Probenecid is known to be a potent inhibitor of renal tubular secretion of several drugs or chemical substances including drugs such as penicillins, and reabsorption of uric acid [12]. On the other hand, probenecid has also been extensively studied for investigating the process of drug excretion in urine by the kidney. In 1963, plasma probenecid concentrations in biological samples from humans and dogs were determined by ultraviolet spectrophotometry but with a relatively complicated manner for preparation of biological samples [5]. Gradually, this method has been replaced by using HPLC as described by Hekman et al. [9]. However, this method uses organic extraction and requires larger volume (1 ml) of biological sample, therefore it is rather time consuming and it has difficulty for the frequent sampling from animal. Therefore, this study was undertaken to introduce a simple, easy, and quick HPLC method for plasma probenecid concentration with high sensitivity, good recovery, linearity and reproducibility. Moreover, in vitro characteristics of plasma protein binding of probenecid, and its in vivo pharmacokinetic (PK) parameters after a single bolus iv injection were determined in dogs. These provide basic information which must be taken into account concerning probenecid involved drug-drug interactions.

MATERIALS AND METHODS

Animals and plasma samples: Healthy normal female dogs (Beagles, 8 mo. old, body weight=7.9 ± 0.4 kg, n=5) were used in this study. They were housed in separate cages in the facility, fed daily and regularly weighed and checked for healthy status by veterinarians. Blood tests including complete blood count (CBC) and biochemistry were also performed before and after experiments. Blood was taken from the cephalic vein with the indwelling catheter into heparinized syringes, centrifuged at 8,000 g for 5 min. Then the supernatant (plasma) was removed and stored at −80°C until the HPLC analysis. Prior to the experiment, this study was approved by the ethical committee for the animal experiment and welfare in Kitasato University, School of Veterinary Medicine and Animal Sciences.

Drugs and chemicals: Probenecid (MW = 285) was obtained from Sigma chemicals (St Louis, MO, U.S.A.). All other chemicals including ammonium carbonate, HCl, acetonitrile, and methanol were of analytical grade.

Determination of plasma probenecid concentration: The applied HPLC system (LC-VP, Shimadzu HPLC system, Kyoto Japan) was composed of a pump (LC-10ADVP), a degasser (DGU-14A), a manual fixed-loop injector (model 7725; Rheodine, Cotani, CA, U.S.A.), a column oven (CTO-10A), a UV detector (SPD-10A), a system controller (SCL-10AVP) and its controlling software (CLASS-VP 5.0) with a personal computer. A C-18 cartridge guard column (NOVA PAK WAT044380, with Sentry Guard Column, Waters, Milford, MA, U.S.A.) and a C-18 cartridge analytical column (NOVA PAK C18, 8NVC18...
4 μl, 8 mm × 100 mm, Waters) with a radial compression module (RCM 8 × 10, Waters) were used together for the determination. The mobile phase was a mixture of 0.5% ammonium carbonate buffer (pH 7.9) and acetonitrile (30:70, v/v). The flow rate of the mobile phase was 1.0 ml/min, the temperature of the column oven was adjusted at 40°C, and applied wavelength of the UV detector was 242 nm. The stored plasma sample (100 μl) was mixed with HCl (3 N, 10 μl) and methanol (100 μl), and vortexed for 15 sec. The mixture was centrifuged for 20 min at 8,000 g, and its supernatant was removed and filtered through a 0.45 μm filter (Chromatodisk 4A, Biofield, Tokyo, Japan), and 10 μl of the filtrate was injected into the HPLC system. All data were analyzed in duplicate and concentrations were calculated based on the chromatographic areas of standards and the recovery of probenecid from plasma.

Prior to the actual determination of probenecid in canine plasma samples, standard solutions together with probenecid-spiked plasma samples were analyzed for the determination of its detection limit and recovery. After this determination, plasma protein binding property of probenecid was also determined by adding 50 μl of fixed concentrations (1 – 2,500 μg/ml) of the standard probenecid solution into plasma (450 μl). Then unbound plasma concentrations were determined after ultrafiltration, using Microcon Centrifugal Filter Units (YM-100, Millipore, Bedford, MA, U.S.A.).

**Plasma protein binding analysis:** Since there were obviously two specific binding characteristics of probenecid to plasma proteins, Eq. 1 was applied for the determination of binding parameters, as follows,

\[
Cb = \frac{Bmax_1 \times Cf}{Kd_1 + Cf} + \frac{Bmax_2 \times Cf}{Kd_2 + Cf} \quad \ldots \text{Eq. 1}
\]

where Cb and Cf are the bound and free concentration, Bmax1 and Kd1 are the maximal binding capacity and dissociation constant of high affinity binding, and, Bmax2 and Kd2 are the maximal binding capacity and dissociation constant of the lower affinity binding, respectively. Each binding parameter in Eq. 1 was calculated by non-linear least squares method by using computer program “MULTI” [13].

**Pharmacokinetic analysis of probenecid:** Probenecid (20 mg/kg body wt) was injected intravenously into the cephalic vein via an indwelling catheter in each animal. Then blood (1 ml) was taken from the other side of cephalic vein via an indwelling catheter with a heparinized syringe before and after probenecid administration at certain time intervals (5, 10, 18, 20, 25, 30, 45, 75, 105, 135, 195, 375, 615, 975, 1455 min) of the injection. Plasma was separated by centrifugation of the blood at 8,000 g for 5 min and stored at −80°C until HPLC analysis of probenecid. After determination of plasma probenecid concentration-time profiles, the pharmacokinetic parameters of probenecid were determined based on a two-compartment open model with the program “MULTI”. The suitability of the compartment model was finally fixed by the maximum likelihood estimation by comparing the Akaike’s information criterion (AIC) [1, 10].

AIC is the method of comparing alternative specifications by adjusting the error sum of squares (ESS) for the sample size (n) and the number of coefficients in the model (K). This criterion is used to decide if the improved fit caused by an additional variable is worth the decreased degrees of freedom and increased complexity caused by the addition, according to Eq. 2.

\[
AIC = \log (\text{ESS/n}) + 2 (K) / n \quad \ldots \text{Eq. 2}
\]

where K is the number of estimated coefficients and n is the number of observations in the sample. The lower AIC is the better specification.

The compartment model was calculated based on Eq. 3.

\[
Cp = A \cdot \exp (-\alpha \cdot t) + B \cdot \exp (-\beta \cdot t) \quad \ldots \text{Eq. 3}
\]

where Cp is plasma concentration of probenecid, A and B are the zero time drug concentration intercept of the disposition curve, respectively, α and β are hybrid rate constants of distribution and elimination phases, respectively, and t is the time after probenecid injection. Non-compartmental (moment) analysis was also applied to calculate area under the concentration-time curve (AUC), area under the moment curve (AUMC), plasma clearance (CLp=dose/AUC), and mean residence time (MRT=AUMC/AUC). The areas (AUC and AUMC) were calculated by the trapezoidal rule from time 0 to the last sampling (24 hr) plus extrapolation of the area from 24 hr until infinity. The volume of central compartment (V1) was calculated by dividing the dose by A+B, while Vd of peripheral compartment (V2) was the product of V1 and a ratio of K12 to K21 which are rate constants between the two compartments. The rate constants K21, K10, and K12 were determined by the following equations (Eq. 4–6), respectively.

\[
K21 = \frac{A \cdot \beta + B \cdot \alpha}{A + B} \quad \ldots \text{Eq. 4}
\]

\[
K10 = \frac{(A+B) \cdot \alpha \cdot \beta}{A \cdot \beta + B \cdot \alpha} \quad \ldots \text{Eq. 5}
\]

\[
K12 = \alpha + \beta - K_{10} \cdot K_{12} = \frac{A \cdot B \cdot (\alpha - \beta)^2}{(A + B) \cdot (A \cdot \beta + B \cdot \alpha)} \quad \ldots \text{Eq. 6}
\]

The distribution volume at steady state (Vdss) was calculated by the product of the dose and MRT divided by AUC, and Vd (area) was the dose divided by AUC and β, as written elsewhere.

**RESULTS**

**Probenecid determination by using HPLC:** The HPLC chromatogram showed good separation of probenecid with no or negligible interfering endogenous substances in canine plasma. The detection limit of the probenecid standard solution was about 10 ng/ml and the quantification limit of probenecid in canine plasma was almost comparable.
to the level of 50 ng/ml at S/N ratio of 3, respectively. The average recovery of plasma probenecid determined by HPLC at concentrations ranging from 100 ng/ml to 100 μg/ml was 101 ± 4.6%. The inter-day variation of the overall recovery of plasma probenecid (CV) by this method was at most 6.7% for 5 days’ determinations. There was a good correlation between the preset concentrations and their determined levels by HPLC ($r^2=0.984$). Both results indicated good linearity between the two values (Fig. 1).

**Plasma protein binding of probenecid:** Although the bound concentration increased as the free concentration increased, the Scatchard plot of protein binding of probenecid showed biphasic property (Fig. 2). The apparent biphasic binding property between probenecid and plasma proteins was due to high-affinity with low-capacity binding and low-affinity with high capacity binding. The binding parameters are listed in Table 1. According to these parameters, the simulation curves of probenecid-protein bindings were drawn in Fig. 3. As shown in Fig. 3, the lower the probenecid total concentrations, the higher the high-affinity binding becomes dominant. And its contribution decreases to be replaced by the low-affinity binding at the higher probenecid concentrations.

**Probenecid pharmacokinetics:** As shown in Fig. 4, the probenecid concentration-time curve in dogs was biphasic, a two-compartment open model was applied for the determination of PK parameters. The lowest AIC value also supported its consistency rather than the other 1- and 3-compartment model. The elimination phase after the distribution phase appeared to start at about 5 hr after injection. The PK parameters are listed in Table 2. The distribution half-life was 2.31 hr, and elimination half-life was 17.7 hr. The estimated apparent volume of distribution at steady-state (Vdss) was $0.460 ± 0.074 \text{ l/kg}$, and plasma clearance was $0.343 ± 0.044 \text{ ml/min/kg}$. Mean residence time was $22.9 ± 6.1$ hr. At 24 hr after administration, the average plasma probenecid concentration in 5 dogs was $13.4 ± 1.4$ mg/ml, which was far above the quantification limit (50 ng/ml). The results of blood tests including CBC and biochemistry before and after probenecid administration were within normal range of values and had no statistical significance in all dogs (results not shown).

**DISCUSSION**

**Chromatographic determination of canine plasma probenecid:** The HPLC procedure applied in this study for the detection of probenecid in canine plasma had high sensitivity (detection limit; 10 ng/ml and quantification limit; 50 ng/ml), good recovery (101 ± 4.6% and 6.7% of inter-day variation) and good linearity over the concentration range (100 ng/ml to 100 μg/ml) applied ($r^2=0.984$, Fig. 1) compared with the other methods reported [2, 3, 5, 8, 9, 11]. The analytical method entailed a simple and the much better procedure; i.e., HCl and methanol to be added to plasma samples and centrifuged, and it was not necessary to dry out the samples as reported previously [5, 8, 9]. Furthermore, the
amount of injectable solution into HPLC system is minimal (10 μl/sample). This indicates that probenecid concentration can be best detectable by this procedure in duplicate as long as 100 μl of plasma is available for analysis.

Protein binding of probenecid in canine plasma: In this study, the protein binding characteristics of probenecid were described by binding parameters including the maximum binding capacity (Bmax) and dissociation constant (Kd). As shown by the Scatchard-plot (Fig. 2), plasma probenecid binding was biphasic and this indicated that probenecid binds to at least two binding sites in dogs, which was in contrast to a previous report in humans [6]. According to the binding parameters determined (Table 1), simulation curve of the binding ratio indicated that probenecid binds mostly to the high affinity binding site at the lower concentrations in plasma (Fig. 3). However, as total concentration increases, saturation of the high-affinity binding site leads to the increase in binding to the lower affinity binding site. Therefore when probenecid concentration exceeds 100 μg/ml, a significant increase in unbound fraction would be expected. Moreover, it was calculated that at a concentration about 210 μg/ml in plasma, the high- and low-affinity binding becomes equal (34%) and total binding ratio is 68%. Chiang and Benet [3] reported probenecid binds from 89–73% over a concentration range of 40–800 μg/ml in humans. Similar observation in humans has been reported by Dayton et al. [5] and Emanuelsson et al. [6]. Therefore, as this study indicated, canine plasma appears to have lower affinity and/or capacity to bind probenecid than human plasma.

Pharmacokinetics of probenecid: Based on the AIC as well as the plasma concentration-time profile (Fig. 4), a two-compartment open model rather than other models best fitted the PK of probenecid in normal dogs. A non-linear PK of probenecid was reported at an intravenous dose of 1,000 mg in monkeys [3]. In our study, it was not clear if non-linear PK observed at an intravenous bolus dose of 20 mg/kg in dogs, since the injected dose might be too low to saturate high-affinity plasma protein binding of probenecid, as indicated in Figs. 2 and 3 and Table 1. However, according to figure 2–3, a higher dose study that exceeds the plasma probenecid concentration of 100 μg/ml may induce non-linear PK in dogs. There are studies in 3 animal species (dog, human [4], and sheep [7]) available for comparison of PK parameters of probenecid. As shown in Table 3, dogs showed the longest elimination half-life (t1/2 β=17.7 ± 5.7 hr) that was more
than 10 times the half-life in sheep, and the highest distribution volume ($V_{dss}=0.460 \pm 0.074 \text{ l/kg}$) among the 3 species.

However, total body clearance of probenecid in dogs ($0.343 \pm 0.044 \text{ ml/min/kg}$) was comparable to that in humans ($0.32 \pm 0.14 \text{ ml/min/kg}$), despite the wide differences in elimination half-life and distribution volume. Although information is not available concerning the protein binding of probenecid in sheep, it appears that the longer half-life in dogs than in the other species is, at least partly, due to the larger distribution volume which may be caused by the lower protein binding in canine plasma and lower urine pH. Since probenecid is a potent inhibitor of renal tubular excretion of acidic drugs and highly binds to plasma proteins, further study is highly likely to provide useful basic information concerning probenecid-related drug-drug interactions.

REFERENCES


