean ± SD of a minimum of three animals

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Table 3: Volume of distribution of tissues in rat and dog

Compound	Species	Vt (L/Kg)			Vss (L/Kg)
		Liver (L/Kg)	Muscle (L/Kg)	Plasma (L/Kg)	
Ibuprofen	Rat	0.008	0.04	0.14	0.18
Indomethacin	Rat	0.007	0.01	0.14	0.16
Losartan	Rat	2.5	0.005	0.14	2.62
Ramatroban	Rat	1.2	0.15	0.14	1.46
Sulfisoxazole	Rat	0.002	0.02	0.14	0.16
Telmisartan	Rat	3.3	0.02	0.14	3.44
Tolmetin	Rat	0.01	0.03	0.14	0.18
AZ1	Rat	1.7	0.02	0.14	1.86
AZ2	Rat	0.5	0.02	0.14	0.71
Ibuprofen	Dog	0.08	0.06	0.14	0.19
Losartan	Dog	0.07	<LOQ	0.14	0.25
Telmisartan	Dog	0.004	0.005	0.14	0.32

Mean values of 2 animals (rat) and 1 animal (dog) from tissue homogenate prepared in triplicate.

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Table 4: In Vitro parameters

Compound	k_{mem}	Rat $f_{med,ss}$	Dog $f_{med,ss}$	fu_{inc}	fu_L	Dog PPB	Rat PPB	Dog B:P	Rat B:P
Ibuprofen	0	0.98	0.98	0.97	0.25	98.8	97.7	0.6	0.6
Indomethacin	0.20	0.65	0.70	0.78	0.08	99.2	99.7	0.6	0.6
Losartan	0.05	0.32	0.67	0.87	0.08	95.2	98.5	1	0.8
Ramatroban	0.22	0.42	0.58	0.71	0.08	95.5	96.3	0.6	0.8
Sulfisoxazole	0.05	0.84	0.80	0.77	0.51	86.6	98.4	0.6	0.6
Telmisartan	0.004	0.33	0.41	0.51	0.04	97.9	99.4	0.9	0.9
Tolmetin	0.04	0.96	0.96	0.96	0.31	95.0	97.1	1.1	1
AZ1	0.14	0.27	0.45	0.80	0.08	99.7	99.7	0.6	0.7
AZ2	0.08	0.25	0.42	0.90	0.06	99.6	99.6	0.6	0.6

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Table 5: In Vitro and In Vivo Intrinsic clearance parameters in Rat

Compound	Ψ	Intrinsic Clearance					
		In Vitro (µL/min/1E6 cells)			In Vivo (mL/min/Kg)		
		$CL_{int,pass}$	$CL_{int,uptake}$	$CL_{int,met}$	$CL_{int,L,pass}$	$CL_{int,L,uptake}$	$CL_{int,L}$
Losartan	23	7.0 ± 2.0	190 ± 1.7	1.5 ± 0.65	7.7	2600	2.3
Ramatroban	5.9	61 ± 44	324 ± 6.2	4.0 ± 1.7	320	7800	21
Telmisartan	2.2	960 ± 4.4	1200 ± 630	9.7 ± 6.2	550	38000	23
AZ1	26	12 ± 2.5	310 ± 120	0.53 ± 0.43	110	16000	10
AZ2	100	4.0 ± 0.78	420 ± 160	0.22 ± 0.24	21	17000	3.8
Mean Correction Factor		**0.83 ± 0.67**	**6.5 ± 3.0**	**1.9 ± 1.7**			

The correction factor between *in vitro* and *in vivo* values is defined as the factor over and above hepatocellularity (117 and 215 million cells/g liver for rat and dog, respectively)

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Table 6. In Vivo predictions in rat and dog

Compound	Species	Hepatic Metabolic Clearance (mL/min/Kg)			Vss (L/Kg)	
		Predicted (method)		Observed	Predicted	Observed
		Cells + Media	Media loss		Media loss	
Ibuprofen	Rat	13.3	5.7	2.4, 1.5	0.17	0.2, 0.2
Indomethacin	Rat	0.9	0.61	0.2, 0.3	0.19	0.1, 0.1
Losartan	Rat	3.9	19	4.1, 4.4	1.5	2.0, 1.2
Ramatroban	Rat	5.1	26	15, 13	1.8	1.3, 1.3
Sulfisoxazole	Rat	0.6	0.23	0.1, 0.1	0.17	0.2, 0.2
Telmisartan	Rat	2.0	4.9	5.8, 7.3	0.7	2.4, 2.7
Tolmetin	Rat	2.0	0.86	1.5 ± 0.4	0.12	0.11 ± 0.03
AZ1	Rat	1.1	3.9	5.6 ± 2.4	1.0	0.9 ± 0.2
AZ2	Rat	0.6	4.7	6.1 ± 1.8	0.83	0.6 ± 0.3
Ibuprofen	Dog	1.3	1.5	0.5, 0.7	0.14	0.08, 0.07
Indomethacin	Dog	2	9.3	3.8, 2.1	0.25	0.09, 0.05
Losartan	Dog	8	30	24, 34	1.1	0.4, 0.3
Ramatroban	Dog	6	17	7.6 ± 3.4	1.2	2.2 ± 1.4
Sulfisoxazole	Dog	7	1.5	0.2 ± 0.2	0.60	0.3 ± 0.1
Telmisartan	Dog	5	1.7	4.1, 2.3	0.60	0.7, 0.4
Tolmetin	Dog	1.7	0.8	0.3, 0.7	0.25	0.1, 0.4
AZ1	Dog	0.7	1.8	1.5, 0.8	0.25	0.2, 0.4
AZ2	Dog	1	2.6	0.9, 2.0	0.45	0.4, 0.3

Figure1

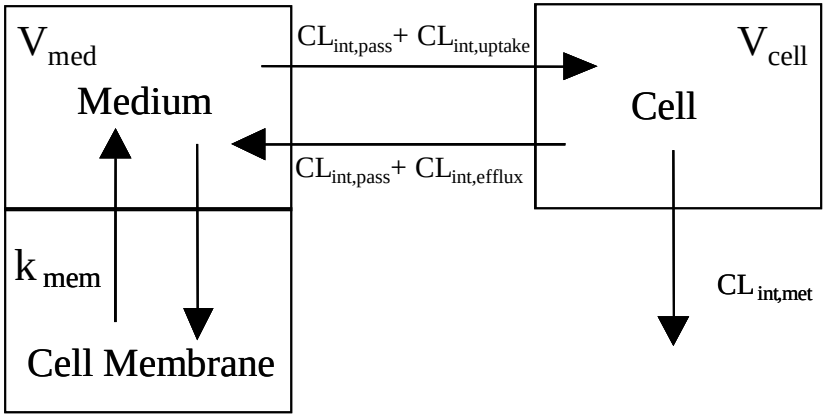
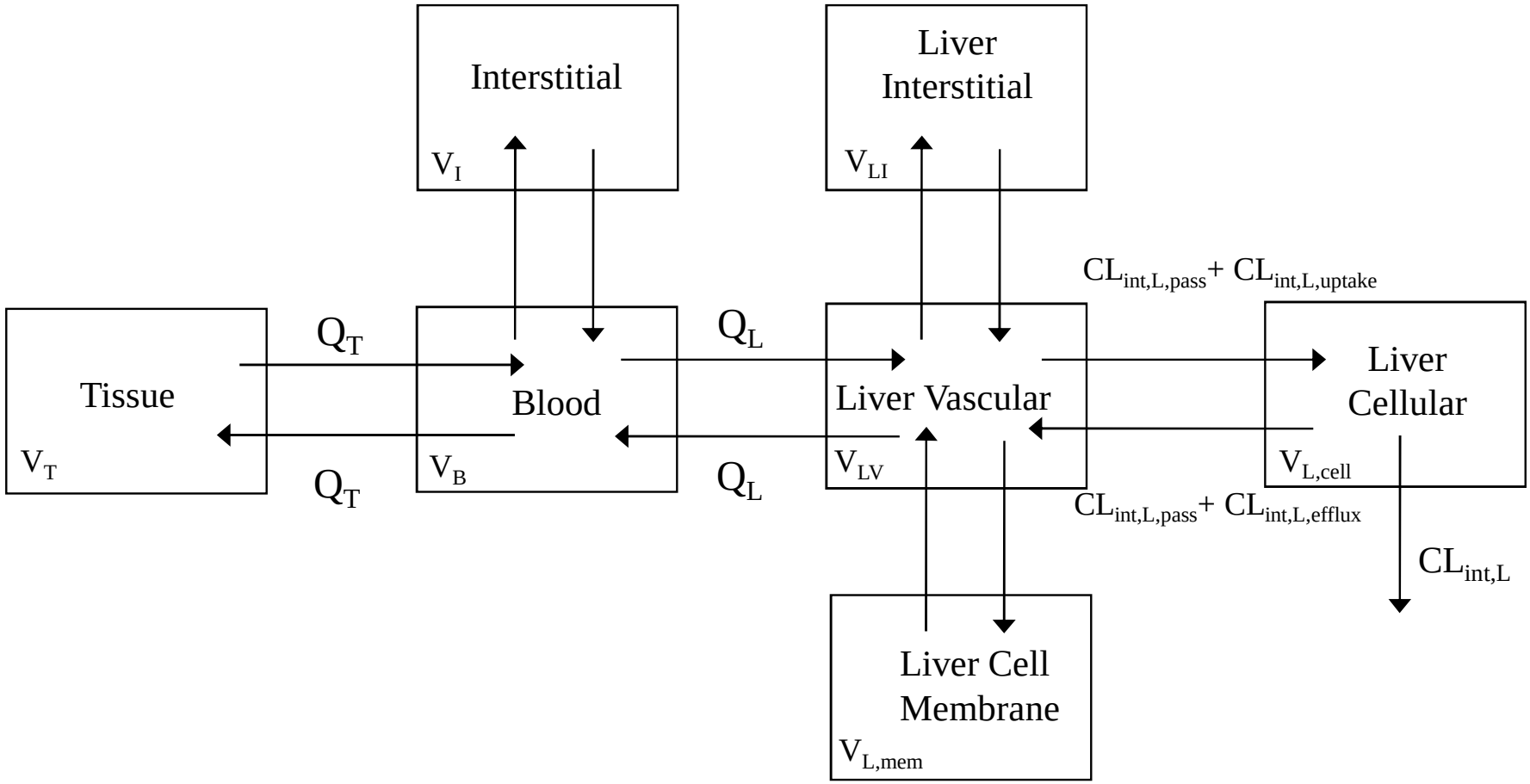


Figure2



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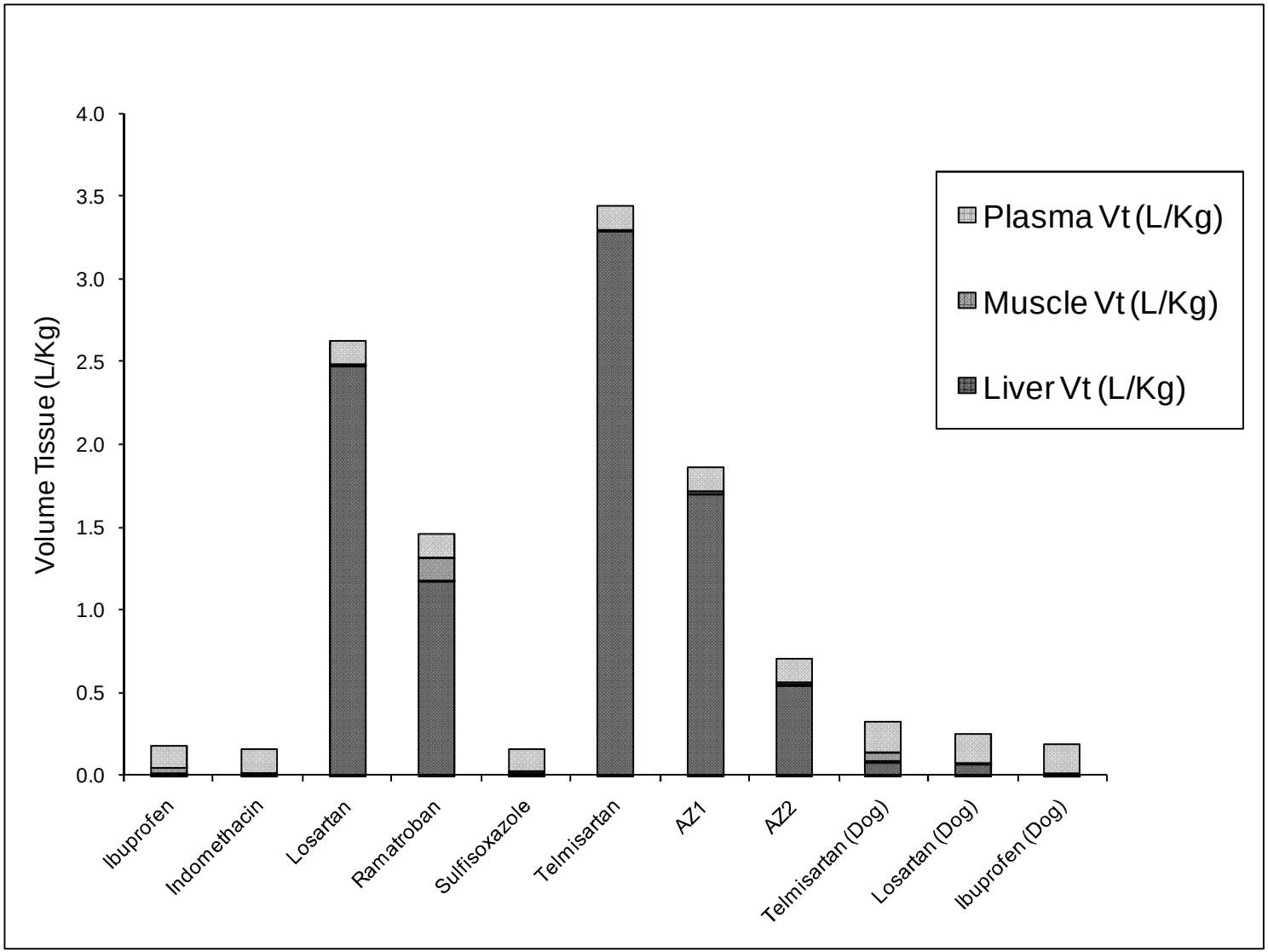


Figure 4

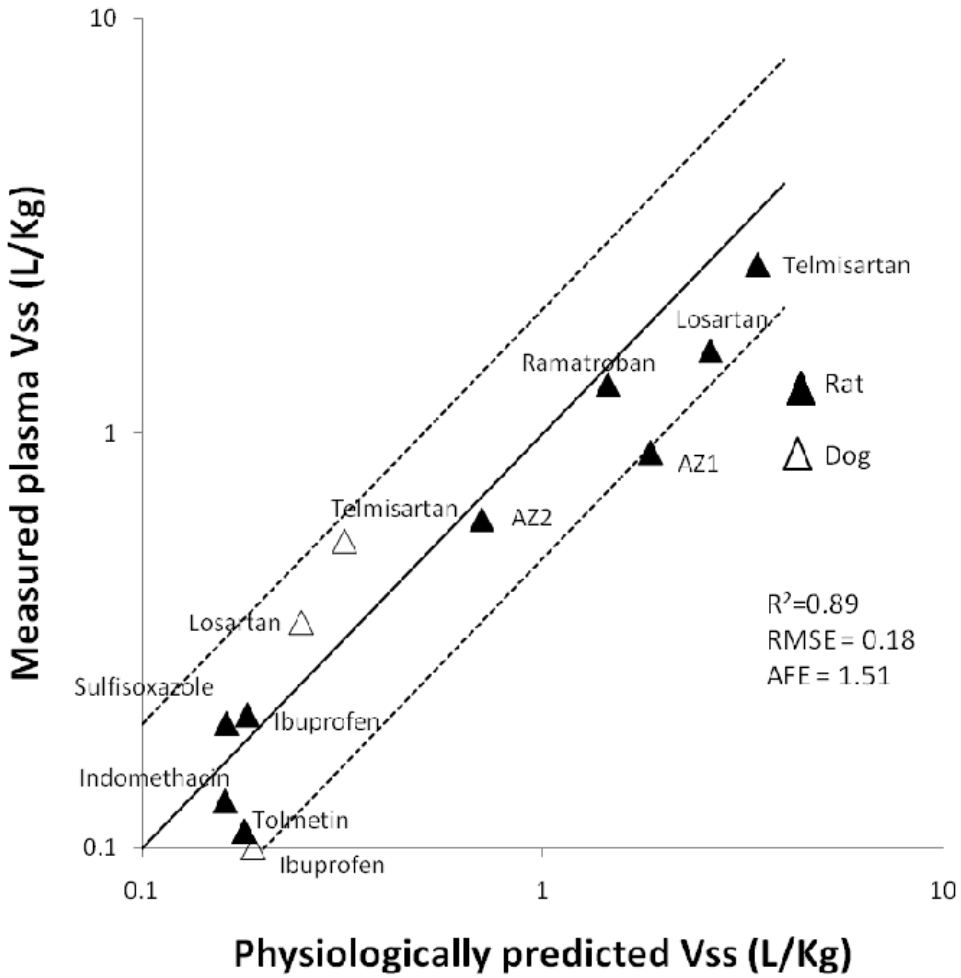
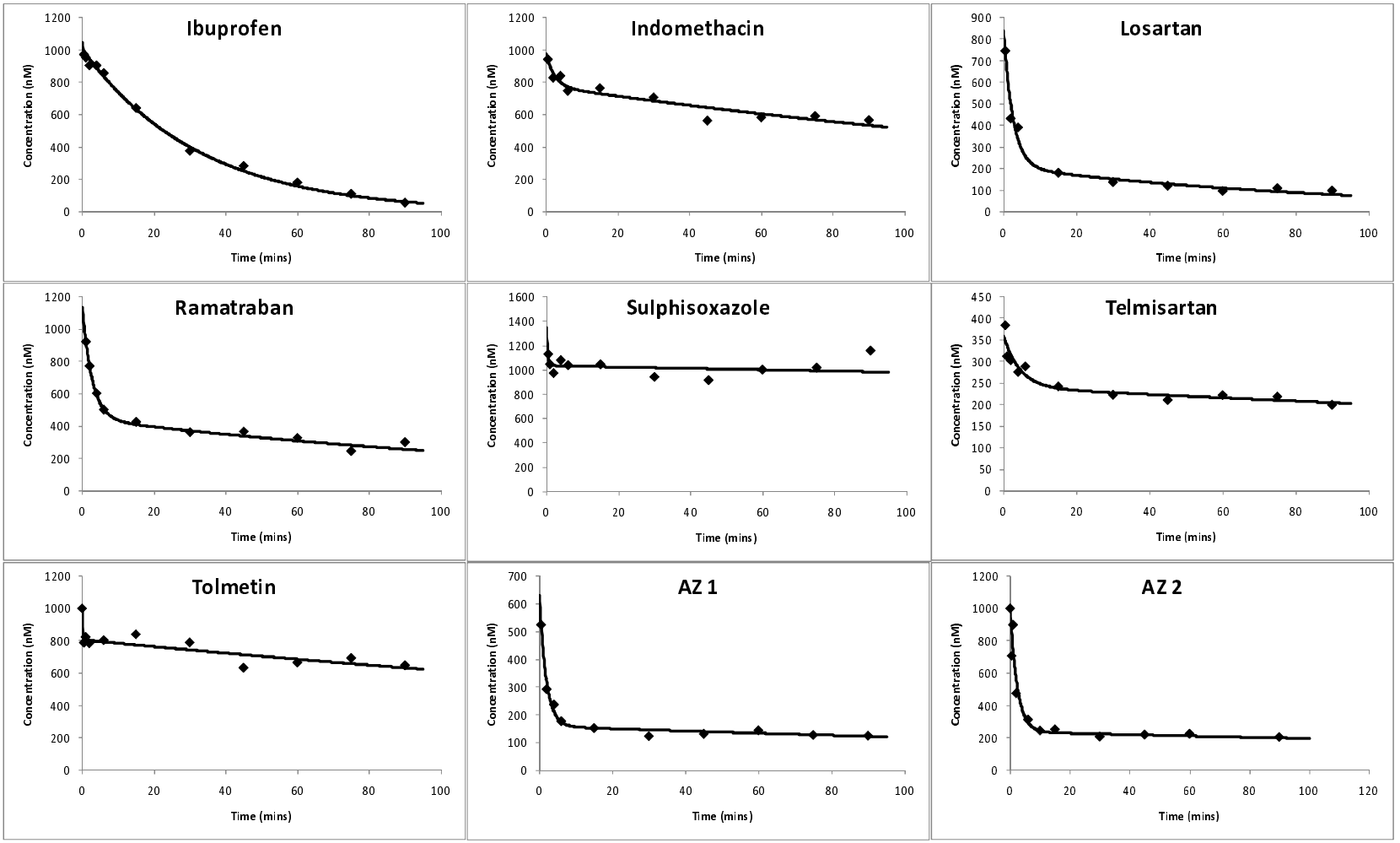


Figure 5



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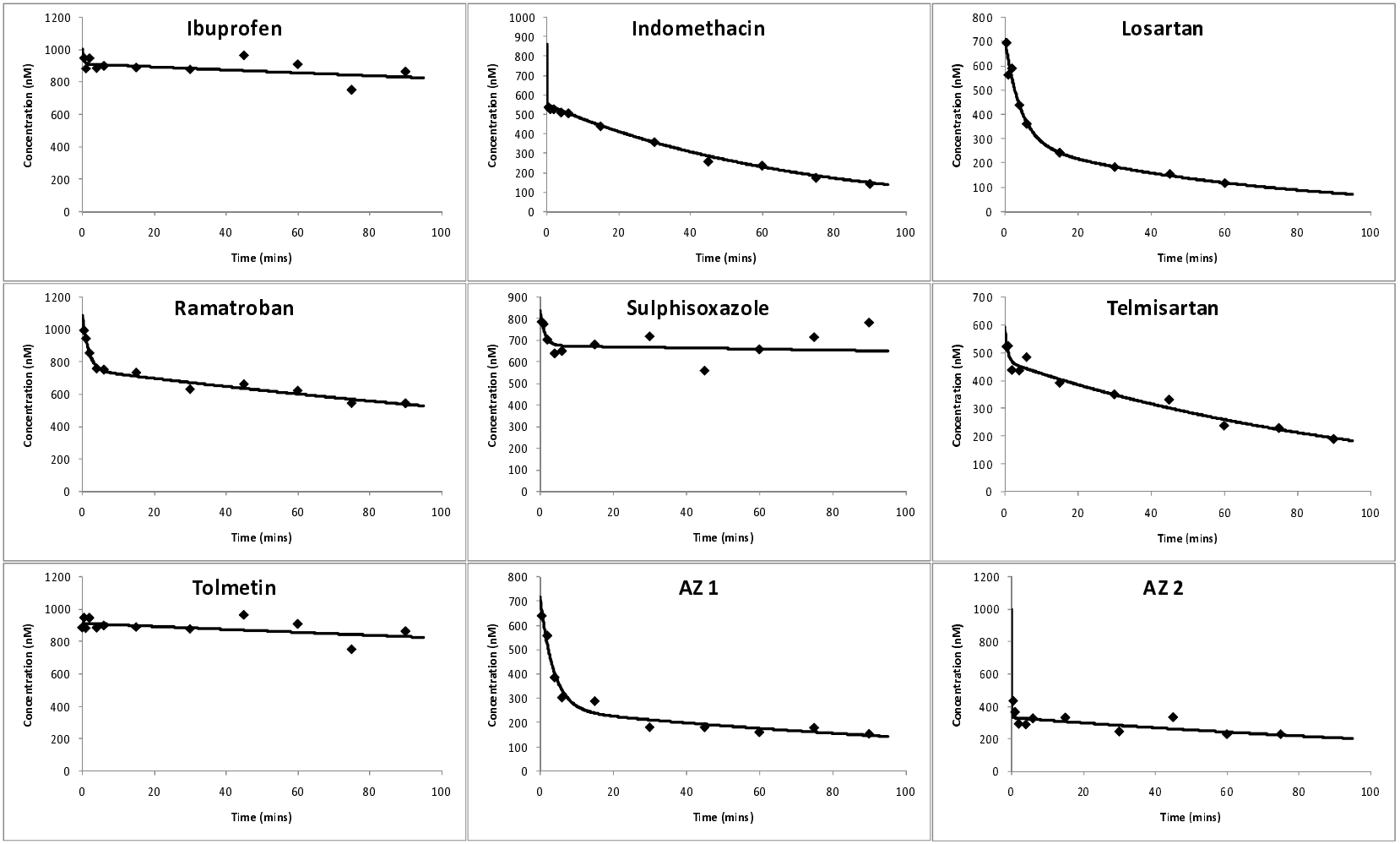

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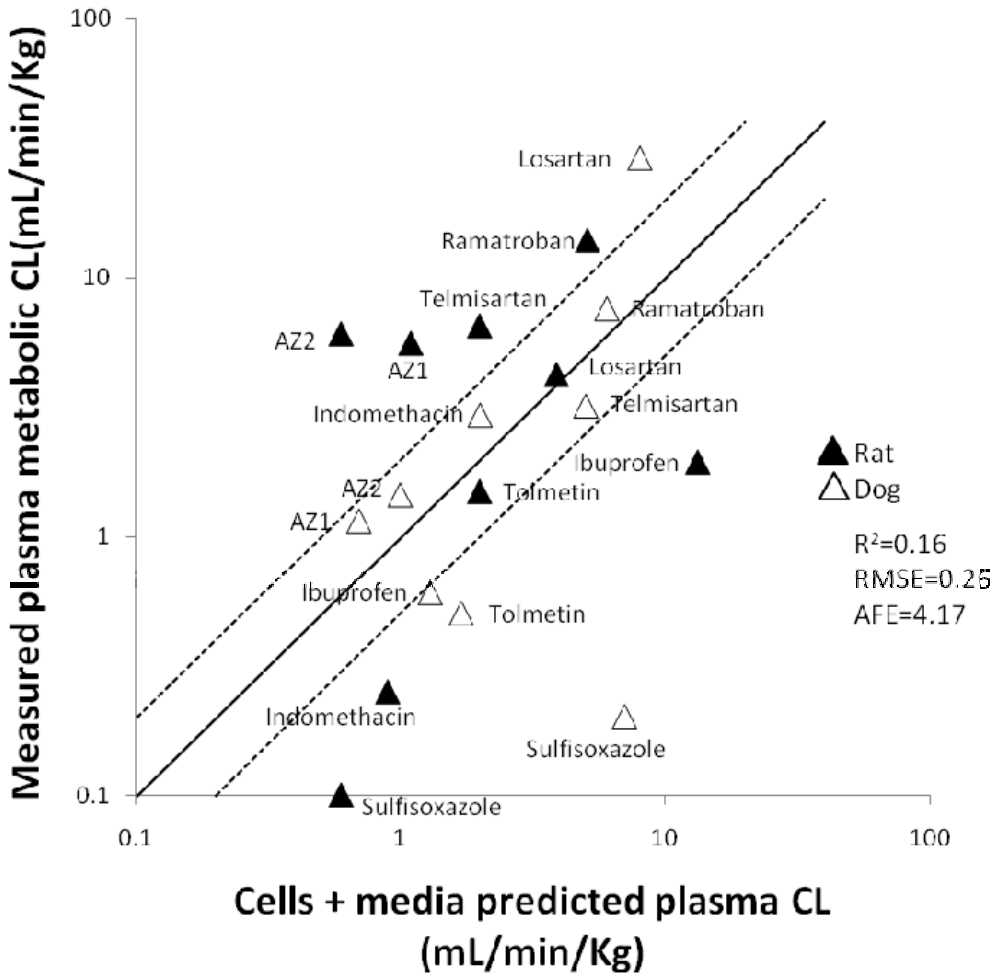

Figure 8

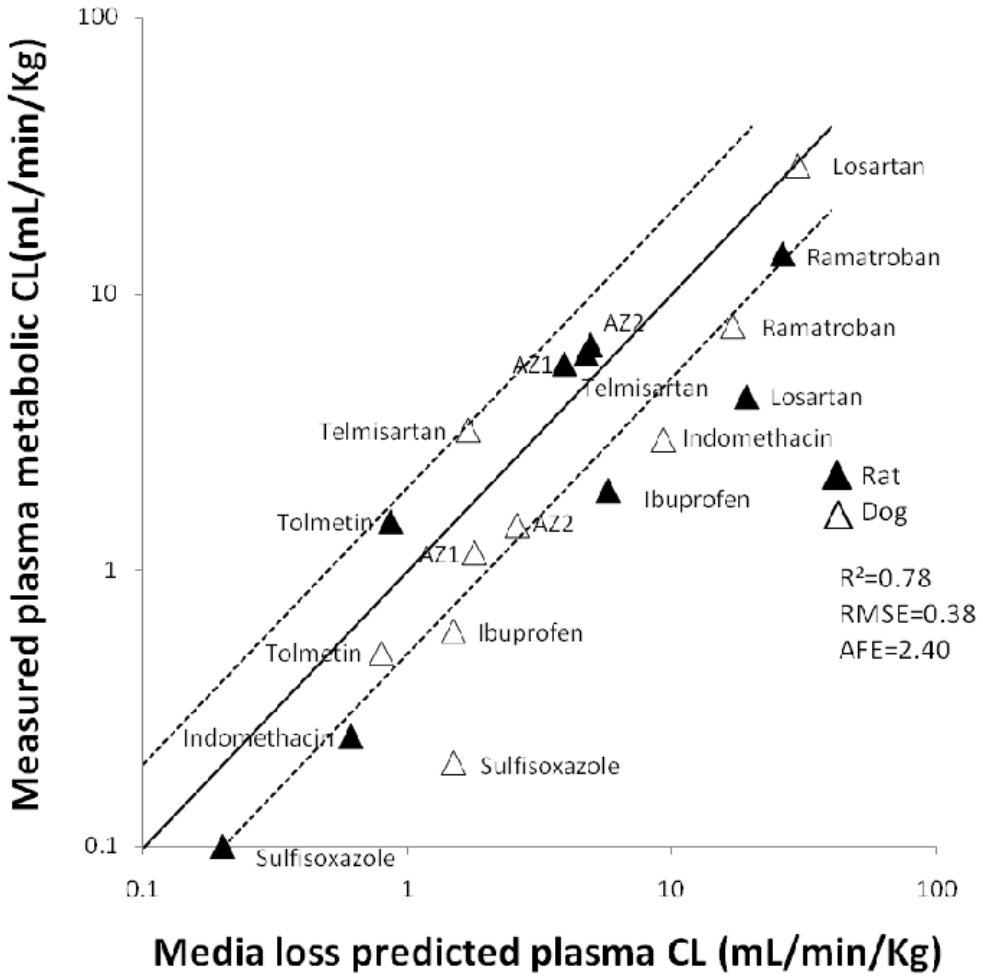

Figure 9