International Journal of Pharmaceutics, 56 (1989) 235–241
Elsevier

IJP 01913

Protein binding characteristics of new bronchodilators, 1-methyl-3-propylxanthine (MPX) and 3-propylxanthine (enprofylline)

Takaaki Hasegawa [1], Ruttikorn Apichartpichean [1], Takafumi Kuzuya [1], Masayuki Nadai [1], Yoshimi Tsunekawa [1], Tadashi Horiuchi [1] and Kenichi Miyamoto [2]

[1] *Department of Hospital Pharmacy, Nagoya University School of Medicine, Tsurumai-cho, Showa-ku, Nagoya (Japan) and*
[2] *Research Laboratory for Development of Medicine, School of Pharmacy, Hokuriku University, Kanazawa (Japan)*

(Received 26 October 1988)
(Modified version received 23 May 1989)
(Accepted 6 June 1989)

Key words: 1-Methyl-3-propylxanthine; Enprofylline; Protein binding; Free fatty acid; Affecting factor; pH-dependency; Albumin concentration

Summary

The binding to human serum albumin (HSA) of two drugs which are chemically related to theophylline, 1-methyl-3-propylxanthine (MPX), a new bronchodilator, and 3-propylxanthine (enprofylline), was investigated in vitro using an ultrafiltration method. The binding process involves one class of binding sites, with $n = 2$ and $K_{d1} = 0.403$ mM and $n = 1$ and $K_{d1} = 0.906$ mM for MPX and enprofylline, respectively. The binding of both drugs to HSA was shown to be dependent on pH with approximately 20% of MPX and 60% of enprofylline unbound at pH 6.60 and 10% and 40%, respectively, unbound at pH 7.45. Albumin concentration also had a significant effect on the binding of both drugs. Elevation of free fatty acid concentrations in the HSA solution resulted in an increase in the free fraction of both drugs. A study using oleic acid as a representative inhibitor revealed that the inhibitory effect of free fatty acid on MPX binding is due to competition for binding sites. These results suggest that free fatty acid may play a role in the decreased binding of MPX. The findings indicate that factors such as these that affect the protein binding of both drugs could be of importance when using the drugs with asthmatic patients in various disease states.

Introduction

It is well-known that 1,3-dimethylxanthine (theophylline) exhibits a strong bronchial smooth muscle relaxant effect, which is much stronger than those of other known xanthine derivatives such as caffeine and theobromine. Furthermore, theophylline is widely used in the treatment of patients with reversible obstructive airway diseases (Mitenko and Ogilvie, 1973; Levy and Koysooko, 1975; Takagi et al., 1986).

As part of a program of research on bronchodilators, we were interested in developing compounds with a stronger relaxant effect by making chemical modifications of the xanthine molecule. We have previously reported the role of the alkyl chain length at the N3 position of the xanthine molecule in the inhibition of cyclic AMP phos-

Correspondence: T. Hasegawa, Department of Hospital Pharmacy, Nagoya University School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466, Japan.

phodiesterase (PDE) in isolated guinea pig tracheal smooth muscle (Takagi et al., 1988). We have also found that a new xanthine derivative, 1-methyl-3-propylxanthine (MPX), possesses a much stronger relaxant effect than either theophylline or enprofylline (Apichartpichean et al., 1988). In the comparative pharmacokinetics of MPX and theophylline, the following characteristics were observed: protein binding of MPX in rat plasma was much stronger than that of theophylline, renal excretion was very low compared to theophylline, and the apparent partition coefficient was remarkably higher than that of theophylline (Apichartpichean et al., 1988).

It is accepted that drugs unbound to serum (plasma) proteins are pharmacologically active and capable of diffusing across biological membranes. This binding can influence drug distribution, elimination kinetics and pharmacologic effects, and may be influenced by many factors such as age, disease, other drugs, blood pH, etc. Thus, information concerning protein binding behavior is useful in interpreting the characteristics and pharmacologic effects of a new drug.

A number of pharmacokinetic and pharmacodynamic studies of enprofylline in human subjects have been published (Borga et al., 1983; Laursen et al., 1983a and b, 1984; Lunell et al., 1984). Only one has described the protein binding of enprofylline in human plasma as being about 47% (Borga et al., 1983). However, the protein binding characteristics of both enprofylline and MPX are still not clearly understood.

In order to evaluate the advantages and disadvantages of MPX and enprofylline when compared to theophylline, the present study aims to identify and evaluate certain variables such as albumin concentration, pH and non-esterified fatty acids that may influence the serum protein binding of MPX and enprofylline.

Materials and Methods

Drugs and chemicals

1-Methyl-3-propylxanthine (MPX) and 3-propylxanthine (enprofylline) were synthesized in our laboratories, with both compounds being identical to those used in previous studies (Takagi et al., 1988; Apichartpichean et al., 1988). Theophylline, non-esterified fatty acid (FFA, oleic acid) and human serum albumin (HSA, Fraction V, A 1887), mol. wt. of 69,000, were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Determination of protein binding

The extent of protein binding of MPX and enprofylline was determined using a commercially available ultrafiltration method, MPS-1 (Amicon Corp., MA, U.S.A.). The membrane filter used retains compounds with molecular weights of more than 30,000. A preliminary experiment, in which drug concentrations in 0.067 M phosphate buffer (pH 7.4) and in the ultrafiltrate were measured, indicated that adsorption of drugs to the device or the membrane was negligible.

In all experiments, each drug in methanolic solution was dried down prior to adding HSA solution and was shaken gently for 2 h at room temperature.

Estimation of binding parameters. The concentration of HSA was adjusted to 579.71 μM (4 g/dl) with a 0.067 M phosphate buffer (pH 7.4), to which each drug was added at various concentrations.

Effect of pH. The pH of the HSA solution was adjusted to the desired pH (between 6.6 and 8.7) with 0.067 M KH_2PO_4 solution or 0.067 M Na_2HPO_4 solution. To the final concentration (579.71 μM) of HSA, each drug was added at 3 different concentrations (5, 10 and 20 μg/ml).

Effect of albumin concentration. Five different concentrations (0.9, 1.8, 3.6, 5.4 and 7.2 g/dl) of HSA were prepared with the same buffer solution, to which each drug had been added at a constant concentration (10 μg/ml).

A possible inhibition of MPX binding was studied both in the presence and absence of free fatty acid (FFA), using oleic acid at 3 different concentrations (0.971, 1.456 and 2.912 mM). The oleic acid was added to the respective drug-spiked HSA solutions (10 μg/ml).

In each of the different experiments, an aliquot (0.4 ml) of the respective HSA solutions was poured into the upper reservoir cup and centrifuged at room temperature for 8 min at 2000 $\times$ g.

The total and free (ultrafiltrate) drug concentrations were measured by high-performance liquid chromatography (HPLC).

In the ultrafiltration method, the unbound drug fraction was calculated by the following equation:

$$F(\%) = \frac{\text{drug concentration in ultrafiltrate}}{\text{drug concentration before ultrafiltration}} \times 100 \quad (1)$$

HPLC assay

The concentration of MPX was determined by the method previously reported (Apichartpichean et al., 1988). The concentration of enprofylline was determined by a modification of the method for determination of MPX, using 8-chlortheophylline as the internal standard. Separation was carried out on a Zorbax ODS column (Du Pont Instruments, U.S.A.) with an eluent of 0.01 M sodium acetate buffer solution (pH 4.0)–acetonitrile (82/18 for MPX and 88/12 for enprofylline v/v). Drug concentrations were calculated from their relative peak height ratios based on a standard curve.

Data analysis

Protein binding data were fitted to the following equation using the non-linear least-squares method program, MULTI, written by Yamaoka et al. (1981).

$$C_b = \frac{n_1 P_1 C_f}{K_{d1} + C_f} \quad (2)$$

where C_b and C_f are the concentrations of the bound drug and the unbound drug, respectively. $n_1 P_1$ is the binding capacity of the first class of binding sites, and K_{d1} is the dissociation constant for the first class of binding sites.

Data obtained with an inhibitor (oleic acid) were fitted to the following Klotz-type model (Klotz and Hunston, 1975; Kragh-Hansen, 1981) assuming one specific binding site:

$$\frac{1}{r_{drug}} = \frac{K_{d'drug}}{n}\left(1 + \frac{C_{f'FFA}}{K_{d'FFA}}\right)\frac{1}{C_{f'drug}} + \frac{1}{n} \quad (3)$$

where r_{drug} is the average number of molecules of bound drug per molecule of HSA; $K_{d'drug}$ and $K_{d'FFA}$ are the dissociation constants of drug and FFA, respectively; n is the number of binding sites; $C_{f'drug}$ and $C_{f'FFA}$ are the respective unbound concentrations of drug and FFA (Kragh-Hansen, 1981). The affinity constants of drug ($K_{a'drug} = 1/K_{d'drug}$) and n were graphically estimated from Eqn. 3 by computer-aided non-linear least-squares regression analysis (Yamaoka et al., 1981).

Apparent partition coefficient

Each drug was dissolved at a concentration of 10 μg/ml in pH 7.4 phosphate-buffered saline (PBS). Five ml of the PBS solution were added to an equal volume of *n*-octanol, and equilibrated at 25 °C by continuous shaking for 2 h. A preliminary test was carried out to confirm the equilibrium of these drug solutions in aqueous phase after 2 h shaking compared to those of 3 h. The concentration of each drug in the aqueous phase was determined by spectrophotometry at 278 nm. Hydrophobicity was expressed as a logarithmic partition coefficient (log PC).

Results and Discussion

Computer estimates of binding parameters calculated by the non-linear least-squares method, assuming one class of independent binding sites, are listed in Table 1. Over a range of 7.60 to 704.37 μM for MPX and 12.1 to 525.5 μM for enprofylline, the free fraction (%) to 579.71 μM HSA remained almost constant. The free fraction mean (±S.D.) values are 27.6 ± 3.4% for MPX

TABLE 1

Binding parameters of MPX and enprofylline to human serum albumin

Drug	$n_1 P_1$ (mM)	K_{d1} (mM)
MPX	1.201 ± 0.143	0.403 ± 0.063
Enprofylline	0.472 ± 0.114	0.906 ± 0.295

Each value represents the computer-estimated parameter ± S.D. The concentration of human serum albumin was 4 g/dl.

and 70.2 ± 1.9% for enprofylline. Scatchard plots for the two drugs are linear, and the bound versus unbound plots, according to Eqn. 2, also showed one class of binding sites with $n = 2$ and $n = 1$ for MPX and enprofylline, respectively. Differences were observed in the binding characteristics to HSA between MPX and enprofylline. That is, the binding affinity of MPX was approximately twice as great as that of enprofylline. In addition, the binding capacity to HSA of MPX was also found to be higher than that of enprofylline. Laznicek et al. (1987) have reported the relationships between plasma protein binding and hydrophobicity in some structurally similar drugs (i.e. the stronger the hydrophobicity, the higher the extent of drug binding to plasma protein). In the present study, we compared the partition coefficients of MPX, enprofylline and theophylline. The order of log PC was MPX (1.022) > enprofylline (0.331) > theophylline (−0.042) which is in agreement with the extent of these drugs' binding to HSA. These facts confirm that the increase of hydrophobicity of structurally similar drugs increases the extent of protein binding.

The effect of pH on the binding of the two drugs at a constant concentration (10 μg/ml) to HSA is shown in Fig. 1. In both drugs, the free fraction increased as pH decreased. Free fraction values increased by approximately 83% and 58% for MPX and enprofylline, respectively, when the pH was decreased from 7.45 to 6.60. In addition, no differences were observed in the increases of free fractions between measurements taken at 10, 5 or 20 μg/ml (data not shown). These results suggest that the binding of both drugs to HSA is also pH-dependent as reported previously in a study on the protein binding of theophylline (Vallner et al., 1979; Shaw et al., 1982; Buss et al., 1983) but not concentration-dependent within the range studied. The effect of pH on the protein binding of theophylline was first reported by Vallner et al. (1979). We have also confirmed that theophylline binding to plasma protein is highly pH-dependent. Therefore, we proposed a calculation equation for predicting free fractions at physiological plasma pH 7.4 from the plasma in asthmatic patients (Johno et al., 1984). The change in the binding caused by pH is surmised to be related to a change in the conformation of the albumin molecule (N–B transition) and/or a change in the ionization of the drug. For example, since theophylline is a weak acid with a pK_a of 8.77, the fraction ionized will decrease considerably as pH is decreased to within the physiological range. In the present study, the binding of both drugs to HSA significantly increased based on pH values ranging from 6.6 to 8.7. This degree of alteration in binding is comparable with the HSA N–B transition, but the fact that both drugs are weak acids makes it unlikely that some alteration in ionization of the drugs was responsible for the changes in binding.

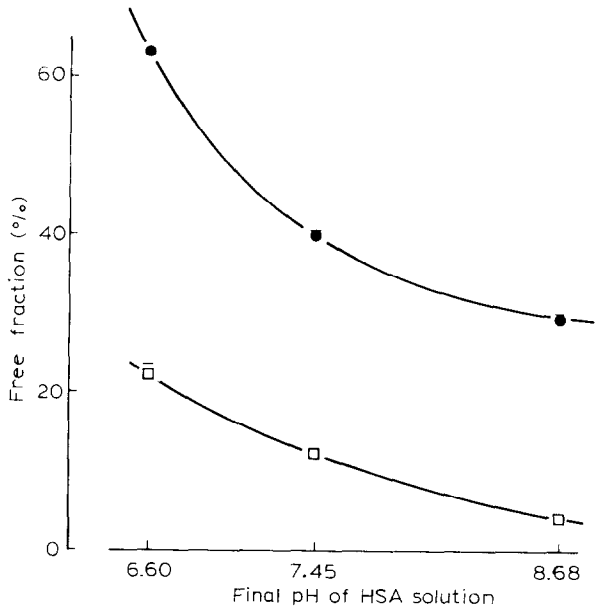

Fig. 1. Effect of pH on protein binding of MPX and enprofylline. The values are the mean ± S.D. of 3 experiments. The concentration of HSA used was 4 g/dl (579.71 μM). Symbols of combined drugs: □, 1-methyl-3-propylxanthine (MPX); ●, enprofylline.

When the binding of the two drugs to HSA was studied with varying albumin concentrations at ranges from 0.9 to 7.2 g/dl (130.4 to 1043.4 μM) at a constant drug concentration (10 μg/ml), the free fraction of the two drugs was shown to increase with decreasing albumin concentration (Fig. 2). The results indicate that the protein binding of both drugs is protein (albumin) concentration-dependent in the same way as theophylline (Buss et

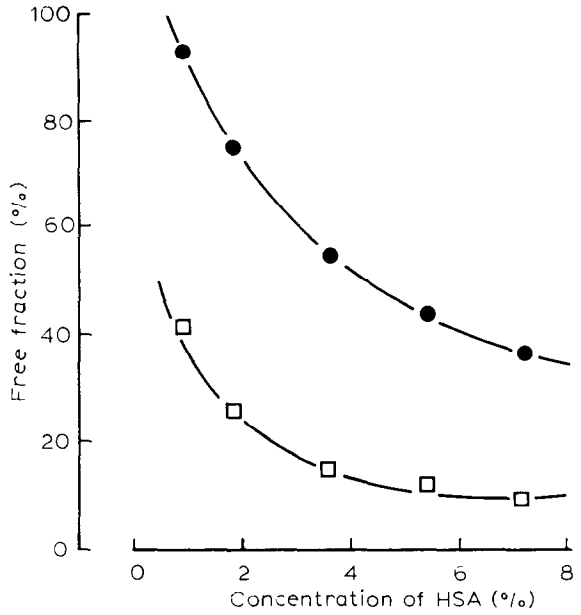

Fig. 2. Effect of concentration of HSA on protein binding of MPX and enprofylline. The concentration of the two drugs used was 10 μg/ml. The values are the mean of 3 experiments. Symbols are the same as Fig. 1.

al., 1983; Ebden et al., 1984). On the basis of these results, the effects of pH and albumin concentration on protein binding of both drugs could be of importance in the use for asthmatic patients with various disease states, just as with theophylline.

Methylxanthines, which are structurally similar to the two drugs, have been reported to elevate the concentration of FFA in plasma (Bellet et al., 1965; Patwardhan et al., 1980). On the other hand, FFA has been shown to affect the plasma protein binding of many drugs (Spector and Santos, 1973; Wood et al., 1979; Giacomini et al., 1980). Therefore, a possible inhibition of the binding of the two drugs to HSA was studied both in the presence and absence of non-esterified fatty acid (FFA; oleic acid), a factor which has also been mentioned in connection with the serum protein binding of theophylline (Buss et al., 1983). The changes in the free fraction of the two drugs in the presence of oleic acid are shown in Fig. 3. Particularly, the free fraction of the two drugs was significantly higher when oleic acid was present in the HSA solution at high concentrations (2.912 mM), and the effect of FFA on MPX binding was much stronger than that of enprofylline within the range studied. These results indicate that the free fraction of the two drugs increased as FFA concentration in the serum increased. The difference in the degree of the effect between the two drugs may be due to the presence and absence of the alkyl group (methyl) in the N1 position of the xanthine molecule or it could be related to the affinity of drug binding sites. In addition, Fig. 4 shows that the inhibitory effect of FFA (oleic acid) is a directly competitive function, with FFA acting as a representative displacer to prevent binding of MPX to protein. The higher the concentration of oleic acid, the greater was the slope of the curve, although the intercepts were not altered. The calculated value of n was shown to be between 2.13 and 3.44 with K_a ranging from 1.71 to 0.13 (1/mM) both in the presence and absence of oleic acid. It is well known that FFA binds to human serum albumin with high affinity and acts to modify the affinity of drug binding sites rather than displace drugs

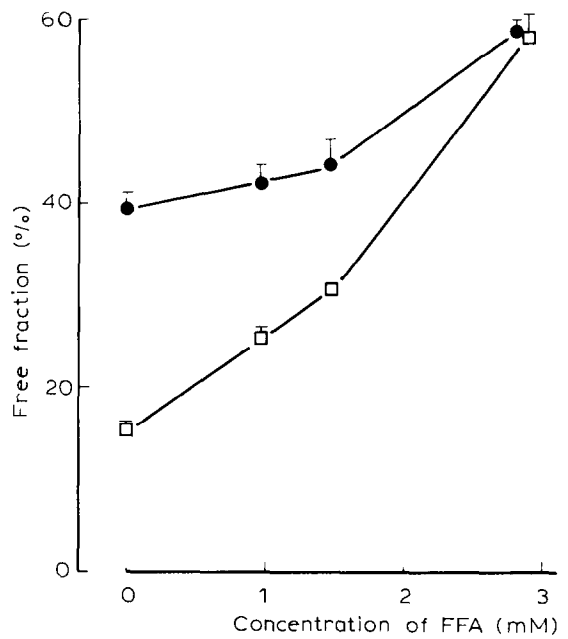

Fig. 3. Effect of free fatty acid on protein binding of MPX and enprofylline. Mean free fractions of the two drugs are shown in the absence and presence of oleic acid with a bar of S.D. of 3 experiments. The concentrations of oleic acid used were 0.971, 1.456 and 2.912 mM, respectively. Symbols are the same as Fig. 1.

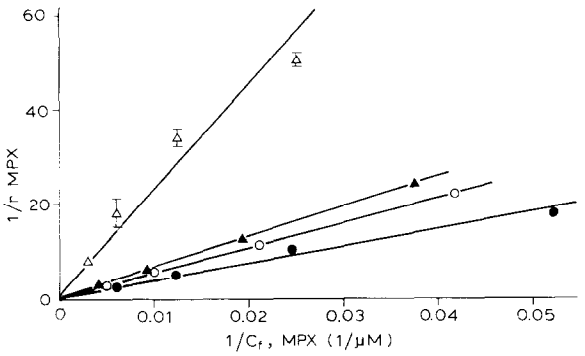

Fig. 4. Double reciprocal (Klotz-type) plot for MPX binding to HSA in the absence and presence of oleic acid. Symbols represent mean values in addition of 0 (●), 0.971 (○), 1.456 (▲) and 2.912 mM (△), respectively, with bars of S.D. of 3 experiments. Data points without bars show that S.D. were smaller than the size of the points.

directly (Spector, 1975). In the present study, the Klotz plot (Klotz et al., 1975; Kragh-Hansen, 1981) was used to determine that the inhibiting action of oleic acid is due to its competition with MPX for binding to HSA. The figure also suggests that the inhibition by oleic acid at higher concentration may be due to other mechanism in addition to competitive inhibition.

It is well known that methylxanthines are inhibitors of cyclic AMP phosphodiesterase and an increase in the level of cyclic AMP in adipose tissue stimulates lipolysis (Butcher and Sutherland, 1962). We speculate, therefore, that the two drugs used in this study elevate FFA since these drugs are much stronger cyclic AMP phosphodiesterase inhibitors than other methylxanthines such as theophylline, theobromine and caffeine (Hasegawa, unpublished data). However, further investigation whether the two drugs also elevate the concentration of FFA in plasma is needed.

This report is the first to study the inhibiting mechanism of FFA on the protein binding of drugs which are structurally similar to theophylline. These results indicate that it is likely that FFA significantly inhibits the binding of MPX by competing for the same binding sites.

In conclusion, FFA may play a significant role in the decreased binding of the two drugs. The present data should prove useful in predicting the role of protein binding on the pharmacokinetics and pharmacodynamics of the two drugs under various conditions.

References

Apichartpichean, R., Takagi, K., Nadai, M., Kuzuya, T., Ogawa, K., Miyamoto, K. and Hasegawa, T., Studies on alkyl-xanthine derivatives II. Pharmacokinetic and pharmacodynamic studies of a new bronchodilator, 1-methyl-3-propylxanthine (MPX). *Jap. J. Pharmacol.*, 48 (1988) 341–347.

Bellet, S., Kershbaum, A. and Aspe, J., The effect of caffeine on free fatty acids. *Arch. Int. Med.*, 116 (1965) 750–752.

Borga, O., Andersson, K.-E., Edholm, L.-E., Fagerstrom, P.-O., Lunell, E. and Persson, C.G.A., Enprofylline kinetics in healthy subjects after single doses. *Clin. Pharmacol. Ther.*, 34 (1983) 799–804.

Buss, D., Leopold, D., Smith, A.P. and Routledge, P.A., Determinations of the plasma protein binding of theophylline in health. *Br. J. Clin. Pharmacol.*, 15 (1983) 399–405.

Butcher, R.W. and Sutherland, E.W., Adenosine 3′,5′-phosphate in biological materials. I. Purification and properties of cyclic 3′,5′-nucleotide phosphodiesterase and the use of this enzyme to characterize adenosine 3′,5′-phosphate in human urine. *J. Biol. Chem.*, 237 (1962) 1244.

Ebden, P., Leopold, D., Smith, A.P., Buss, D. and Routledge, P.A., Free and total plasma theophylline concentrations in chronic airflow obstruction. *Thorax*, 39 (1984) 352–355.

Giacomini, K.M., Giacomini, J.C. and Blaschke, T.F., Absence of effect of heparin on the binding of prazosin and phenytoin to plasma proteins. *Biochem. Pharmacol.*, 29 (1980) 3337–3340.

Johno, I., Ogura, Y., Hasegawa, T., Yokochi, Y., Hasegawa, M., Kitazawa, S., Takagi, K. and Satake, T., Clinical evaluation of theophylline binding to plasma protein in bronchial asthmatic patients. *J. Clin. Hosp. Pharm.*, 9 (1984) 231–239.

Klotz, I.M. and Hunston, D.L., Protein interactions with small molecules: relationships between stoichiometric binding constants, site binding constants, and enapirical binding parameters. *J. Biol. Chem.*, 250 (1975) 3001–3009.

Kragh-Hansen, U.K., Molecular aspects of ligand binding to serum albumin. *Pharmacol. Rev.*, 33 (1981) 17–53.

Laursen, L.C., Johannesson, N., Fagerstrom, P-O. and Weeke, B., Intravenous administration of enprofylline to asthmatic patients. *Eur. J. Clin. Pharmacol.*, 24 (1983a) 323–327.

Laursen, L.C., Johannesson, N., Dirksen, A., Djurup, R., Munch, E.P., Taudorf, E. and Weeke, B., Enprofylline – effects of a new bronchodilating xanthine derivative in asthmatic patients. *Allergy*, 38 (1983b) 75–79.

Laursen, L.C., Johannesson, N. and Weeke, B., Continuous treatment of asthmatic patients with enprofylline and theophylline. *Eur. J. Respir. Dis.*, 65 (1984) 504–508.

Laznicek, M., Kvetina, J., Mazak, J. and Krch, V., Plasma protein binding-lipophilicity relationships: interspecies comparison of some organic acid. *J. Pharm. Pharmacol.*, 39 (1987) 79–83.

Levy, G. and Koysooko, R., Pharmacokinetic analysis of the effect of theophylline on pulmonary function in asthmatic children. *J. Pediatr.*, 86 (1975) 789–793.

Lunell, E., Andersson, K.-E., Persson, C.G.A and Svedmyr, N., Intravenous enprofylline in asthma patients. *Eur. J. Respir. Dis.*, 65 (1984) 28–34.

Mitenko, P.A. and Ogilvie, R.I., Rational intravenous doses of theophylline. *N. Engl. J. Med.*, 289 (1973) 600–603.

Patwardhan, R.V., Desmond, P.V., Johnson, R.F., Dunn, G.D., Robertson, D.H., Hoyumpa, A.M. and Schenker, S., Effects of caffeine on plasma free fatty acids, urinary catecholamines, and drug binding. *Clin. Pharmacol. Ther.*, 28 (1980) 398–403.

Shaw, L.M., Fields, L. and Mayock, R., Factors influencing theophylline serum protein binding. *Clin. Pharmacol. Ther.*, 30 (1982) 490–496.

Spector, A.A. and Santos, E.C., Influence of free fatty acid concentration on drug binding to plasma albumin. *Ann. N.Y. Acad. Sci.*, 226 (1973) 247–258.

Spector, A.A., Fatty acid binding to plasma albumin. *J. Lipid Res.*, 16 (1975) 165–179.

Takagi, K., Nosaka, H., Satake, T., Hasegawa, T., Ogura, Y. and Mizukami, Y., Studies on the pharmacokinetics of theophylline and its effect on respiratory impedance in asthmatic patients after multiple oral dosing of a sustained-release theophylline tablet formulation. *Jpn. J. Allergol.*, 35 (1986) 320–327.

Takagi, K., Hasegawa, T., Kuzuya, T., Ogawa, K., Watanabe, T., Satake, T., Miyamoto, K., Wakusawa, S. and Koshiura, R., Structure–activity relationship in N^3-alkyl-xanthine derivatives. *Jap. J. Pharmacol.*, 46 (1988) 373–378.

Vallner, J.J., Speir, W.A., Kolbeck, R.C., Harrison, G.N. and Bransome, E.D., Effect of pH on the binding of theophylline to serum proteins. *Am. Rev. Respir. Dis.*, 120 (1979) 83–86.

Wood, M., Shand, D.G. and Wood, A.J.J., Altered drug binding due to the use of indwelling heparinized cannulas (heparin lock) for sampling. *Clin. Pharmacol. Ther.*, 25 (1979) 103–107.

Yamaoka, K., Tanigawara, Y., Nakagawa, T. and Uno, T., A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobio-Dyn.*, 4 (1981) 879–885.