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MUTUAL DISPLACEMENT INTERACTIONS IN THE BINDING OF TWO DRUGS TO HUMAN SERUM ALBUMIN BY FRONTAL AFFINITY CHROMATOGRAPHY

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SUMMARY

A continuous frontal analysis chromatographic method was developed for studying the simultaneous binding of two drugs or ligands with an immobilized macromolecule. The usefulness of this method was demonstrated in the interactions of sulphamethizole and salicylic acid with human serum albumin (HSA). The mutual inhibitory effect on the binding of one drug of the presence of the other was directly shown to be due to displacement of the bound drug from HSA by the other. On the basis of a double-reciprocal plot analysis, these two drugs are interpreted as competing for the same primary binding sites.

INTRODUCTION

Of the many causes of drug interactions, the interaction thought to be caused by displacement of one drug from serum albumin by another is recognized to be clinically important in multiple dose therapy[1]. Because the concentrations of the free or unbound fraction of two drugs administered concurrently may increase by mutual displacement from albumin over those when administered alone, the consequences of this interaction can affect many aspects of drug disposition and lead to enhanced pharmacological and toxicological responses. One of the well known examples of such an interaction is that between phenylbutazone and warfarin[2,3]. A marked argumentation of hypothrombinaemia and fatal haemorrhages were reported when phenylbutazone was coadministered with warfarin[4].

The displacement interactions are interpreted as either competitive or non-competitive[5]. In competitive displacement, two drugs are considered to share the same binding sites, whereas in non-competitive displacement the binding of the displacing drug takes place at sites other than those of the displaced drug and induces structural changes in the tertiary conformation of albumin (also called allosteric mechanism[6]). Such a distinction is often made on the basis of the Scatchard plot or double-reciprocal plot and the indication of an equal number of binding sites is taken as a criterion for competitive binding or displacement[7–10]. The limitation of this approach, unless the experimental procedures permit the concentrations of the free

displacing drug to be constant, has been fully discussed[11,12]. For instance, in subtractive methods[13] such as equilibrium dialysis, it is difficult to keep the concentrations of the free displacing drug constant and experiments have to be carried out in the presence of excess amounts of the displacing drug so that this drug will not be displaced to any significant extent by the original drug. Under these conditions, however, effect of the original drug on the interaction of the displacing drug could not be observed. Only a few experimental procedures which permit the concentration of displacing drug to be set at a pre-determined value have been utilized to study the binding of two drugs with albumin. These include a continuous ultrafiltration technique[14], flow equilibrium dialysis[15] and Hummel and Dreyer's gel filtration technique[16].

We have previously demonstrated[17–19] the use of immobilized bovine and human serum albumin in the quantitative studies of serum albumin–single drug interactions by a frontal affinity chromatographic procedure and observed that the binding capacity of the albumins was retained on immobilization when a six-carbon-atom spacer was introduced between the albumin molecule and gel matrix. This study extends this technique to the interactions of two drugs with human serum albumin (HSA). The method allows a simple and direct manifestation of the mutual displacement phenomena and also facilitates the quantitative treatment of binding data of two-drug interactions by the conventional double-reciprocal plot. This is because in the present method the concentrations of the free fraction of both drugs are set at pre-determined values and the amounts of drugs bound are simultaneously determined for the two drugs.

As one of two drugs we selected salicylic acid, as its binding to serum albumin *in vitro* has been extensively studied and it is reported to be displaced by many drugs[20–22], including sulphonamides, sulphonylureas, warfarin[12] and indomethacin[15]. Further, when aspirin is administered, salicylic acid is expected to be present in plasma owing to its rapid hydrolysis. Sulphamethizole, a urinary tract antiseptic, was also chosen because of the possibility of its co-administration with aspirin and its analytical convenience.

EXPERIMENTAL

Materials

Human serum albumin (HSA) was purchased from Sigma (St. Louis, Mo., U.S.A.) (Fraction V, essentially fatty acid free, lot no. 76C-7480). It was fractionated in 0.1 *M* sodium chloride solution on Sephadex G-200 obtained from Pharmacia (Uppsala, Sweden) as described elsewhere[19]. The monomer fraction which contained over 93% of monomer was used for coupling to agarose beads. Activated CH-Sepharose 4B was purchased from Pharmacia.

Salicylic acid from Wako (Osaka, Japan) was recrystallized from hot water. Sulphamethizole from Eizai (Tokyo, Japan) was recrystallized from a mixture of water and methanol. All other chemicals were of reagent grade. Water was deionized and doubly distilled, with the second distillation performed in an all-glass apparatus.

Affinity columns

The monomeric HSA was coupled to activated CH-Sepharose 4B at pH 8

according to the procedure recommended by the manufacturer by reacting them for 1 h at room temperature[19]. The HSA-coupled gel was packed into a Pharmacia column (K16/20) with flow adaptors so that the direction of flow could be varied. Usually upwards elution was carried out by means of Pharmacia Model P-3 peristaltic pump at a rate of about 10 ml/h. The temperature of the gel was maintained at 4° with water circulated through the jacket of the column by a Haake Model FK 10 constant-temperature circulator.

Determination of drugs bound by frontal affinity chromatography for two-drug interactions

All binding experiments were carried out in 0.05 *M* Tris–hydrochloric acid buffer containing 0.1 *M* sodium chloride, ionic strength 0.142. The pH of this buffer was 7.40 ± 0.01 at 20° and 7.87 ± 0.01 at 4°. When the columns were not in use they were constantly washed with the above buffer containing 0.01 % of sodium azide. Prior to use they were pre-equilibrated with the buffer for at least 16 h.

Three types of frontal analyses (I–III) were performed and the eluate was collected in 4.4–6.1-ml fractions by means of a LKB Model 2112 Redirack fraction collector until the concentrations of both drugs applied to the column reached those of the applied solution (see Fig. 1).

In Type I (simultaneous application), a solution containing both salicylic acid and sulphamethizole at known concentrations was applied to the column. In Type II (displacement of sulphamethizole by salicylic acid), a solution containing sulphamethizole at a known concentration was applied to the column followed by a solution containing sulphamethizole at the same concentration as that of the preceding solution and salicylic acid at a known concentration. In Type III (displacement of salicylic acid by sulphamethizole), the same procedure as Type II was used except that a salicylic acid solution was applied first followed by a solution containing both.

In each instance the concentrations of sulphamethizole and salicylic acid in the eluate were determined by dual-wavelength spectrophotometry on a Shimadzu Model UV-300 double-beam spectrophotometer using either 2- or 10-mm path length cells, depending on the concentrations of the two drugs. For sulphamethizole the two wavelengths were 260.0 and 323.0 nm, and for salicylic acid 230.0 and 295.8 nm.

The amounts of drug X bound, $(D_b)_x$, by the above three procedures are indicated in Fig. 1; the areas were determined by planimetry. For S-shaped elution patterns, $(D_b)_x$ was also determined by the formula $(V - V_0) \cdot (D_f)_x$, where $(D_f)_x$ is the concentration of drug X applied to the column, V is the elution volume of drug X determined as the volume of eluate corresponding to the concentration that reached 50% of that of the applied solution and V_0 is the void volume of the column, which is the sum of the volume of gel bed available for eluting solutions and that of the tubing. The void volume was determined for each column by eluting the column with 0.1% sodium azide solution[19]. The elution volume of this solution agreed to within about 2% with the calculated value obtained by measuring the volume of gel bed and internal volume of tubing.

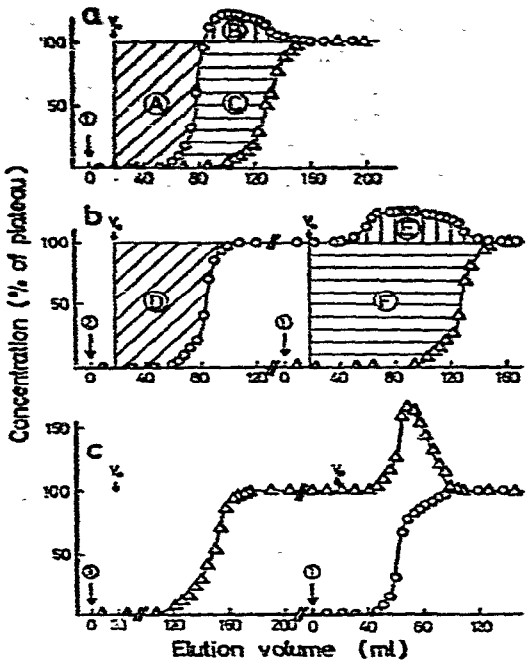

Fig. 1. Frontal analysis diagrams to illustrate mutual displacement interactions in the simultaneous binding of sulphamethizole and salicylic acid to immobilized HSA in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87 at 4°). (a) Type I analysis. Solution 1, containing sulphamethizole ($2.94 \cdot 10^{-5}$ *M*) and salicylic acid ($2.17 \cdot 10^{-5}$ *M*), was applied to column I (see Table I for details). (b) Type II analysis. Solution 2, containing sulphamethizole ($2.94 \cdot 10^{-5}$ *M*), was applied to the column, followed by solution 1. (c) Type III analysis. Solution 3, containing salicylic acid ($2.17 \cdot 10^{-5}$ *M*), was applied to the column, followed by solution 1. ○, Elution of sulphamethizole; △, elution of salicylic acid. The amounts of sulphamethizole and salicylic acid bound were determined as follows: for Type I analysis, $(D_b)_{SMZ} = (A - B) \cdot 10^{-5} \cdot (D_t)_{SMZ}$ and $(D_b)_{SA} = (A + C) \cdot 10^{-5} \cdot (D_t)_{SA}$; for Type II analysis, $(D_b)_{SMZ} = (D - E) \cdot 10^{-5} \cdot (D_t)_{SMZ}$ and $(D_b)_{SA} = F \cdot 10^{-5} \cdot (D_t)_{SA}$; for Type III analysis, analogous to Type II analysis, where A – E denote the areas indicated and V_0 is the void volume of the column.

Continuous frontal analyses

After the column had been equilibrated with an applied solution, instead of completely washing the column with buffer to release all of the drugs bound, continuous frontal analysis was carried out by combining Types II and III.

Treatment of data

When the binding of drug 1 is inhibited by the presence of drug 2 by competitive binding to a single class of n binding sites, the number of moles of drugs 1 and 2 bound per mole of albumin are given by eqns. 1 and 2, respectively[23]:

$$r_1 = \frac{n K_1 (D_t)_1}{1 + K_1 (D_t)_1 + K_2' (D_t)_2} \quad (1)$$

$$r_2 = \frac{n K_2 (D_t)_2}{1 + K_2' (D_t)_1 + K_2 (D_t)_2} \quad (2)$$

where K_1 and K_2 are the binding constants of drugs 1 and 2, respectively, K_1' and K_2' are the inhibition constants of drugs 1 and 2, respectively, and $(D_f)_1$ and $(D_f)_2$ are the concentrations of free or unbound drugs 1 and 2, respectively.

Rearrangement of eqn. 1 gives eqn. 3, according to which the double-reciprocal plots were constructed for drug 1:

$$\frac{1}{r_1} = \frac{1}{n} + \frac{1 + K_2'(D_f)_2}{n K_1} \cdot \frac{1}{(D_f)_1} \tag{3}$$

The values of K_2' were determined from eqn. 4:

$$K_2' = \left(\frac{S}{S_0} - 1\right) \frac{1}{(D_f)_2} \tag{4}$$

where S and S_0 are the slopes of eqn. 3 when $(D_f)_2 \neq 0$ and $(D_f)_2 = 0$, respectively. The equation for K_1' was similarly derived from eqn. 2.

In the plots, the data points were fitted to straight lines by linear regression analysis using the values on the abscissa as the independent variable and the values on the ordinate as the dependent variable.

RESULTS AND DISCUSSION

Fig. 2 shows the binding characteristics of sulphamethizole and salicylic acid to the immobilized HSA monomer in the form of a Scatchard plot. As the pK_a values of salicylic acid and sulphamethizole are reported to be 3.0[24] and 5.4[25], respectively, both drugs exist as anions at pH 7.87. Because these plots are hyperbolic, they are likely to bind to more than one class of site. For the simulutaneous binding of these drugs, the experiments were therefore limited to low r values ($r < 1.6$), so that the plots can be considered to be linear and to represent mainly the binding to one class of high-affinity site.

The amounts of both drugs bound determined from the three frontal analysis diagrams shown in Fig. 1 corresponding to Types I–III for two pairs of fixed concentrations of both drugs are presented in Table I.

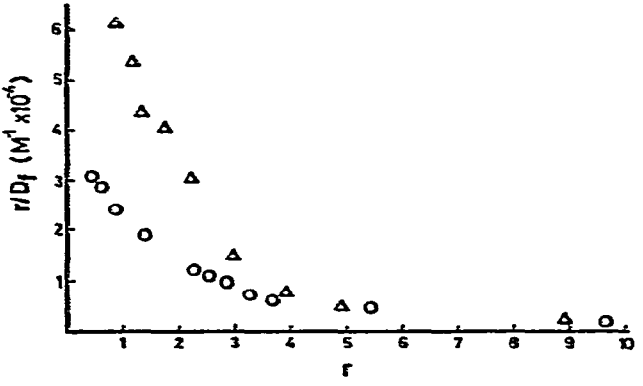

Fig. 2. Scatchard plots for single-drug interactions with immobilized HSA in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87 at 4°). ○, Sulphamethizole; △, salicylic acid.

TABLE I

COMPARISON OF DRUGS BOUND, D_b (MOLES × 10^6), OBTAINED FROM THREE FRONTAL ANALYSIS DIAGRAMS*

D_b determined at 4° in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH = 7.87 at 4°). SMZ and SA denote sulphamethizole and salicylic acid, respectively.

Type of frontal analysis	*Column I** $[(D_t)_{SMZ} = 2.94 \cdot 10^{-5}$ *M*, $(D_t)_{SA} = 2.17 \cdot 10^{-5}$ *M*]		*Column II** $[(D_t)_{SMZ} = 4.41 \cdot 10^{-5}$ *M*, $(D_t)_{SA} = 2.17 \cdot 10^{-5}$ *M*]	
	$(D_b)_{SMZ}$	$(D_b)_{SA}$	$(D_b)_{SMZ}$	$(D_b)_{SA}$
I, simultaneous application (Fig. 1a)	1.49	2.42	1.31	1.44
II, displacement of SMZ by SA (Fig. 1b)	1.49	2.37	1.33	1.37
III, displacement of SA by SMZ (Fig. 1c)	1.47	2.43	1.28	1.41

* The gel volumes of columns I and II were 13.7 and 9.1 ml, respectively.

Fairly good agreement of these values for each pair of concentrations of free drugs indicates a reversible nature of the binding of these drugs and the amount of the drugs bound can be determined from any of these three diagrams. Type I analysis (Fig. 1a) gives the usual frontal analysis diagram for two solutes[26] and shows that salicylic acid, with a stronger affinity, elutes behind the less strongly bound sulphamethizole, which is displaced by salicylic acid as the solution moves up the column. Therefore, this is analogous to Type II analysis (Fig. 1b), in which the displacement of sulphamethizole by salicylic acid is shown by the area indicated as E. The displacement of salicylic acid with sulphamethizole is shown in Fig. 1c by the appearance of the peak. The advantage of Types II and III over Type I is that the actual amount of drug displaced by another can be directly determined.

As the amounts of both drugs bound were independent of the three types of elution analysis, all binding studies were carried out continuously by the combination of Types II and III. Part of such a continuous analysis diagram is shown in Fig. 3. This sequence of applications of six different solutions containing either sulphamethizole or salicylic acid alone or together permitted the determinations of drugs

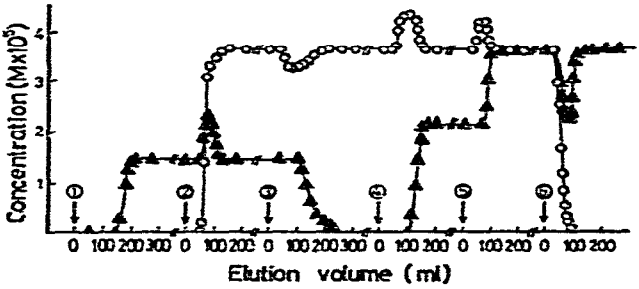

Fig. 3. Part of a continuous frontal analysis diagram from which the amounts of drugs bound were determined for both single-drug and two-drug interactions with immobilized HSA in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87 at 4°). ○, Elution of sulphamethizole; ▲, elution of salicylic acid. The arrows indicate where solutions 1–6 were applied: 1, $1.45 \cdot 10^{-5}$ *M* salicylic acid; 2, 1 + $3.65 \cdot 10^{-5}$ *M* sulphamethizole; 3, $3.65 \cdot 10^{-5}$ *M* sulphamethizole; 4, 3 + $2.17 \cdot 10^{-5}$ *M* salicylic acid; 5, 3 + $3.62 \cdot 10^{-5}$ *M* salicylic acid; 6, $3.62 \cdot 10^{-5}$ *M* salicylic acid.

bound in the corresponding solutions. In the presence of both drugs, the concentration of free sulphamethizole was kept constant while that of salicylic acid was varied. The peak or trough around the concentration of sulphamethizole which was kept constant ($3.65 \cdot 10^{-5}$ *M*) (Fig. 3) indicates the amount of the drug released or bound, respectively, with respect to the amount of the drug bound in equilibrium with the preceding solution. Further, by interposing an application of a single drug solution between solutions containing both drugs, as shown in Fig. 3 (solution 3 or 6, the following advantages are apparent: (1) the time lag between single-drug and two-drug is shortened; this is important if the leakage of the immobilized albumin is appreciable; and (2) when a solution containing two drugs was replaced with a single-drug solution, the analysis for the drug removed allows the determination of the amount of the drug released. Thus, a check can be made on the amount of the drug bound in equilibrium with the preceding solution.

Table II gives the results of both single-drug and two-drug interactions with the immobilized HSA. As this method allows the concentrations of free drugs to be maintained constant and both drugs were analysed simultaneously, the mutual displacement interaction can be readily seen by decreasing *r* values for the drug, the concentration of which was kept constant, with increasing concentration of the other.

TABLE II

MOLES OF DRUGS BOUND PER MOLE OF IMMOBILIZED HUMAN SERUM ALBUMIN MONOMER, *r*

Determined at 4° in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH = 7.87 at 4°). SMZ and SA denote sulphamethizole and salicylic acid, respectively. Some of these values are averages of two determinations and they were reproducible to within 5%.

$(D_f)_{SMZ}$ ($M \times 10^5$)	$(D_f)_{SA}$ ($M \times 10^5$)							
	0		1.45		2.17		3.62	
	r_{SMZ}	r_{SA}	r_{SMZ}	r_{SA}	r_{SMZ}	r_{SA}	r_{SMZ}	r_{SA}
0	—	—	—	0.891	—	1.16	—	1.57
1.46	0.450	—	0.360	0.799	0.332	1.07	0.284	1.47
2.19	0.629	—	0.495	0.760	0.457	1.03	0.401	1.42
3.65	0.876	—	0.732	0.698	0.678	0.969	0.601	1.36
7.30	1.38	—	1.19	0.604	1.13	0.829	1.03	1.19

Fig. 4 shows the double-reciprocal plots for both drugs according to eqn. 3. The binding and inhibition parameters that were calculated on the basis of these plots are summarized in Table III. All of the regression lines shown in Fig. 4 appear to show a common intercept on the ordinate and indicate that this displacement interaction is competitive. The degree of variation in the values of the intercepts is shown in Table III as the variation in the values of *n* which were obtained for each line as the reciprocals of the intercepts on the ordinate, together with the binding and inhibition constants for the two sets of data. The values of the inhibition constant of salicylic acid determined from the analyses of sulphamethizole in the presence of three different concentrations of free salicylic acid are comparable to its binding constant. The same can be said for the values of the inhibition constant of sulphamethizole determined from the salicylic acid data.

TABLE III

BINDING AND INHIBITION PARAMETERS CALCULATED FROM THE DOUBLE-RECIPROCAL PLOTS FOR INTERACTIONS OF SULPHAMETHIZOLE OR/AND SALICYLIC ACID WITH IMMOBILIZED HUMAN SERUM ALBUMIN MONOMER

Studied at 4° in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH = 7.87 at 4°). SMZ and SA denote sulphamethizole and salicylic acid, respectively. All parameters were calculated by regression analysis.

Interaction	*Sulphamethizole*				*Salicylic acid*			
	$(D_f)_{SA}$ $(M \times 10^5)$	K_{SMZ}* $(M^{-1} \times 10^{-4})$	K'_{SA}* $(M^{-1} \times 10^{-4})$	n	$(D_f)_{SMZ}$ $(M \times 10^3)$	K_{SA}* $(M^{-1} \times 10^{-4})$	K'_{SMZ}* $(M^{-1} \times 10^{-4})$	n
Single-drug interaction	0	1.4	—	2.7	0	2.7	—	3.2
Two-drug interaction	1.45	—	2.1	2.6	1.46	—	1.2	3.4
	2.17	—	2.0	2.6	2.19	—	1.2	3.4
	3.62	—	2.0	2.7	3.65	—	1.2	3.8
					7.30	—	0.93	3.4

* K and K' are the binding constant and inhibition constant, respectively.

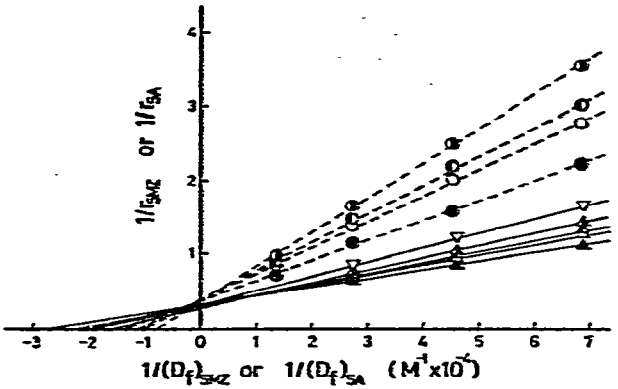

Fig. 4. Double-reciprocal plots according to eqn. 3 at low r values ($r < 1.6$) for single-drug and two-drug interactions with immobilized HSA in 0.05 M Tris buffer, containing 0.1 M NaCl (pH 7.87 at 4°). Broken lines indicate sulphamethizole data: in the absence of salicylic acid (●) and in the presence of free salicylic acid at $1.45 \cdot 10^{-5}$ M (○), $2.17 \cdot 10^{-5}$ M (◐) and $3.62 \cdot 10^{-5}$ M (◑). Solid lines indicate salicylic acid data: in the absence of sulphamethizole (▲) and in the presence of free sulphamethizole at $1.46 \cdot 10^{-5}$ M (△), $2.19 \cdot 10^{-5}$ M (◭), $3.65 \cdot 10^{-5}$ M (◮) and $7.30 \cdot 10^{-5}$ M (▽).

Therefore, this simple and fairly crude treatment of the data suggests that the mutual displacement interaction is attributable to the competitive binding of these two drugs to the same primary binding sites.

CONCLUSION

In view of potential hazards of coadministration of drugs related to mutual displacement from albumin, a simple and direct method in screening for scuh interactions is desirable at an early phase of drug development. The proposed method may be useful for this purpose as it gives a direct indication of the amount of drug displaced. The experimental procedure is very simple. Moreover, immobilized albumin can be used repeatedly.

For quantitative studies such as those presented here, the major concern is the stability of the covalent linkage between albumin and the gel matrix. All the present binding data obtained by continuous frontal analysis were obtained within 3 weeks on one column. The column remained stable during this period. However, the stability of the column varied from column to column, some being stable for several months and others for about 1 month. One of the solutions to this problem would be to shorten the analysis time further, for instance by performing the analysis under high pressure. Another possibility is to develop a method that allows corrections for the leaked albumin.

REFERENCES

1 P. D. Hansten, *Drug Interactions*, Lea and Febiger, Philadelphia, Pa., 3rd ed., 1976.
2 P. M. Aggeler, R. A. O'Reilly, L. Leong and P. E. Kowitz, *N. Engl. J. Med.*, 276 (1967) 496.
3 R. A. O'Reilly, *Ann. N.Y. Acad. Sci.*, 226 (1973) 293.
4 J. Koch-Weser and E. M. Sellers, *N. Engl. J. Med.*, 285 (1971) 487; 547.

5 J. Koch-Weser and E. M. Sellers, *N Engl. J. Med.*, 294 (1976) 526.
6 B. J. Soltys and J. C. Hsia, *J. Biol. Chem.*, 253 (1978) 3029.
7 P. Hsu, J. K. H. Ma and L. A. Luzzi, *J. Pharm. Sci.*, 63 (1974) 27.
8 H. M. Solomon, J. J. Schrogie and D. Williams, *Biochem. Pharmacol.*, 17 (1968) 143.
9 S. Goto, T. Hara and H. Yoshitomi, *Yakugaku Zasshi*, 96 (1976) 565.
10 Y. Kaneo, A. Nishikawa, Y. Kato and S. Kiryu, *Chem. Pharm. Bull.*, 27 (1979) 1335.
11 L. P. Simonson, H. P. Bar, B. Jastorff and R. Hitkar, *Biochem. Biophys. Acta*, 379 (1975) 114.
12 L. J. Aarons, W. L. Schary and M. Rowland, *J. Pharm. Pharmacol.*, 31 (1979) 322.
13 J. Steinhardt and J. A. Reynolds, *Multiple Equilibria in Proteins*, Academic Press, New York, 1968, p. 39.
14 D. S. Champion and R. Olsen, *J. Pharm. Sci.*, 63 (1974) 249.
15 D. Hultmark, K. O. Borg, E. Elofsson and L. Palmer, *Acta Pharm. Suecica*, 12 (1975) 259.
16 B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.*, 167 (1978) 159.
17 N. I. Nakano, T. Oshio, Y. Fujimoto and T. Amiya, *J. Pharm. Sci.*, 67 (1978) 1005.
18 N. I. Nakano, T. Oshio, N. Sato, Y. Shimamori and S. Yamaguchi, *Chem. Pharm. Bull.*, 27 (1979) 2048.
19 N. I. Nakano, S. Yamaguchi, Y. Shimamori and Y. Fujimoto, *99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo. 1979.*
20 M. Otagiri, and J. H. Perrin, *Biochem. Pharmacol.*, 26 (1977) 283.
21 Y. Kaneo, A. Nishikawa, Y. Kato and S. Kiryu, *Yakuhaku Zasshi*, 98 (1978) 1452.
22 A. Kober, B. Ekman and I. Sjöholm, *J. Pharm. Sci.*, 67 (1978) 107.
23 I. M. Klotz, H. Triwush and F. M. Walker, *J. Amer. Chem. Soc.*, 70 (1948) 2935.
24 A. Albert and E. P. Serjeant, *The Determination of Ionization Constants*, Chapman and Hall, London, 2nd ed., 1971, p. 89.
25 G. S. Avery (Editor), *Drug Treatment*, Adis Press, Sydney, 1976, p. 892.
26 S. Claesson, *Ann. N.Y. Acad. Sci.*, 49 (1948) 183.