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Cytoplasmic binding and disposition kinetics of diclofenac in the isolated perfused rat liver

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1 The binding kinetics of diclofenac to hepatocellular structures were evaluated in the perfused rat liver using the multiple indicator dilution technique and a stochastic model of organ transit time density.

2 The single-pass, *in situ* rat liver preparation was perfused with buffer solution (containing 2% albumin) at 30 ml min^{-1}. Diclofenac and [^{14}C]-sucrose (extracellular reference) were injected simultaneously as a bolus dose into the portal vein (six experiments in three rats). An analogous series of experiments was performed with [^{14}C]-diclofenac and [^{3}H]-sucrose.

3 The diclofenac outflow data were analysed using three models of intracellular distribution kinetics, assuming (1) instantaneous distribution and binding (well-mixed model), (2) 'slow' binding at specific intracellular sites after instantaneous distribution throughout the cytosol (slow binding model), and (3) 'slowing' of cytoplasmic diffusion due to instantaneous binding (slow diffusion model).

4 The slow binding model provided the best description of the data. The rate constants for cellular influx and sequestration were 0.126 ± 0.026 and 0.013 ± 0.009 s^{-1}, respectively. The estimated ratio of cellular initial distribution volume to extracellular volume of 2.82 indicates an almost instantaneous distribution in the cellular water space, while the corresponding ratio of 5.54 estimated for the apparent tissue distribution volume suggests a relatively high hepatocellular binding. The non-instantaneous intracellular equilibration process was characterized by time constants of the binding and unbinding process of 53.8 and 49.5 s, respectively. The single-pass availability of diclofenac was 86%. The results obtained with [^{14}C]-diclofenac and [^{3}H]-sucrose were not statistically different.

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Abbreviations: CL_{BT}, membrane permeation clearance; d, relaxation time of the diffusional equilibration process; D_{eff}, effective diffusion coefficient; F, hepatic availability; f_{mob}, fraction of mobile particles in cytosol; k_{in}, k_{out}, influx and efflux rate constants; k_{on}, k_{off}, binding and unbinding rate constants; MSC, model selection criterion; Q, flow rate; RTD, residence time density; SB, slow binding; SD, slow diffusion; TTD, transit time density; τ, time constant corresponding to rate constant ($=1/k$); V_B, extracellular volume; V_C, initial distribution volume of the cellular phase ($v_c = V_C/V_B$); V_T apparent cellular distribution volume ($v = V_T/V_B$); WM, well-mixed

Introduction

The investigation of tissue binding in pharmacokinetic studies is generally limited to the estimation of steady-state parameters such as the apparent volume of distribution or partition coefficient, which provide no information on the binding kinetics (i.e., rate constants of binding and unbinding) of drugs. Most models of hepatic distribution and elimination kinetics of drugs are based on the assumption of an instantaneous cytoplasmic distribution equilibrium. However, this assumption is unrealistic in that binding during the diffusion process reduces the effective diffusion constant (Crank, 1975). Hence, instantaneous binding or immobilization of molecules at cellular binding sites contradicts the assumption that distribution throughout the cytoplasm is instantaneous. Conversely, a slow partition or binding process is inconsistent with an instantaneous equilibration at the cellular level. Only a limited number of studies have examined the assumption of non-instantaneous intracellular distribution in models of hepatic drug disposition. A slow binding process to sites in hepatocytes was required to adequately analyse the cytoplasmic distribution kinetics of L-lactate (Schwab, 1984), enalaprilat (Schwab *et al*., 1990), and acetaminophen (Pang *et al*., 1995) in the isolated perfused rat liver. While the 'intracellular two pool' or, as defined in the present study, 'slow binding' model implies a rapid distribution throughout the cytoplasm after cellular entry, a model representing slow radial hepatocellular diffusion with instantaneous binding is characterized by a concentration gradient in the liver cell (Weisiger, 1996). This approach was successfully used to analyse liver outflow data of triiodothyronin (Luxon & Weisiger, 1992) and fatty acids (Luxon, 1996; Luxon *et al*., 1998) after bolus input. Thus, the cytoplasmic distribution of a solute after cellular entry can be described with at least three modelling approaches: (i) a quasi-instantaneous tissue equilibration due to rapid diffusion and binding; (ii) a slowing of diffusion of the solute in the cell due to instantaneous binding to immobile cellular structures and mobile carrier proteins; (iii) a slow binding to sites in the cell after instantaneous distribution throughout the cytosol.

Model (i), also called barrier-limited tissue distribution, is conventionally used in most organ models of solute disposition such as the two-compartment dispersion model (Roberts *et al*.,

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1988; Yano *et al*., 1989). There is, however, increasing evidence that this conventional approach inadequately describes the tail part of outflow curves (Pang *et al*., 1995; Schwab *et al*., 1990; Luxon & Weisiger, 1992; Schwab, 1984; Tirona *et al*., 1998; Weiss *et al*., 1997). More realistic models, such as (ii) and (iii) described above, recognize that intracellular solute distribution is not instantaneous. One of us has recently used a theoretical analysis to show that these models may be indistinguishable on the basis of hepatic outflow curves alone (Weiss, 1999). Since the choice of an appropriate model has important consequences in terms of understanding drug disposition and as model discrimination is best for solutes with moderate partition coefficients and relatively low extraction ratios (Weiss, 1999), we studied the hepatocellular distribution processes of diclofenac using models (i)–(iii).

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) that is widely used as a therapeutic agent against rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and acute gouty arthritis. Diclofenac binds extensively to plasma proteins. The influence of plasma protein binding on hepatic disposition kinetics of diclofenac has been extensively studied in the isolated perfused rat liver using the two-compartment dispersion model (Evans *et al*., 1991; 1993; Hussein *et al*., 1993). In this model, the effects of catheters are convoluted with a dispersion model representation of intrasinusoidal distribution kinetics and solutes are assumed to be limited in their permeation from the sinusoid to the cytoplasm by the hepatocyte cell wall and the cytoplasm is assumed to be well-mixed. In this study, we use a more general model of hepatic distribution and elimination kinetics which accounts for both a non-instantaneous radial equilibration in the cellular phase after permeation across the hepatocyte membrane and the intrasinusoidal distribution kinetics at long times. The model is an extension of the stochastic two-phase model of organ transit time distributions (Weiss & Roberts, 1996). We sought therefore to apply this model and perfused whole-organ sucrose and diclofenac outflow profiles after bolus administration to define the hepatocellular distribution kinetics of diclofenac. This process enables intracellular diclofenac binding kinetics to be defined under more physiological conditions than possible by the classical *in vitro* approach (e.g., Barlow *et al*., 1994).

The main aim of this paper was to examine the hypothesis that the slow binding model (ii) is the most appropriate model to describe the disposition of diclofenac in the liver. The initial emphasis in this study was on defining diclofenac disposition using a specific and sensitive analytical method (HPLC) for unchanged diclofenac. In later studies, radiolabelled ^{14}C-diclofenac was administered and both bile and perfusate outflow collected to enable a monitoring of the metabolism and excretion of diclofenac and its metabolites. This study design allowed an independent assessment of diclofenac disposition kinetics since all previous experiments and data analysis were based on this method (Evans *et al*., 1993; Hussein *et al*., 1993).

Theory

The two-phase organ model (Weiss & Roberts, 1996), which describes vascular mixing, membrane permeation, and intracellular distribution, has been extended to describe liver disposition kinetics by recognizing the hepatocellular sequestration of solutes (Weiss *et al*., 1997; Weiss, 1999). Figure 1 shows the two phases in the model. Phase 1 represents the extracellular region (sinusoid and Disse space) which is surrounded by one plate of cells (phase 2). Drug transport takes place in phase 1 by convective blood flow and in phase 2 by intracellular distribution subsequent to cellular entry, i.e., transfer across the permeability barrier (plasma membrane). The model recognizes 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, transit time distributions (TTD), or residence time distributions (RTD). The model equations can be formulated in the Laplace domain, where $\hat{f}(s) = L\{f(t)\}$ denotes the Laplace transform of the function $f(t)$. The transit time density function $\hat{f}(s)$ of drug molecules across the liver is derived in terms of the extracellular transit time density of non-permeating reference molecules $\hat{f}_B(s)$, using in this study sucrose ($\hat{f}_{sucr}\}(s)$), and the density function of successive sojourn times $\hat{f}_y(s)$ of the drug molecules into the cellular space. Describing cellular uptake by the influx rate constant k_{in} from the sinusoid into the hepatocyte, the hepatic TTD is given by (Weiss & Roberts, 1996)

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

where $k_{in} = CL_{BC}/V_B$, CL_{BC} is the permeation clearance ($CL_{BC} = f_{uB}PS$), PS is the permeability-surface product, f_{uB} is free fraction in perfusate), and V_B is the extracellular volume. Although the density function $\hat{f}_B(s) = \hat{f}_{sucr}(s)$ characterizes the intravascular mixing kinetics, all information relevant to the intracellular pharmacokinetics (i.e., the cellular distribution and elimination processes of diclofenac) is defined by hepatocellular residence time density function $\hat{f}_y(s)$.

We therefore need to find an appropriate model to describe $\hat{f}_y(s)$ for diclofenac. Experimentally, the outflow concentration–time data of diclofenac is defined by equation (2):

$$C(t) = \frac{Dose}{Q} L^{-1}\{\hat{f}_{cath}(s)\hat{f}(s)\} \quad (2)$$

where Q is the perfusate flow rate and $\hat{f}_{cath}(s)$ denotes the impulse response of the catheter system. Solving equations (1) and (2) using empirical functions for $\hat{f}_{cath}(s)$ and $\hat{f}_B(s)$ ($=\hat{f}_{sucr}(s)$) yields the cellular distribution kinetic function $\hat{f}_y(s)$. We now consider the forms of this function corresponding to the various models of intracellular distribution kinetics (cellular RTD) being considered in this study.

Well-mixed model (WM)

The simplest model of cellular RTD is based on the assumption of quasi-instantaneous intracellular distribution

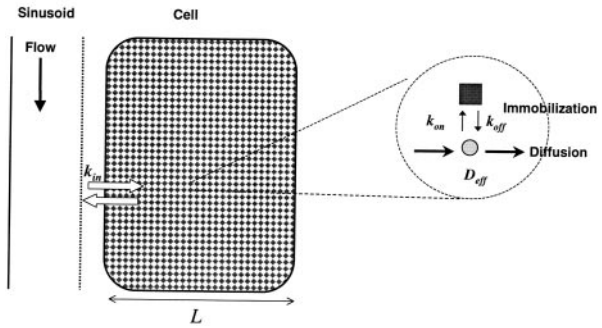

Figure 1 Schematic presentation of hepatocellular drug distribution. The well-mixed model (WM) assumes rapid diffusion ($D_{eff} \approx D_{water}$) and almost instantaneous binding. The slow binding model (SB) assumes rapid diffusion but 'slow' binding ($D_{eff}/L^2 >> k_{on}$) to various intracellular binding sites (which may be distributed heterogeneously). In the 'slow' diffusion model (SD) the apparent diffusion coefficient D_{eff} is reduced due to almost instantaneous immobilization ($D_{water}/L^2 << k_{on}$). Rapid diffusion in cell water is characterized by a distributional relaxation time of about 1 s ($\approx L^2/D_{water}$).

equilibrium, i.e., a cellular space which is well-mixed perpendicular to flow direction (Figure 1). The mathematical consequence of perfect mixing is a monoexponential cellular RTD (Weiss *et al*., 1997)

$$f_y(t) = k_{out} e^{-(k_{out}+k_e)t} \tag{3}$$

where k_{out} and k_e are the efflux and elimination rate constants. Note that the WM Model implies both instantaneous binding to intracellular components and instantaneous diffusional equilibrium. The apparent cellular distribution volume V_T then exceeds the accessible cellular water space of the drug and the efflux rate constant, $k_{out} = CL_{BC}/V_T$, is determined by the permeation clearance CL_{BC} and the distribution volume V_T. After Laplace transformation and reparameterization, one obtains from equation (3)

$$\hat{f}_y(s) = \frac{k_{in}/v}{k_{in}/v + k_e + s} \tag{4}$$

where $k_{out} = k_{in}/v$ and v denotes the fractional tissue distribution volume $v = V_T/V_B$. Note that with respect to the assumptions on cellular distribution kinetics the WM Model is identical with the two-compartment dispersion model (Roberts *et al*., 1988; Yano *et al*., 1989; Evans *et al*., 1993).

Slow binding model (SB)

This model assumes rapid intracellular diffusion but slow binding of molecules to immobile intracellular sites (Figure 1), with the consequence that the cytoplasmic equilibration process is determined by the binding and unbinding rate constants k_{on} and k_{off} following instantaneous distribution in volume V_c. The RTD of the corresponding two-compartment cell model can be readily obtained applying standard compartment theory (Wagner, 1975) and the definition of RTDs in linear systems (Weiss, 1991). Recalling that the cellular initial distribution volume V_c, plays the role of the central compartment and $(k_{out}+k_e)$ represent the irreversible loss from this compartment when f_y (t) characterizes the RTD of a molecule after a single excursion in the cellular space, we obtain

$$\hat{f}_y(s) = \frac{(s+k_{off})k_{out}}{(s+\lambda_1)\ (s+\lambda_2)} \tag{5}$$

$$\lambda_{1,2} = \frac{1}{2}\left(k_{on}+k_{off}+k_{out}+k_e \pm \sqrt{(k_{off}+k_{on}+k_{out}+k_e)^2 - 4k_{off}(k_{out}+k_e)}\right) \tag{6}$$

Assuming passive transmembrane transport processes, we have $k_{out}\ V_c = k_{in}V_B$, and in terms of normalized volume $v_c = V_c/V_B$, we can write $k_{out} = k_{in}/v_c$. The rate constants of solute binding and unbinding, k_{on} and k_{off} determine the apparent tissue distribution volume V_T which exceeds the initial volume V_c where quasi-instantaneous cytoplasmic diffusional equilibration occurs:

$$V_T = V_C(1 + k_{on}/k_{off}) \tag{7}$$

Note that the apparent initial distribution volume V_c can become larger than the accessible cellular water space of the drug V_w since 'quasi-instantaneous' distribution is not well-defined. In the fitting procedure, equations (6) and (7) are used in a reparameterized form to estimate v_c, v/v_c, k_{in}, k_{on} and k_c, substituting $k_{out} = k_{in}/v_c$ and $k_{off} = k_{on}/(v/v_c - 1)$ where $v = V_T/V_B$ is the ratio of the apparent cellular distribution volume to the extracellular volume.

Slow diffusion model (SD)

If the rate of immobilization is very large compared with the rate of diffusion ($1/k_{on} >> d = L^2/D$ in Figure 1), the equilibrium between the mobile and immobile concentration C_{mob} and C_{imo} is established almost instantaneously and the intracellular distribution process is governed by a diffusion process which is characterized by an apparently reduced diffusion coefficient (Crank, 1975); i.e., 'slow diffusion' means that binding (immobilization) reduces the diffusion coefficient by the factor $f_{mob} = C_{mob}/(C_{mob} + C_{imo})$, the fraction of mobile molecules. The cellular radial diffusion process can then be treated like an ordinary diffusion process in one dimension with a diffusion coefficient $D_{eff} = D_{mob}f_{mob}$ where D_{eff} denotes the apparent or effective diffusion coefficient of the binding molecule and D_{mob} is the diffusivity in cytoplasma of the corresponding mobile particle (Luxon & Weisiger, 1992; 1993; Crank, 1975; Weiss, 1999). The cellular RTD for the Model SD is obtained as

$$\hat{f}_y(s) = \frac{1}{1 + \frac{v}{k_{in}d}\sqrt{d(s+k_e)}\tanh\sqrt{d(s+k_e)}} \tag{8}$$

where $d = L^2/D_{eff}$ is the relaxation time of the diffusional equilibration process which is determined by the effective cytoplasmic diffusion constant D_{eff} and the cell diameter $L \approx 20\ \mu$m. (Note the equation (8), which is based on the two-phase organ model (Weiss & Roberts, 1996), has parallels to the model developed by Luxon & Weisiger (1992)).

Sensitivity analysis

A measure of the sensitivity of the impulse response function $C(t)$ to parameters are the sensitivity coefficients

$$S_{Pi}(t) = \frac{p_i}{C(t)}\frac{\partial C(t)}{\partial p_i} = \frac{p_i}{C(t)} L^{-1}\left[\frac{\partial \hat{C}(s)}{\partial p_i}\right] \tag{9}$$

which determine the relative change in $C(t)$ caused by a small change in the ith model parameter p_i. Substituting equations (5) and (6) into equation (2), the sensitivity functions were calculated for Model SB with the help of MAPLE 5 (Waterloo Maple Inc, Waterloo, ON, U.S.A.) after implementing a numerical method of inverse Laplace transformation (Schalla & Weiss, 1999).

Methods

Materials

Diclofenac-sodium, $CaCl_2 \cdot 2\ H_2O$, D-glucose, KCl, KH_2PO_4, $MgSO_4 \cdot 7\ H_2O$, MOPS (3-[N-Morpholino] propanesulphonic acid), NaCl, NaOH, and BSA were obtained from Sigma (Deisenhofen, Germany), sodium acetate anhydrous from Fluka (Neu-Ulm, Germany), and acetonitrile from E. Merck, Darmstadt, Germany. [^{14}C]-diclofenac was purchased from E. Kocher (Germany); [^{14}C]-and [^{3}H]-sucrose were obtained from Amersham (U.K.). Phosphoric acid (85%) was produced by Laborchemie, Apolda, Germany. Xylazine came from Bayer Australia (Pymble, Australia), heparin-sodium from David Bull Laboratories (Australia), and ketamin-hydrochloride from Parnell Laboratories Pty. (Alexandria, Australia).

Liver perfusion experiments

The isolated perfused liver system was essentially that described previously (Cheung *et al*, 1996; Waynforth & Flecknell, 1992; Wolkoff *et al*., 1987). Wistar rats (230–480 g), fed on a normal laboratory diet and free access to water, were used. In brief, the rats, under anaesthesia (peritoneal injection of xylazine 10 mg kg^{-1} and ketamin-hydrochloride 80 mg kg^{-1}), were heparinized (500 Uml^{-1}) *via* the inferior vena cava. The portal vein and thoracic inferior vena cava were cannulated with polyethylene tubing (about 7 cm in length), using a 16-gauge intravenous catheter (16G, i.d. 1.3×32 mm; Terumo Medical Corporation, Elkton, U.S.A.). The animal was placed in a temperature controlled perfusion cabinet at 37°C. Oxygen consumption, bile flow, perfusion pressure, and macroscopic appearance assessed liver viability (bile flow: 840 ± 55 $\mu g\ min^{-1}$; oxygen consumption: 1.9 ± 0.2 $\mu mol\ min^{-1}$).

The perfusion medium consisted of MOPS buffer (pH 7.4), 2% bovine serum albumin (BSA), 0.2% D-glucose and was saturated with 95% O_2/5% CO_2 (Cheung *et al*., 1996). After a stabilization period of 10 min at a perfusate flow of 30 ml min^{-1}, 50 μl of perfusate solution, containing diclofenac (0.53 mg) and [^{14}C]-sucrose (2 μCi), were injected into the tubing (about 7 cm proximal to the portal vein cannula). The total hepatic effluent was collected by using a fraction-collector at 1-s intervals (for the first 30 s) and 2.5-s intervals (31–150 s), respectively. Two experiments were performed in each rat ($n=3$). An analogous series of experiments ($n=3$) were performed injecting a mixture of [^{3}H]-sucrose (3×10^6 d.p.m.) and [^{14}C]-diclofenac (1.5×10^6 d.p.m.). Bile was collected for 10 min for each bolus after injection.

MOPS buffer

Solution A: 138.98 g NaCl, 7.1 g KCl and 3.24 g KH_2PO_4 were solved in 2000 ml distilled water. Solution B: 11.6 g $MgSO_4 \cdot 7\ H_2O$, solved in 1000 ml distilled water, and solution C consisted of 18.74 g $CaCl_2 \cdot 2\ H_2O$ (solved in 50 ml distilled water). 206 ml A, 53 ml B and 1 ml C were mixed together and filled up with distilled water to 1000 ml. 10.46 g MOPS and 4 g D-glucose were added and filled up with distilled water to 2000 ml. With 5 M NaOH the pH was adjusted to 7.4. After adding 40 g BSA the pH was controlled and readjusted to 7.4. Finally, the perfusate was filtered (Whatman 541, Whatman Int., Maidstone, U.K.) and used within 2 or 3 days (stored at 4°C). The solutions A, B and C were stored at 4°C for weeks until use.

Assay procedure

The concentration of diclofenac was measured by high-performance liquid chromatography (HPLC) as described previously (Kuhlmann & Krauss, 1997). The samples were stored until analysis at −20°C. For HPLC determination, 100 μl of perfusate were deproteinized with 100 μl acetonitrile. After centrifugation for 5 min at $100 \times g$, the supernatant was injected onto the column. The [^{14}C]- and [^{3}H]-sucrose and [^{14}C]-diclofenac samples were taken for scintillation counting using a MINAXI beta TRI-CARB 4000 series liquid scintillation counter (Packard Instruments Co., U.S.A.).

Data analysis

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 applying the non-linear regression program SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT, U.S.A.). Data were analysed by a sequential procedure: First, the fractional outflow curve $C_{sucr}(t)$ of the extracellular marker [^{14}C]-sucrose was fitted by equation (10), whereby $\hat{f}_{cath}(s)$ accounts for the catheter and large vessel transit time

$$C_{sucr}(t) = \frac{Dose}{Q} L^{-1}\{\hat{f}_{cath}(s)\hat{f}_B(s)\} \tag{10}$$

and the TTD of the non-permeating indicator is given by

$$\hat{f}_B(s) = p\hat{f}_1(s) + (1-p)\hat{f}_2(s) \tag{11}$$

with

$$\hat{f}_i(s) = exp\left\{ \frac{1}{CV_i^2} - \left[\frac{MT_i}{CV_i^2/2} \left(s + \frac{1}{2MT_iCV_i^2} \right) \right]^{1/2} \right\} \tag{12}$$

Equation 12 is the Laplace transform of the inverse Gaussian density function with mean MT_i and relative dispersion CV_i^2 ($i=1$, 2). [It was shown previously, that equations (10) to (12) adequately describe the TTD of vascular markers in perfused rat livers (Weiss *et al*., 1997; 1998; Weiss, 1997).] Second, utilizing this information the outflow concentration data of the permeating drug diclofenac, $C(t)$, were analysed, i.e., the parameters MT_i, CV_i^2 ($i=1$, 2), p, and V_B of the individual fits are substituted as fixed parameters in $\hat{f}_B(s)$ of the diclofenac model (equation (1)). Equation (2) is fitted to the diclofenac data using $\hat{f}_y$ (s) in $f(s)$ according to the cellular distribution model: Equation (4) for the WM, equation (5) for the SB, and equation (8) for the SD Model, respectively.

The TTD of the extracellular indicator $\hat{f}_B$ (s)$=\hat{f}_{sucr}(s)$ accounts for the effect of intrasinusoidal mixing in the model of hepatic diclofenac disposition. The mean transit time of the extracellular reference, $MTT_B = \int_0^\infty t f_B(t)dt$, is given by

$$MTT_B = pMT_1 + (1-p)MT_2 \tag{13}$$

Note that MTT_B is a model-independent parameter given by $MTT_B = V_B/Q$, where the volume, accessible to sucrose, is the sum of the sinusoidal plasma space and the Disse space, $V_B = V_{Plasma} + V_{Disse}$, and Q denotes the flow rate.

The hepatic availability F ($F=1-E$, where E denotes the single-pass extraction ratio) is obtained from equations (10) and (5) (Model SB) or (8) (Model SD), respectively, for $s \to 0$.

Statistical analysis

All results are presented as mean ± s.d. The model selection criterion (MSC) provided by SCIENTIST, a modified Akaike Information Criterion (normalized to the number and scaling of data points), is used to compare the models with regard to 'goodness of fit'; the most appropriate model – from a pure statistical point of view – is that with the largest MSC. Information on parameter statistics was obtained from the approximate coefficients of variation of each estimate and the correlation matrix. A high coefficient of variation and/or correlation between parameters suggest unreliable estimates.

Results

Model discrimination was based on the outflow concentration-time profiles for unchanged diclofenac analysed by HPLC after bolus injection with [^{14}C]-sucrose co-administered as an extracellular reference solute. Figure 2 shows a typical outflow concentration-time profile for the [^{14}C]-sucrose and its fit using a mixture of two inverse Gaussian functions [equations (11) and (12)], used as an empirical model of the intrasinusoidal distribution process. Also included in Figure 2 are the diclofenac data collected at the same time as the sucrose data. It is apparent that whilst the curves are similar initially, a much slower decline of the diclofenac curve is evident after about 15 s. Figure 3A shows the fit of an outflow curve of diclofenac from a representative liver by the slow binding (SB) and well-mixed (WM) models, respectively. It is apparent that Model SB perfectly fitted the data while Model WM failed to describe the tail part of the curve. A difference in the fits obtained by the SD and SB models was only visible in the early portion of the outflow curve where Model SD underestimated the peak (Figure 3B). Table 1 summarizes the estimated and derived parameters for all three models. The MSC criterion confirmed the inability of Model WM in describing the data. There are apparent systematic deviations of Model SD in the small peak region of the curve (Figure 3B). However, the poor estimation of model parameters is probably of greater concern than the quality of the 'goodness of fit'. The parameters v and d are 'poorly determined' as evidenced by a strong parameter interaction (as indicated by relatively high values of the correlation coefficients (>0.95) between v and d), these parameters and the high approximate coefficients of variations of d (generally $>50\%$). The estimation of the ratio d/v instead of d also resulted in imprecise and highly correlated estimates. Thus, our results suggest that Model SB produces both the best goodness of fit to experimental data, appropriate coefficients of variation for the individual parameter estimates v_c, v/v_c, k_{in}, k_{on}, and k_e and an approximate correlation matrix, in which the off-diagonal elements were less than 10% and 0.8, respectively. Figure 4 shows the effect of model parameter

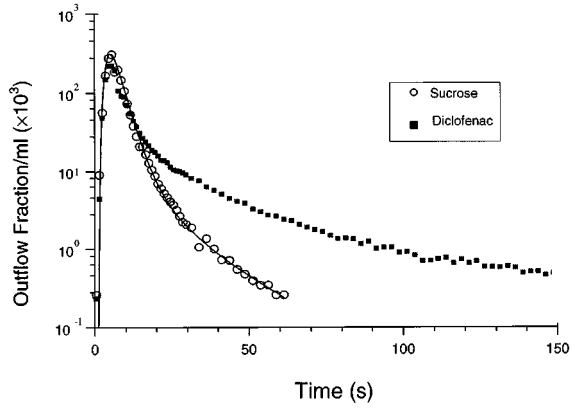

Figure 2 Typical [^{14}C]-sucrose outflow profile with the fit obtained by equations (10) to (12) shown together with the corresponding diclofenac data collected simultaneously.

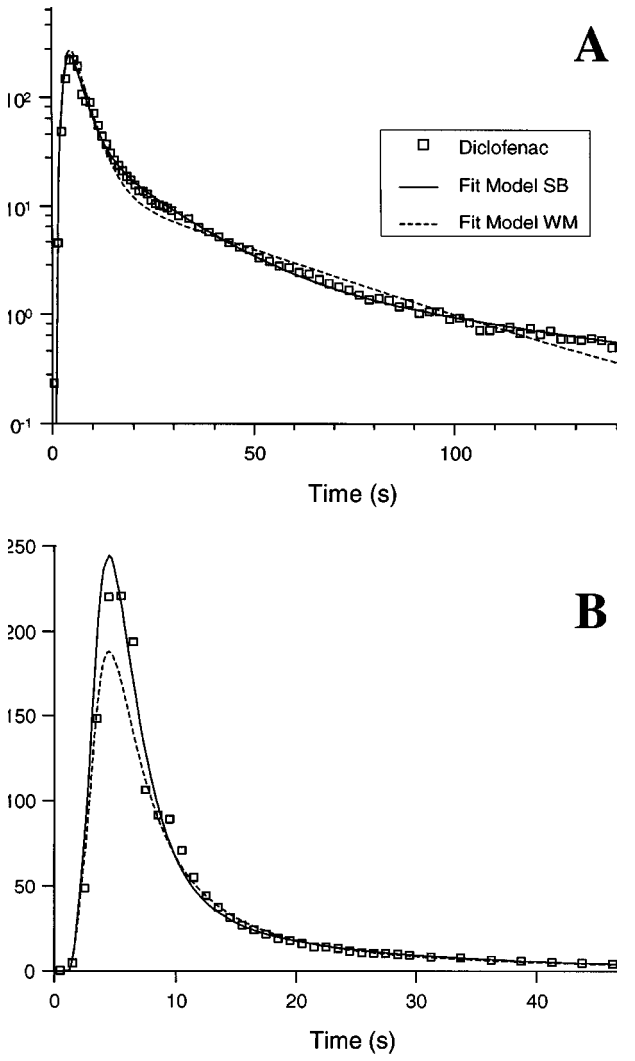

Figure 3 Comparison of experimental data and fitted diclofenac outflow curve in rat 1 for Model SB and Model WM (A). The early differences between Model SB and Model SD are only visible in a linear scale (B).

Table 1 Estimated and derived parameters by the stochastic two-phase liver model using three models of cytoplasmic distribution kinetics

Model	k (s^{-1})	v_c	v/v_c	k_{on} (s^{-1})	v	k_e (s^{-1})	d (s)	V_C (ml)	V_T (ml)	CL_{BC} (ml s^{-1})	F	MSC
WM[a]	0.088				3.20	0.008			10.4	0.284	0.89	2.71
	(0.058)				(2.79)	(0.009)			(9.2)	(0.179)	(0.11)	(0.31)
SD[a]	0.280				24.47	0.008	372.8		78.61	0.762	0.70	3.44
	(0.080)				(13.63)	(0.003)	(119.2)		(41.93)	(0.459)	(0.16)	(0.51)
SB[a]	0.126	2.82	1.95	0.020	5.54	0.013		8.94	17.55	0.395	0.86	3.48
	(0.026)	(1.55)	(0.07)	(0.007)	(3.15)	(0.009)		(5.11)	(10.32)	(0.091)	(0.11)	(0.42)
SB[b]	0.190	3.36	2.06	0.116	7.03	0.022		11.82	25.29	0.561	0.76	3.41
	(0.107)	(0.56)	(0.40)	(0.078)	(2.23)	(0.007)		(5.62)	(14.63)	(0.154)	(0.07)	(0.34)

[a]From, diclofenac outflow data after bolus input: mean ($\pm$s.d.); $n=3$, with two experiments in each rat. [b]From total [^{14}C]-radioactivity outflow data after [^{14}C]-diclofenac input: mean ($\pm$s.d.); $n=3$, with two experiments in each rat.

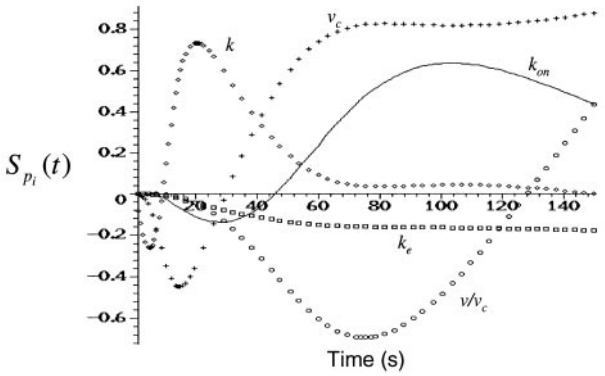

Figure 4 Normalized model sensitivities for Model SB based on the means of estimated parameter values. The sensitivity function $S_{p_i}(t)$ describes the relative change in the outflow curve predicted by the model in response to a change in the value of parameter p_i.

variations on the outflow concentration – time curve as defined by equation 9. It is apparent that there is a high sensitivity with respect to k in the early phase and an increasing sensitivity to v/v_c in the terminal phase.

The fractional cellular initial distribution volume of diclofenac, $V_C/V_B = 2.82 \pm 1.55$, is in good agreement with the value of the fractional intracellular water space of 3.0 reported by Pang *et al*. (1995). Consequently, the ratio of the apparent distribution volume V_T to V_C, $V_T/V_C = 1.95 \pm 0.07$, can be interpreted as cellular equilibrium partition coefficient. Together with the estimate of the rate constant k_{on}, this leads to time constants ($\tau = 1/k$) of cellular binding and unbinding processes of $\tau_{on} = 53.8 \pm 16.6$ s and $\tau_{off} = 49.5 \pm 13.0$ s, respectively. A value of $CL_{BC} = 0.395 \pm 0.091$ ml s^{-1} was derived for the permeation clearance or permeability surface area product ($CL_{BC} = f_{uB}PS$). The elimination rate constant of $k_e = 0.013 \pm 0.009$ s^{-1} corresponds to an intrinsic clearance ($CL_{int} = k_e \ V_C$) of 0.15 ± 0.15 ml s^{-1}. The model-derived hepatic availability of $F = 0.86 \pm 0.11$ is slightly lower than the value of 0.93 ± 0.13 estimated by numerical integration using mono-exponential curve extrapolation.

The outflow radioactivity data following bolus injection of [^{14}C]-diclofenac (with [^{3}H]-sucrose as extracellular reference) could also adequately be described by Model SB and the parameter estimates were not significantly different from those obtained in the experiments with cold diclofenac (Table 1). Since a contribution of radioactive metabolites cannot be excluded (despite the relatively low extraction of diclofenac), the data were not used for model discrimination. A dose fraction of $0.34 \pm 0.07\%$ was recovered in the bile within 10 min after injection (as unchanged drug and/or generated metabolites) corresponding to 2.4% of the drug eliminated by the liver (for $E = 14\%$).

Discussion

The present experiments were designed to test three models of hepatocellular distribution kinetics for diclofenac. The suitability of these models has been examined using the three principal criteria for a satisfactory model. The first is that the form of the experimental response should be satisfactorily represented by the theoretical response, as evidenced by a satisfactory 'goodness of fit'. However, whilst 'goodness of fit' is necessary, it is not a sufficient criterion for the validity of a model. The second requirement is the consistency of parameter estimates (model identifiability) as reflected, for example, by reasonable coefficients of variation of the individual estimates and sufficiently low correlation coefficients between parameters. The third criterion is that the results should be in accordance with the *a priori* knowledge of the underlying physical process.

Model WM was clearly inadequate for describing hepatocellular distribution kinetics of diclofenac. A strong argument supporting the validity of Model SB (slow binding) is the finding that the estimated fractional volume $v_c = V_C/V_B$, which characterizes instantaneous cytoplasmic distribution of diclofenac, is in good agreement with the value of the fractional intracellular water volume reported by Pang *et al*. (1995). The fractional distribution volume $v = V_T/V_B$ estimated by Model WM is smaller than that estimated by Model SB (3.20 vs 5.54), as a consequence of its failure to adequately fit the tail of the outflow curve. Since Models SB and SD fitted the data equally well, steady-state parameter estimates should theoretically be independent of the underlying model of distribution dynamics. However, Model SD yielded an unexpectedly high value of $v = 24.5$, probably due to an 'instability' in the ability of the estimation procedure to identify some parameters as a result, for instance, of a high correlation between v and d. A comparison of the current parameter estimates of Model WM with the results obtained previously using the two-compartment dispersion model (Evans *et al*., 1993; Hussein *et al*., 1993) cannot readily be made as the tail of the outflow concentration-time profile was not adequately described in that model. We have shown that a single inverse Gaussian density function (as implied by the dispersion model) is not flexible enough to describe the tail of outflow concentration-time profiles of extracellular indicators (Weiss *et al*., 1997). The consequences of this model misspecification (i.e., using a single instead of a double inverse Gaussian function for $f_B(t)$ in equation (11)) have been discussed in detail elsewhere (Weiss *et al*., 1997; Tirona *et al*., 1998). For a flow rate of 15 ml min^{-1} and 2.5% albumin in perfusate values, $F = 93\%$ and $k_e = 0.0042$ s^{-1} are reported by Evans *et al*. (1993). While the model-dependent k_e estimates are not directly comparable, a ratio CL_{BT}/CL_{int} between 4 and 8 observed by Evans *et al*. (1993) is in agreement with our estimate of 6.9. Note that their experimental design was inappropriate to detect the long tail of the diclofenac outflow curve since data truncation at 100 s for $Q = 15$ ml min^{-1} corresponds to a truncation at 50 s in our case ($Q = 30$ ml min^{-1}). This may have led to an underestimation of the apparent hepatic distribution volume ($v = 2.7$ vs 3.2 estimated by Model WM).

The sensitivity analysis for Model SB (Figure 4) shows the time intervals during the experiment, which provide the best information for defining a particular parameter. Permeability (k_{in}) is best defined by data below about 50 s, with less available information on intracellular distribution kinetics (v_c, v/v_c, and k_{on}) and on elimination (k_e). The relative sensitivity for k_e remains comparably low. That sensitivity functions do not change proportionally (or inversely proportionally) suggests that the parameters are not highly correlated.

It should be emphasized that our support for Model SB, in which the cellular distribution kinetics of diclofenac is characterized by instantaneous distribution in cell water followed by slow binding to immobile cellular components, is heuristic rather than rigorous. Model SD fitted the data almost equally well (with exception of the early peak). The third criterion states that the results should be in accordance with the *a priori* knowledge of the underlying physical process. Evidence supporting this criterion would be an independent confirmation (e.g., *in vitro* studies) of the time constants of binding and unbinding processes for diclofenac in the liver cell

(τ_{on} = 53.8 and τ_{off} = 49.5 s). It should be noted that the values for τ_{on} and τ_{off} are similar to the values of 66.7 and 42.7 s reported by Schwab *et al*. (1990) for enalaprilat. One might argue that a hybrid model (slow binding and slow diffusion) should appear most appropriate since Models SB and SD present opposite extremes with regard to binding dynamics. However, in addition to problems arising from its mathematical complexity, such a model would be hardly identifiable (and probably indistinguishable) from the two limiting cases (SB and SD) on the basis of the given data. Thus, the principle of parsimony is in favour of Model SB. That the binding sites are probably heterogeneously rather than homogeneously distributed in the cellular space is another argument for Model SB; both the hybrid and diffusion (SB) model assume homogeneity as depicted in Figure 1, while no assumptions on the location of binding sites are made with Model SB.

Initial emphasis was given in this study to the analysis of diclofenac disposition in the liver using a specific and sensitive analytical (HPLC) method. In later studies, a number of additional experiments were conducted by administering radiolabelled diclofenac and collecting total radioactivity outflow data as described previously by Evans *et al*. (1993). The data were best fitted with model SB, supporting its validity. In addition, the parameter estimates from model SB were not significantly different from those for the studies measuring unchanged diclofenac (Table 1), suggesting that the contribution of metabolites is negligible (Evans *et al*., 1993). The low measured fraction of total radioactivity due to diclofenac and its metabolites in bile and the minimal recovery of diclofenac metabolites in the perfusate suggest that the hepatocellular kinetic events account for the results in this work. However, due to the relatively short sampling period of 10 min, the measured fraction of radioactivity excreted in the bile and recovered in the perfusate may not be representative for the total fraction that would be eventually recovered.

The present analysis, which overcomes the logical inconsistencies of the well-stirred cell model for drugs with cellular binding, emphasizes the need to examine the intracellular distribution kinetics of diclofenac and other solutes. We also showed that a misspecification of the cytoplasmic distribution model leads to biased parameter estimates. It is suggested that the underestimation of the terminal portion of venous outflow curves observed in previous studies (e.g., for salicylic acid (Hussein *et al*., 1994) and diazepam (Diaz-Garzia *et al*., 1992)) arises, in part, as a consequence of assuming a well-stirred model of hepatocellular distribution applied in these studies.

The knowledge obtained on the hepatocellular binding kinetics of diclofenac may also assist in better understanding the relationship between the reversible binding of diclofenac to cellular membranes and its uncoupling effect on oxidative phosphorylation (Petrescu & Tarba, 1997). Such a relationship has also been suggested in relation to the hepatocellular binding observed for the NSAID niflumic acid in single-pass liver perfusion studies (Kelmer-Bracht *et al*., 1993). In analysing diclofenac competition for triiodothyronine-binding sites, Barlow *et al*. (1994) found a 500 fold greater relative potency for cytosolic binding than for nuclear binding. In this context, a $K_{eq} = V_T/V_c$ of 1.95 and a $v = V_T/V_B$ of 5.54 indicate a relatively high hepatocellular binding, especially as diclofenac also shows a strong competitive extracellular binding to albumin, as evidenced by 99.7% of diclofenac being bound in perfusate with an albumin concentration of 2.5% (Hussein *et al*., 1993).

In conclusion, the present study suggests that the hepatic distribution kinetics of diclofenac is characterized by transmembrane exchange (permeability barrier) and the intracellular binding process. The model development also indicates that further efforts to analyse cellular transport processes of drugs are warranted.

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