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The Effects of *N*-Benzoyl-β-alanine, a New Nephroprotective Drug, on the Distribution and Renal Excretion of Enprofylline in Rats

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Abstract—The effects of the new nephroprotective drug *N*-benzoyl-β-alanine (BA) on the disposition and renal excretion of the bronchodilator enprofylline, which is actively secreted in urine, were investigated in rats. Enprofylline was administered intravenously at a dosage of 2·5 mg kg^{-1} under three different steady-state plasma BA concentrations (100, 200 and 400 μg mL^{-1}) which were achieved by constant infusion rates. Pharmacokinetic parameters for both total and unbound enprofylline were estimated by model-independent methods. The presence of BA (400 μg mL^{-1}) increased the systemic clearance by 25% and the volume of distribution at steady-state by 90%. A significant increase in the dissociation constant, which is the protein binding parameter of enprofylline was observed in the presence of BA (400 μg mL^{-1}), indicating that BA competitively inhibits the protein binding of enprofylline. However, BA significantly decreased the systemic clearance and volume of distribution for unbound enprofylline. These results suggest that BA, the organic anion transport inhibitor, inhibits renal excretion of enprofylline with a high affinity for renal tubular secretion, although the unbound concentration of enprofylline increases with administration of BA. We conclude that BA decreases the renal tubular secretion of enprofylline probably by reducing the affinity of the tubular transport system, and that these changes have marked effects on the pharmacokinetic behaviour of enprofylline.

The organic anion transport inhibitor *N*-benzoyl-β-alanine (BA) is a new clinical drug used to protect against nephrotoxicity induced by panipenem, a newly synthesized carbapenem antibiotic. Since BA is known to inhibit the renal cortical uptake and tubular toxicity of panipenem (Naganuma et al 1991), it may be a useful inhibitor against drug-induced nephrotoxicity based on the knowledge that a carrier-mediated transport system is closely related to the renal cortical uptake and nephrotoxicity of drugs (Tune 1972). However, there is a possibility that pharmacokinetic and pharmacodynamic interactions occur when BA is coadministered with drugs mainly excreted by the kidney. As far as we know, there is no reported data on the influence of BA on the disposition and renal handling of concomitant drugs.

Enprofylline, which is a potent bronchodilator with a low affinity for adenosine A_1 receptors in comparison with theophylline, can be substituted for theophylline (Persson & Kjellin 1981; Persson et al 1982; Ogawa et al 1989; Hasegawa et al 1990b). Enprofylline has been shown to be excreted mainly into the urine by an active tubular secretion mechanism both in man and in animals (Borgå et al 1986; Apichartpichean et al 1991; Nadai et al 1991). It is possible that BA affects the disposition and renal excretion of enprofylline.

The present study was conducted as part of a programme to develop guidelines for the safe use of BA in combination with enprofylline to asthmatic patients. Therefore, we have investigated the possibility that when the active transport inhibitor BA is coadministered with enprofylline, it may affect the disposition and renal handling of enprofylline in rats.

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Materials and Methods

Chemicals

Enprofylline and 1-methyl-3-ethylxanthine (MEX) were synthesized in our laboratory as described previously (Hasegawa et al 1990a, b, 1991; Apichartpichean et al 1991; Nadai et al 1991). *N*-Benzoyl-β-alanine (BA) was obtained from Tokyo Kasei Co. Ltd (Tokyo, Japan). All other agents and reagents used in the experiments were obtained commercially without requiring further purification. Enprofylline and BA were suspended in isotonic saline, and NaOH (0·5 M) was added in drops to create a clear solution. The solution was neutralized with HCl (pH 6·9).

Animal experiments

Eight-to-ten-week-old Wistar strain male rats (Japan SLC, Hamamatsu, Japan), 270–300 g, were used for all experiments.

In order to investigate the effects of different BA dosages on the pharmacokinetics of enprofylline, rats under light ether anaesthesia were cannulated in the right jugular vein for blood sampling and in the left femoral vein for drug administration. After surgical preparations were complete, BA (treated group) or saline (control) was infused before, during and after intravenous administrations of enprofylline at a dose of 2·5 mg kg^{-1}. The desired range of steady-state plasma concentrations of BA (100–400 μg mL^{-1}) could be attained by constant infusion rates (3·99, 7·99 and 16 mg min^{-1} kg^{-1}, respectively) over 3 h. Dosages of BA were calculated using data obtained from preliminary studies. The desired steady-state plasma concentrations of BA were attained within 30 min in each animal. Blood samples (0·25 mL) were collected at regular intervals (5, 10, 20, 30, 45, 60 and 75 min) after the administration of enprofylline. Plasma

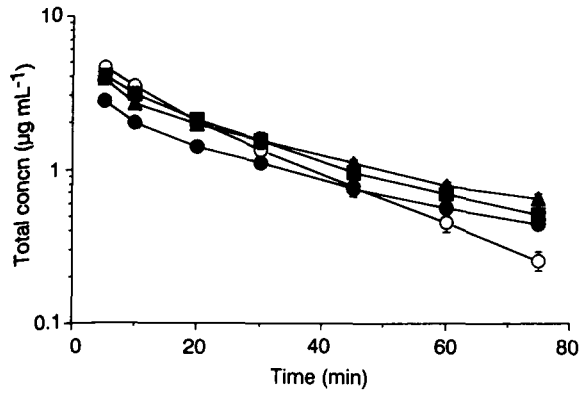

FIG. 1. Semilogarithmic plots of total plasma concentration-time data of enprofylline after a single intravenous administration in the presence (▲ 100, ■ 200, ● 400 μg mL^{-1}) or absence (○) of BA in rats. Each plot represents mean ± s.e. (n = 4–5). When the standard error is small, it is included in the symbol.

samples were obtained by centrifugation at 6000 *g* for 5 min. To determine urine recovery of unchanged enprofylline, urine was collected from plastic metabolism cages (Natsume KN-326, Tokyo, Japan) for 180 min after administration of enprofylline. Plasma and urine samples were stored at −40°C until analysis.

Drug analysis

Plasma and urine concentrations of enprofylline and BA were measured by HPLC using the Shimadzu LC-6A system (Shimadzu Company, Kyoto, Japan) which consists of an LC-6A liquid pump, an SPD-6A UV spectrophotometric detector and an SIL-6A autoinjector. A Cosmosil $5C_{18}$ packed column (4·6 × 150 mm; Nacalai Tesque, Kyoto, Japan) was also used. The mobile phase was a 30 mM phosphate buffer (pH 4·0):methanol (85:15 v/v), used at a flow rate of 1·0 mL min^{-1}. The elution was carried out at 40°C, and the effluent column was monitored at 274 nm.

In a plastic centrifuge tube, 50 μL plasma or urine diluted with distilled water, and 0·35 mL of the internal standard solution (MEX 1·0 μg mL^{-1}) were vortexed and centrifuged at 6000 *g* for 2 min. The obtained supernatant was then completely evaporated under a gentle stream of nitrogen at 40°C. The residue was reconstituted with 0·2 mL of the mobile phase. The reconstituted solution (110 μL) was then injected into the column. The two drugs were measured over ranges of 0·1–20 μg mL^{-1} for enprofylline and 10–800 μg mL^{-1} for BA. The detection limit for each drug was 0·05 and 5 μg mL^{-1}, respectively. The intraday and interday coefficients of variation for the HPLC assay were less than 7%. Recoveries for enprofylline and BA ranged from 95 to 101%. Blank plasma and urine samples did not interfere with any of the peaks corresponding to each compound. The concentration of albumin in plasma was determined by the bromocresol green method using a commercial kit (Iatron Albumin Kit, Iatron Laboratories, Tokyo, Japan).

Protein binding

Because the binding behaviour of enprofylline to albumin is concentration-dependent (Hasegawa et al 1991; Nadai et al 1991), the effect of BA on the plasma protein binding of enprofylline was examined by equilibrium dialysis using a cellulose membrane (Visking Sheet, Sanplatec Corp., Osaka, Japan) with molecular weight cut off set at 10000–20000. Blood samples were obtained from saline-treated rats by exsanguination from the abdominal aorta under light ether anaesthesia. Plasma samples were immediately prepared by centrifugation. Fresh plasma samples (0·4 mL) containing added enprofylline (20–330 μM) were immediately dialysed against an equal volume of isotonic phosphate buffer (pH 7·4) in a concentration of 400 μg mL^{-1} BA at 37°C for 6 h to attain equilibrium. Concentrations of enprofylline on both sides of the membrane were measured by HPLC. Assuming that only one binding site exists for enprofylline in plasma, protein binding data were fitted with the following equation using the nonlinear least-squares method program MULTI (Yamaoka et al 1981).

$$C_b = \frac{nP \cdot C_u}{K_d + C_u} \quad (1)$$

where C_b and C_u are the concentrations of the bound drug and the unbound drug, respectively. nP is the binding capacity of the first class of binding sites, and K_d is the dissociation constant.

Pharmacokinetic analysis

Plasma concentration-time data for each drug were analysed using model-independent methods and a nonlinear least-squares method program. The area under the plasma concentration-time curve (AUC) and the area under the first moment curve (AUMC) were calculated by the trapezoidal rule with extrapolation to infinity. Systemic clearance (CL_{sys}) was calculated as the dose divided by AUC. The volume of distribution at steady-state (Vd_{ss}) was calculated as $Vd_{ss} = CL_{sys} \cdot MRT$, where MRT is the mean residence time calculated as MRT = AUMC/AUC. The corresponding

Table 1. Pharmacokinetic parameters of total enprofylline during constant infusion of saline or BA in rats.

		Steady-state plasma concn BA (μg mL^{-1})		
Parameter	Saline	100	200	400
CL_{sys} (L h^{-1} kg^{-1})	1·161 ± 0·015	1·043 ± 0·075	1·052 ± 0·050	1·379 ± 0·037*‡
Vd_{ss} (L kg^{-1})	0·471 ± 0·016	0·644 ± 0·030†	0·630 ± 0·031†	0·905 ± 0·043†‡§
MRT (h)	0·405 ± 0·013	0·626 ± 0·039†	0·614 ± 0·056†	0·654 ± 0·038†

Values represent mean ± s.e. (n = 4–5). *† Significantly different from saline at $P < 0·05$, 0·01, respectively. ‡ Significantly different from BA (100 μg mL^{-1}) at $P < 0·01$. § Significantly different from BA (200 μg mL^{-1}) at $P < 0·01$.

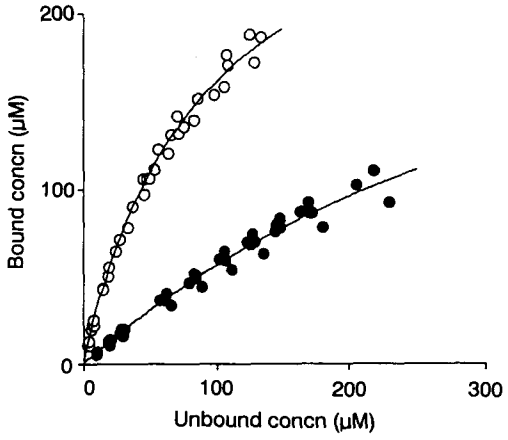

FIG. 2. Protein binding profiles of enprofylline to fresh rat plasma in the presence (●) or absence (○) of BA (400 μg mL^{-1}). Solid lines represent computer-fitted curves taken from equation 1.

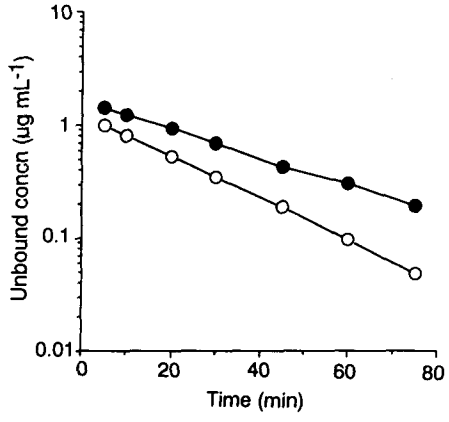

FIG. 3. Semilogarithmic plots of unbound plasma concentration-time data of enprofylline after a single intravenous administration in the presence (●) or absence (○) of BA (400 μg mL^{-1}) in rats. Data were calculated from total plasma concentration data and binding parameters. Each plot represents mean ± s.e. Standard error is included in the symbol.

pharmacokinetic parameters ($CL_{sys,u}$, $Vd_{ss,u}$ and MRT_u) for the unbound drug were estimated in the same manner as those for total concentrations, while the unbound concentration was calculated in a rearrangement of equation 1 using total plasma concentration data and binding parameters obtained from the protein binding experiments. The renal clearance of enprofylline was calculated as:

$$CL_R = CL_{sys} \times f_e \quad (2)$$

where f_e represents the fraction of unchanged drug excreted into the urine in 180 min. $CL_{R,u}$ ($CL_{R,u} = CL_{sys,u} \times f_e$) for the unbound drug was estimated in the same manner as $CL_{sys,u}$, $Vd_{ss,u}$ and MRT_u.

Data analysis

Values are expressed as mean ± s.e. for the indicated number of experiments. Statistical comparisons between the control and treated rats were conducted by analysis of variance. Tukey's test was used to detect differences among individual groups. Statistical significance was set at $P < 0.05$.

Results

The desired steady-state plasma concentrations of BA were achieved. The average plasma concentrations were 100·1 ± 0·9 (n = 5), 199·9 ± 1·8 (n = 5) and 414·9 ± 20·8 μg mL^{-1} (n = 4) at constant infusion rates of 3·99, 7·99 and 16 mg min^{-1} kg^{-1}, respectively. Fig. 1 shows semilogarithmic data for plasma disappearance of enprofylline after a single intravenous administration (2·5 mg kg^{-1}) in the presence of different steady-state plasma concentrations of BA. Plasma disappearance of enprofylline declined biexponentially in both the control and treated rats. The corresponding pharmacokinetic parameters of enprofylline are shown in Table 1. Significant changes in some pharmacokinetic parameters were observed between the control group and the BA-treated group, when data were analysed independently and statistically. The presence of BA (400 μg mL^{-1}) significantly increased the systemic clearance (CL_{sys}). In addition, the volume of distribution at steady-state (Vd_{ss}) of enprofylline significantly increased regardless of the dose of BA.

Table 2. Protein binding parameters of enprofylline.

Treatment	nP (μM)	K_d (μM)	P (μM)
Saline	302·98 ± 10·04	88·51 ± 2·85	540·13 ± 22·50
BA	327·71 ± 9·97	486·37 ± 15·19*	518·94 ± 5·58

Values represent mean ± s.e. (n = 4–5) and was calculated on the basis of human serum albumin with a mol. wt 69000.

*Significant difference was noted between the control and the treated rats ($P < 0.01$). nP = binding capacity. P = albumin concentration.

The protein binding profiles for enprofylline in rat plasma in the presence (400 μg mL^{-1}) and absence of BA are shown in Fig. 2. In the presence of BA, the simulated curve shifts to the right and is nearly linear as illustrated. Computer estimates of binding parameters, as calculated by the nonlinear least-squares method using equation 1, are summarized in Table 2. A significant increase in the dissociation constant (K_d) was observed in the presence of BA, but there were no significant differences in the binding capacity (nP) or albumin concentration (P) between the treated and control rats.

Because significant changes in the systemic clearance for enprofylline were observed only in the presence of BA (400 μg mL^{-1}), the unbound plasma concentration data for enprofylline were calculated using the protein binding parameters (nP and K_d) and total plasma concentration-time data of enprofylline in a manipulation of equation 1. The mean unbound plasma concentrations of enprofylline were plotted against time (Fig. 3). When the corresponding pharmacokinetic parameters for unbound enprofylline were estimated from the unbound enprofylline concentration-time curve data in the same manner as for total enprofylline, significant differences in these parameters for the unbound drug were observed between the control and treated rats (Table 3); the presence of BA decreased the systemic clearance of unbound enprofylline by 50% (5·0 to 2·5 L h^{-1} kg^{-1}) and the volume of distribution by about 25% (2·0 to 1·5 L kg^{-1}). No significant difference was observed in the urinary

Table 3. Pharmacokinetic parameters of unbound enprofylline during constant infusion of saline or BA in rats.

Parameter	Saline	BA treatment
$CL_{sys,u}$ (L h^{-1} kg^{-1})	5·002±0·066	2·542±0·011*
$V_{ss,u}$ (L kg^{-1})	1·999±0·073	1·539±0·065*
MRT_u (h)	0·398±0·012	0·605±0·021*
$CL_{R,u}$ (L h^{-1} kg^{-1})	4·117±0·055	1·999±0·037*
Urinary recovery (%)	82·247±1·430	78·592±0·714

Values represent mean±s.e. (n=4–5). Each parameter was estimated by unbound concentration-time data calculated using total plasma concentration data and binding parameters.
*Significant difference was noted between the control and the treated rats ($P<0·01$).

recovery of enprofylline regardless of the presence of BA (78·6% compared with 82·2% in control). Renal clearance of unbound enprofylline ($CL_{R,u}$), however, decreased by 50% (4·1 L h^{-1} kg^{-1} compared with 2·0 L h^{-1} kg^{-1}).

Discussion

It is generally accepted that protein binding plays a key role in determining the pharmacokinetics including both distribution and rate of elimination and the potency of the pharmacological effects of drugs, since only the unbound drug is capable of being diffused across various biological membranes to be distributed into target organ tissues, as well as being subject to hepatic metabolism and renal excretion. It is possible that the binding and displacement interactions among drugs or between drugs and various proteins may alter the pharmacokinetic characteristics of drugs in the body and lead to serious clinical events. Earlier studies have demonstrated that enprofylline is extensively bound to albumin in rat plasma and is affected by factors such as pH, endogeneous substances, albumin concentration, species and salicylic acid (Tegner et al 1983; Hasegawa et al 1989). Enprofylline, therefore, is probably displaced by other acidic drugs strongly bound to albumin in plasma.

In this study, a significant increase in the volume of distribution for total enprofylline was observed in the presence of BA. This result can be explained by the knowledge that the apparent volume of distribution generally increases as the unbound drug concentrations in plasma increase. The volume of distribution for total enprofylline in the control rats was higher than that obtained from previous studies (Hasegawa et al 1990a; Apichartpichean et al 1991; Nadai et al 1991). This difference may be explained by considering the increase in total body water produced by hydration from a constant infusion of saline. Our previous studies demonstrated that enprofylline exhibits concentration-dependent protein-binding behaviour. We also found that changes in this behaviour affect volume of distribution of enprofylline in rats (Nadai et al 1991). In protein-binding experiments of this study, the presence of relatively high concentrations of BA (400 μg mL^{-1}) strongly inhibited the protein binding of enprofylline in a competitive inhibition manner. Enprofylline was displaced from albumin by BA as the dissociation constant (K_d) of enprofylline increased more than fivefold in the presence of BA (Fig. 2, Table 3). However, a decrease in the volume of distribution for unbound enprofylline was observed in the presence of BA. The decrease in the volume of distribution of unbound enprofylline by BA may be caused by the inhibition of BA on the distribution of enprofylline into organs and tissues. This possible explanation is supported by findings that probenecid restricts the tissue distribution of enprofylline in man (Borgå et al 1986).

Decreases in the systemic clearance of unbound enprofylline in the presence of BA were caused by a reduction in the renal clearance of unbound enprofylline, since no significant difference in urinary excretion of enprofylline was observed between the treated and control groups. Renal clearance for unbound enprofylline ($CL_{R,u}$) in the control rats (4·1 L h^{-1} kg^{-1}) was much greater than the glomerular filtration rate, indicating that active tubular secretion plays a major role in the renal excretion of enprofylline as was previously reported (Apichartpichean et al 1991; Nadai et al 1991). In contrast, the presence of a steady-state BA concentration at 400 μg mL^{-1} significantly decreased the $CL_{R,u}$ of enprofylline by 50% (2·0 L h^{-1} kg^{-1}). These results indicate that a decrease in the $CL_{R,u}$ caused by BA is responsible for the decrease in tubular secretion of enprofylline, possibly through competitive inhibition in the tubular proximal cells, since BA is unlikely to affect glomerular filtration. Also, since the removal of enprofylline from the kidneys is particularly dependent upon the unbound drug concentration and the capacity for tubular secretion (Nadai et al 1991), changes in renal handling of enprofylline observed in this study are, at least partly, associated with an increase in the unbound fraction of enprofylline. These earlier studies may serve to confirm that BA modifies the renal tubular secretion mechanism of enprofylline in addition to affecting the protein-binding behaviour and organ tissue binding.

In summary, BA elevated the unbound fraction of enprofylline by markedly changing its protein-binding behaviour. The decreased systemic and renal clearances for unbound enprofylline were caused by the competitive inhibition of BA against the active renal tubular secretion of enprofylline and by BA-induced changes in the protein-binding behaviour of enprofylline. The results obtained in this study suggest that when BA is coadministered with acidic drugs that are mainly excreted by the kidney and possess relatively high protein-binding behaviour, the renal excretion of those drugs may be inhibited and lead to side-effects. This study indicates that care should be taken in prescribing preparations with BA to patients receiving chronic enprofylline therapy.

References

Apichartpichean, R., Hasegawa, T., Nadai, M., Kuzuya, T., Nabeshima, T. (1991) Structure-pharmacokinetic relationships among the N^1,N^3-alkylxanthines in rats. J. Pharm. Pharmacol. 43: 262–269

Borgå, O., Larsson, R., Lunell, E. (1986) Effects of probenecid on enprofylline kinetics in man. Eur. J. Clin. Pharmacol. 30: 221–223

Hasegawa, T., Apichartpichean, R., Kuzuya, T., Nadai, M., Tsunekawa, Y., Horiuchi, T., Miyamoto, K. (1989) Protein binding characteristics of new bronchodilators, 1-methyl-3-propylxanthine (MPX) and 3-propylxanthine (enprofylline). Int. J. Pharm. 56: 235–241

Hasegawa, T., Nadai, M., Kuzuya, M., Muraoka, I., Apichartpichean, R., Takagi, K., Miyamoto, K. (1990a) The possible

mechanism of interaction between xanthines and quinolone. J. Pharm. Pharmacol. 42: 767–772

Hasegawa, T., Miyamoto, K., Wakusawa, S., Takagi, K., Apichartpichean, R., Kuzuya, T., Nadai, M., Horiuchi, T. (1990b) Relationship between hydrophobicity and biological activities of xanthine derivatives in guinea pig tracheal smooth muscle. Int. J. Pharm. 58: 129–134

Hasegawa, T., Takagi, K., Nadai, M., Miyamoto, K. (1991) Protein binding of xanthine derivatives to guinea pig serum albumin. J. Pharm. Sci. 80: 349–352

Nadai, M., Hasegawa, T., Muraoka, I., Takagi, K., Nabeshima, T. (1991) Dose-dependent pharmacokinetics of enprofylline and its renal handling in rats. J. Pharm. Sci. 80: 648–652

Naganuma, H., Tokiwa, H., Hirouchi, Y., Kawahara, Y., Fukushige, J., Fukami, M., Hirota, K., Muramatsu, S., Takahagi, H., Inui, K., Tanigawara, Y., Yasuhara, M., Hori, R., Kuwahara, S. (1991) Nephroprotective effect and its mechanism of betamipron (I)—relationships of renal transport. Chemotherapy (Tokyo) 39: 166–177

Ogawa, K., Takagi, K., Satake, T. (1989) Mechanism of xanthine-induced relaxation of guinea-pig isolated trachealis muscle. Br. J. Pharmacol. 97: 542–546

Persson, C. G. A., Kjellin, G. (1981) Enprofylline, a principally new antiasthmatic xanthine. Acta Pharmacol. Toxicol. 49: 313–316

Persson, C. G. A., Karisson, J. A., Erjefalt, I. (1982) Differentiation between bronchodilation and universal adenosine antagonism among xanthine derivatives. Life Sci. 30: 2181–2189

Tegner, K., Borgå, O., Svensson, I. (1983) Protein binding of enprofylline. Eur. J. Clin. Pharmacol. 25: 703–708

Tune, B. M. (1972) Effect of organic acid transport inhibitor on renal cortical uptake and proximal tubular toxicity of cephaloridine. J. Pharmacol. Exp. Ther. 181: 250–256

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T. (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. J. Pharmacobiodyn. 4: 879–885