Influence of Age on the Disposition and Renal Handling of Enprofylline in Rats

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Abstract—The effects of ageing on the pharmacokinetics, renal handling and protein binding of enprofylline were investigated in 6-, 13- and 18-month-old male Fischer 344 rats. Concentrations of enprofylline in plasma and urine were determined by HPLC, and pharmacokinetic parameters were estimated by model-independent methods. No significant differences in the volume of distribution, systemic clearance of enprofylline or urinary recovery of unchanged enprofylline ($>85\%$) were observed among any of the groups of rats. The dissociation constant and free fatty acid concentration in plasma increased with age. Age-dependent decreases in the systemic clearance for unbound drug were observed, and the volume of distribution for unbound drug tended to decrease with age. The ratio of systemic clearance for unbound drug to the glomerular filtration rate (GFR) decreased with ageing. Ageing was associated with decreases in the apparent maximum capacity of transport (V_{max}) (223·33, 160·24 and 142·98 μg min^{-1} kg^{-1} for 6-, 13- and 18-month-old rats, respectively) and in the tubular secretory intrinsic clearance (V_{max}/K_m) of enprofylline (75·45, 51·03 and 44·13 mL min^{-1} kg^{-1}, respectively), while a slight change in the Michaelis–Menten constant (K_m) was observed. These results indicate that the mechanism responsible for age-related changes in the disposition and renal handling of enprofylline may be responsible for a decrease in the ability of the tubular anion transport system.

Certain physiological and biochemical parameters, including glomerular filtration, hepatic and renal plasma flow, albumin concentration in plasma, and hepatic metabolism, gradually decrease with age, in both man and animals. Age-related changes in drug disposition also occur in both man (Vestal et al 1979; Vestal & Wood 1980; Greenblatt et al 1982; Barbhaiya et al 1992) and animals (Coleman et al 1977; Tsuji et al 1985; van Bezooijen & van Oorschot 1989; Belpaire et al 1990; Satterwhite & Boudinot 1991).

Enprofylline, a new bronchodilator proven to be a more potent relaxant than theophylline, can be substituted for theophylline in the treatment of asthma (Persson & Kjellin 1981). Theophylline is extensively metabolized in the liver, whereas the metabolism of enprofylline has been shown to be minimal, and the drug is primarily excreted into the urine by glomerular filtration and an active tubular secretion mechanism in both man (Borgå et al 1986) and animals (Apichartpichean et al 1991). Previous studies in our laboratories have also shown that enprofylline exhibits dose-dependent pharmacokinetics and concentration-dependent protein binding behaviour (Hasegawa et al 1991; Nadai et al 1991). In clinical studies, the bronchodilating plasma concentration range of enprofylline has been suggested to be 2–5 μg mL^{-1} (Lunell et al 1982), which is markedly narrow compared with theophylline. Theophylline metabolism in the liver is dependent on age (Grygiel & Birkett 1980; Antal et al 1981). On the other hand, Lunell & Borgå (1987) have demonstrated that systemic and renal clearances of enprofylline decrease with age. Ageing may result in plasma concentrations above the therapeutic range of enprofylline and thus increase the possibility of toxicity. Whether ageing modifies the disposi-

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tion and renal handling of enprofylline remains to be elucidated.

Rats are the most commonly used animals for the study of pharmacokinetic characteristics of drugs. A series of our earlier studies showed that an estimate of the basic pharmacokinetics of enprofylline in man can be obtained from data in various experimental animals, including rats (Tsunekawa et al 1992). Moreover, several studies have proposed that Fischer 344 rats are a good model for evaluating the effects of ageing on drug pharmacokinetics in man (Coleman et al 1977; Satterwhite & Boudinot 1991).

The purpose of the present study was to investigate the possibility that ageing may affect the disposition and renal handling of enprofylline in Fischer 344 rats.

Materials and Methods

Chemicals

Enprofylline (3-propylxanthine) was synthesized in our laboratory to the same specifications as those used in previous experiments (Apichartpichean et al 1991). Inulin was purchased from Nacarai Tesque (Kyoto, Japan). Commercially available reagents of analytical grade were also used. Enprofylline was suspended in an isotonic saline solution and drops of sodium hydroxide were added to produce a clear solution.

Animals and experiments

Male Fischer 344/DuCrj rats (Charles River, Ibaraki, Japan) were used for all experiments. Six-month-old rats, 330–372 g, 13-month-old rats, 396–488 g, and 18-month-old rats, 392–506 g, were selected and were fed a commercial food diet with water freely available. In studies on the effects of ageing on the pharmacokinetics of enprofylline, rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone

(25 mg kg^{-1}) and then cannulated with a polyethylene catheter in the right jugular vein. One day after surgical preparation, enprofylline was administered intravenously at a dose of 2·5 mg kg^{-1}. Blood samples of approximately 0·25 mL were collected at pre-designated intervals of 10, 20, 30, 45, 60, 75, 90 and 105 min after administration. Urine samples were collected over 24 h.

Renal clearance experiments were performed by the single injection method as previously described (Nadai et al 1990). While the animals were under sodium pentobarbitone anaesthesia, the right jugular vein, left carotid artery and urinary bladder were cannulated for drug administration, blood sampling and urine collection, respectively. After surgical preparations were completed, all rats received a constant-rate infusion of inulin dissolved in isotonic saline containing 4% mannitol in addition to a bolus-loading dose, to estimate the glomerular filtration rate (GFR). Enprofylline was then administered intravenously at a rate of 15 mg kg^{-1} by single injection. Urine samples were collected at 10- or 20-min intervals over the subsequent period of 180 min. Blood samples were taken 1 min before the midpoint of urine collection intervals. Urine volume was measured gravimetrically assuming a specific gravity of 1·0. Plasma samples, obtained by centrifugation (11 000 rev min^{-1}, 5 min) and urine samples were stored at −40°C until analysis.

Protein-binding experiments

The effects of ageing on the plasma protein-binding properties of enprofylline were measured by equilibrium dialysis using a cellulose membrane (Visking sheet, Sanplatec Co., Osaka, Japan); the molecular cut-off was set at 10 000–20 000 Da. Blood samples were obtained from each rat by exsanguination from the abdominal aorta under light ether anaesthesia, and plasma samples were immediately extracted by centrifugation. Four hundred microlitre samples containing desired concentrations (5–100 μg mL^{-1}) of enprofylline in pH 7·4, isotonic phosphate buffer were dialysed against an equal volume of fresh plasma at 37°C for 6 h (Apichartpichean et al 1991). Enprofylline has been shown to possess only one binding site in plasma (Hasegawa et al 1989, 1991; Nadai et al 1991); protein-binding data were then fitted to the following equation using the nonlinear least-squares method, MULTI (Yamaoka et al 1981):

$$C_b = \frac{nP \cdot C_u}{k_d + C_u} \qquad (1)$$

where C_b and C_u represent the concentrations of the bound and unbound drug, respectively, P represents the concentration of albumin in plasma, and nP represents the binding capacity of the first class of binding sites and k_d represents the dissociation constant. Each value was calculated on the basis of human serum albumin, with a mol. wt of 69 000 Da.

Drug analysis

The concentration of enprofylline in plasma and urine, on both sides of the dialysis membrane, was measured by HPLC as described previously (Nadai et al 1991). HPLC analysis was performed by a Shimadzu LC-6A HPLC apparatus equipped with a Cosmosil 5C$_{18}$ column (150 × 4·6 mm i.d., Nacalai Tesque, Kyoto, Japan), a Shimadzu SPD-6AV UV-

vis spectrophotometric detector (274 nm), and an SIL-6A autoinjector.

Inulin was measured by the standard colorimetric method (Dische & Borenfreund 1951). The concentration of albumin in plasma was determined by the bromocresol green method using a commercial kit (Iatron Albumin Kit, Iatron Laboratories, Tokyo, Japan). Concentrations of free fatty acids (FFA) in plasma were measured using the commercially available kit (NEFA Kit-U, Nippon Shoji Kaisha, Ltd, Osaka, Japan).

Pharmacokinetic analysis

Plasma concentration-time data of enprofylline were analysed using model-independent methods. AUC and AUMC were calculated using the trapezoidal rule with extrapolation to infinity. Systemic clearance (CL$_{sys}$) was calculated by dividing the dosage by the AUC. Mean residence time (MRT) was calculated as MRT = AUMC/AUC. The volume of distribution at steady-state (Vd$_{ss}$) was calculated as Vd$_{ss}$ = CL$_{sys}$ × MRT.

Because tubular reabsorption of enprofylline is negligible in rats (Nadai et al 1991), the Michealis–Menten constant (K$_m$) and maximum velocity (V$_{max}$) for tubular secretion were estimated by using the following equation:

$$CL_{ru} = GFR + \frac{V_{max}}{K_m + C_u} \qquad (2)$$

where CL_{ru} is the renal clearance of unbound drug during each urine collection period. The GFR was determined as renal clearance of inulin. C_u was recalculated using the mean values of the protein-binding parameters obtained from
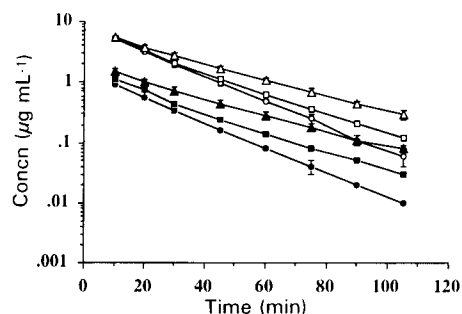

Fig. 1. Mean semilogarithmic plots of plasma concentration-time data of total (O, □, △) and unbound (●, ■, ▲) enprofylline for 6-, 13- and 18-month-old rats, respectively. Each plot represents mean ± s.e.m. of four rats. When the standard error is small, it is included in the symbol.

Table 1. Pharmacokinetic parameters of enprofylline in 6-, 13- and 18-month-old rats.

Age (months)	Vd$_{ss}$ (L kg^{-1})	CL$_{sys}$ (L h^{-1} kg^{-1})	MRT (h)
6	0·278 ± 0·014	0·844 ± 0·059	0·331 ± 0·015
13	0·323 ± 0·008	0·800 ± 0·005	0·403 ± 0·010
18	0·348 ± 0·045	0·663 ± 0·057	0·525 ± 0·047[a,b]

Each value represents mean ± s.e.m. (n = 4). Vd$_{ss}$, steady-state volume of distribution; CL$_{sys}$, systemic clearance. [a] Significantly different from 6-month-old rats ($P < 0.05$). [b] Significantly different from 13-month-old rats ($P < 0.05$).

Table 2. In-vitro plasma protein-binding parameters of enprofylline in 6-, 13- and 18-month-old rats.

Age (months)	nP (μM)	k_d (μM)	Albumin (μM)	FFA (μEq L^{-1})
6	454·99 ± 26·22	93·42 ± 9·19	728·02 ± 8·71	407·88 ± 95·16
13	492·49 ± 31·61	134·89 ± 7·68[a]	711·18 ± 17·56	627·74 ± 75·55
18	414·45 ± 7·13	149·89 ± 8·51[a]	676·31 ± 5·30[a]	830·50 ± 82·74[a]

Each value represents mean ± s.e.m. (n = 3–4). nP, binding capacity; k_d, dissociation constant; FFA, free fatty acids. [a] Significantly different from 6-month-old rats ($P < 0.05$).

protein-binding experiments and total drug concentration levels found in plasma incorporating them in a rearrangement of equation 1. The CL_{ru} was calculated by dividing the amount of unchanged enprofylline excreted into the urine during urine collection periods by the C_u. All computer analyses were performed using the nonlinear least-squares regression program, MULTI (Yamaoka et al 1981).

Statistical analysis

Values are expressed as mean ± s.e.m. for the indicated number of experiments. Statistical comparisons among the three groups of different-aged rats were assessed by one-way analysis of variance. Statistical significance was defined as $P < 0.05$ in accordance with Tukey's multiple comparison procedure (Tukey 1949).

Results

Mean semilogarithmic plasma concentration-time curves of enprofylline in 6-, 13- and 18-month-old rats after a single intravenous administration at a dose of 2·5 mg kg^{-1} are shown in Fig. 1. Plasma disappearance of enprofylline declined monoexponentially in all three groups of rats. The corresponding pharmacokinetic parameters of enprofylline are summarized in Table 1. No significant differences in the volume of distribution (Vd_{ss}) and systemic clearance (CL_{sys}) of enprofylline were observed. There were also no significant differences in the percentage of enprofylline recovered from urine over a 24 h period (> 85%) among all three groups, so systemic clearance represents essentially renal clearance.

The protein-binding parameters of enprofylline and concentrations of albumin and FFA in plasma are summarized

in Table 2. The albumin concentrations decreased, whereas the dissociation constant (k_d) and FFA significantly increased with ageing. The unbound plasma concentration-time data for enprofylline among rat groups were recalculated using the respective protein-binding parameters and total plasma concentration-time data taken from a manipulation of equation 1 (Fig. 1). Age-dependent decreases in the systemic clearance for the unbound drug (CL_{sysu}) were observed, although the volume of distribution for the unbound drug (Vd_{ssu}) tended to decrease with age (Table 3).

The relationship between plasma concentrations of unbound enprofylline and its renal clearance among rat groups is shown in Fig. 2. The renal secretion parameters calculated by the Michaelis–Menten equation and GFR are listed in Table 4. There was little or no change in the values of the Michaelis–Menten constant (K_m), while age-related decrease in the maximum velocity (V_{max}) was observed. The unbound secretory intrinsic clearances (V_{max}/K_m) were 75·45, 51·03 and 44·13 mL min^{-1} kg^{-1} for 6-, 13- and 18-month-old rats, respectively.

Table 3. Pharmacokinetic parameters of unbound enprofylline in 6-, 13- and 18-month-old rats.

Age (months)	Vd_{ssu} (L kg^{-1})	CL_{sysu} (L h^{-1} kg^{-1})	MRT_u (h)
6	1·563 ± 0·083	4·816 ± 0·345	0·327 ± 0·014
13	1·449 ± 0·035	3·636 ± 0·022[a]	0·399 ± 0·010
18	1·264 ± 0·168	2·441 ± 0·215[a,b]	0·518 ± 0·047[a,b]

Each value represents mean ± s.e.m. (n = 4). Vd_{ssu}, steady-state volume of distribution for unbound enprofylline; CL_{sysu}, systemic clearance for unbound enprofylline; MRT_u, mean residence time for unbound enprofylline. [a] Significantly different from 6-month-old rats ($P < 0.05$). [b] Significantly different from 13-month-old rats ($P < 0.05$).
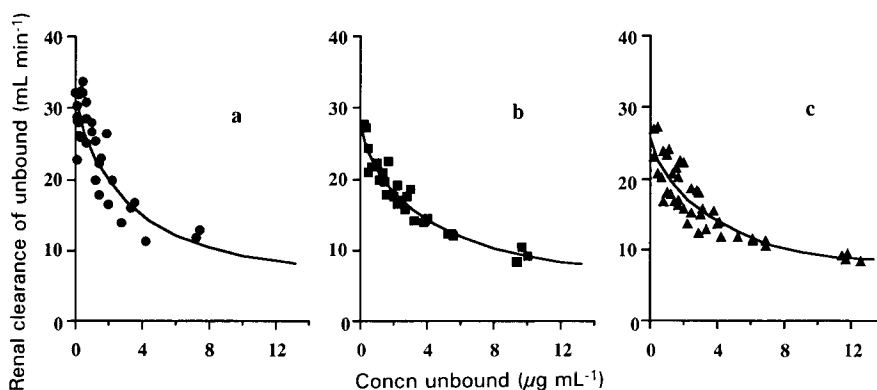

Fig. 2. Relationship between renal clearance of unbound enprofylline and its plasma concentration obtained from 6-month-old rats (a), 13-month-old rats (b) and 18-month-old rats (c). The solid line is the computer-simulated curve.

Table 4. Renal secretion parameters of enprofylline in 6-, 13- and 18-month-old rats.

Age (months)	K_m^a (μg mL^{-1})	V_{max}^a (μg min^{-1} kg^{-1})	GFR[b] (mL min^{-1} kg^{-1})
6	2·96 ± 0·49	223·33 ± 11·30	8·65 ± 0·33
13	3·14 ± 0·31	160·24 ± 5·07	8·28 ± 0·04
18	3·24 ± 0·49	142·98 ± 7·25	8·11 ± 0·15

[a] Each value represents the computer-estimated mean and s.d. calculated by nonlinear least-squares regression program, MULTI. [b] Each value was obtained experimentally as renal clearance of inulin and represents mean ± s.e.m. (n = 4). K_m, Michaelis–Menten constant; V_{max}, maximum velocity; GFR, glomerular filtration rate.

Discussion

The present study demonstrated that the systemic clearance for unbound enprofylline (CL_{sysu}) decreases with age, probably due to a reduced capacity of the tubular transport system, and that the decrease in active tubular cell secretion ability is greater than that in passive GFR with increasing age. This study also showed that there are no saturable elimination kinetics of enprofylline and no significant differences in the urinary recovery of unchanged drug in the rat after dosages of 2·5 mg kg^{-1} among the groups, a finding which concurred with our previous studies using Sprague-Dawley and Wistar rats (Nadai et al 1991; Muraoka et al 1992). These findings suggest that there are no strain-related differences in the pharmacokinetic characteristics of enprofylline. In the present study, plasma protein-binding behaviour of enprofylline and FFA in plasma changed with ageing. FFA is known to inhibit competitively the plasma protein-binding of drugs and to increase substantially in older rats (Terasaki et al 1986). In this study, both plasma concentration levels of FFA and the molar ratio of FFA to albumin in 18-month-old rats were somewhat lower than in other studies (Terasaki et al 1986) using 100-week-old male Wistar rats. This discrepancy may be explained by strain-related differences in plasma concentrations of FFA. Significant relationships were observed between the dissociation constant of enprofylline and plasma concentration of FFA, and the molar ratio of FFA/albumin in different-aged rats (r = 0·865 and 0·868, respectively, $P < 0·01$). From these observations, it may be suggested that this age-related alteration in the plasma-protein binding behaviour of enprofylline was probably caused either by the increase in FFA plasma concentration or in FFA/albumin.

The present study demonstrated that the Vd_{ssu} for enprofylline tends to decrease with age. Age-related reductions in the volume of distribution for theophylline and enprofylline have been noted in man (Antal et al 1981; Lunell & Borgå 1987). The Vd_{ssu} values were larger than the total body fluid in rats of 0·7 L kg^{-1} (Altman & Dittmer 1964). These results indicate that the unbound enprofylline can be distributed throughout the body water and body tissues. Tendencies toward age-related decreases in the Vd_{ssu} are probably caused by decreases in tissue binding and total body fluid levels. It has also been shown that intracellular fluid decreases with ageing in man, while extracellular fluid in both man (Ritschel 1988; Rowland & Tozer 1989) and rats (Tsuji et al 1985) does not. Further, enprofylline is distributed in extracellular fluid in the same manner as methylxanthines (Apichartpichean et al 1991). These findings suggest that the distribution of unbound enprofylline may be unaffected by changes in intracellular fluid.

Decrease in the systemic clearance of unbound enprofylline (CL_{sysu}) in older rats observed in this study was consistent with results in man (Lunell & Borgå 1987). These results indicate that the renal clearance of enprofylline also decreases with age, since no significant differences in the urinary recovery of unchanged enprofylline among 6-, 13- and 18-month-old rats were found. Renal plasma flow and GFR, which affect the renal handling of drug, are generally known to decrease with age in both man and animals. It has also been reported that the change in renal plasma flow rate has no significant effect on alteration of renal clearance of drugs of low extraction ratio (Lesser & Markofsky 1979). The decrease in the CL_{sys} of enprofylline observed in this study is not caused by reduction in renal plasma flow with ageing, since enprofylline is a low extraction ratio drug (Nadai et al 1991). The present study showed that the GFR slightly decreased with ageing, as shown also by other investigators (Gregory & Barrows 1969; Wabner & Chen 1987). Slight decrease in GFR with increasing age contrasts with human data. The difference between rat and man may be explained by vascular sclerosis of frequent occurrence in man (Coleman et al 1977; Bennett 1990). These results suggest that the age-related decrease in the CL_{sysu} for enprofylline is primarily responsible for decreases in tubular secretion ability, which plays a major role in the renal handling of enprofylline.

To clarify the influence of ageing on the ability of the tubular anion transport of enprofylline, the relationship between renal clearance for unbound enprofylline and its plasma concentrations was analysed using the single injection renal clearance method. Because reabsorption of enprofylline at the renal tubule is negligible (Borgå et al 1986; Nadai et al 1991), the secretion clearance was estimated by employing the Michaelis–Menten equation without applying a term for reabsorption as previously described (Nadai et al 1991). In the present study, the estimated values for V_{max} in 13- and 18-month-old rats were markedly decreased compared with the 6-month-old rats, although a slight change in K_m was observed. These results indicate that ageing causes some alterations in tubular proximal cell ability where the secretion of enprofylline is concerned. Wabner & Chen (1987) have demonstrated that age-related changes in active tubular function for p-aminohippurate in Fischer 344 rats are responsible for decrease in active transport capacity, a decrease in the number of receptor sites for p-aminohippurate. They have also reported that renal plasma flow, but not GFR, was slightly changed with ageing (12–27-month-old)

and that decline in tubular maximal activity for p-aminohippurate transport was greater than decline in the renal plasma flow rate. Reckelhoff et al (1992) have demonstrated no age-related changes in GFR and in glomerular blood pressure in rats. In addition, Corman et al (1985) have reported that the impairment of renal function with ageing was not caused by a decline in the number of nephrons. These findings suggest that age-related changes are greater in tubular transport function than in the glomerular haemodynamics function. The present data, which set renal secretion parameters for enprofylline expressed by body weight, may reveal changes in the functional capacity per kidney unit, since Wabner & Chen (1987) and Kiebzak & Sacktor (1986) have demonstrated that the ratio of kidney weight to body weight with increasing age in rats was almost constant. The K_m values obtained in this study were similar to those reported previously using Wistar and Sprague-Dawley rats (Nadai et al 1991; Muraoka et al 1992). It is unlikely that a strain-related difference exists in the affinity for enprofylline secretion in tubular proximal cells. Further studies are also required to clarify the precise mechanism for age-related reductions in the secretion ability of enprofylline.

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