Glycyrrhizin Binding Site on Human Serum Albumin

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The binding site of glycyrrhizin (GLZ) on human serum albumin was detected through competitive displacement experiments with GLZ and ibuprofen (IBU) (diazepam site), warfarin (WAR), salicylate (SAL) (digitoxin site), or deoxycholic acid (DCA) by means of an ultrafiltration technique. The specific binding of GLZ was subject to competitive inhibition by IBU, WAR, SAL, or DCA (1 or 4 mM). The extent of displacement was in the order of: DCA > IBU > WAR > SAL. Conversely, the specific bindings of WAR and DCA and the low-affinity bindings of IBU and SAL were subject to competitive inhibition by GLZ (1 or 4 mM). The extent of inhibition by GLZ was in the order of DCA > IBU > WAR ≒ SAL. In addition, the low-affinity IBU binding and the specific DCA binding showed mutual competitive inhibition at 4 mM, with almost identical displacements. It was concluded that the specific GLZ binding site on human serum albumin may be located mostly within the low-affinity IBU binding site area (probably the same as the specific DCA binding site area) and partially within the specific WAR binding site area and the low-affinity SAL binding site area.

Keywords glycyrrhizin; human serum albumin; binding site; ultrafiltration technique; competitive inhibition; ibuprofen; warfarin; salicylate; deoxycholic acid

Introduction

Previously we reported that glycyrrhizin (GLZ) binds to specific and nonspecific binding sites in human serum at concentrations respectively lower and higher than approximately 2 mM.[1] It was further found that the binding sites of GLZ in human serum exist mainly on albumin, and more than 99% of the drug binds to albumin over the serum concentration range of 2—60 μg/mg following i.v. administration of 80—200 mg/man.[1] If GLZ and other durgs can displace each other from human serum albumin (HSA) binding sites, such displacements may be of clinical importance, assuming that the increase of unbound drug results in a greater pharmacological effect. Many drugs have been shown to bind primarily to one of three distinct binding sites on HSA (diazepam site, warfarin site, or digitoxin site).[2-8] It has also been shown that bile acids may bind to a novel binding site on HSA which is different from the above three sites, fatty acid, or bilirubin binding sites.[9]

The purpose of this study was to identify the binding site of GLZ on HSA through competitive displacement experiments with GLZ and ibuprofen (diazepam site),[2,3] warfarin, and salicylate (digitoxin site)[8] as well as deoxycholic acid.

Experimental

Materials Monoammonium glycyrrhizinate (GLZ-NH_4) was kindly supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan). Ibuprofen (IBU), warfarin (WAR), sodium salicylate (SAL), and the total bile acids test kit were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Sodium deoxycholate (DCA) and HSA (fatty acid-free, fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were commercial products of analytical grade.

Binding of Ligands to HSA (1) HSA binding of GLZ in the presence of IBU, WAR, SAL, or DCA was determined by the same method (an ultrafiltration technique) as reported previously for GLZ alone.[1] IBU and WAR were dissolved initially in a small volume of 0.1 M NaOH. The final pH of the drug solutions was in the range of 7.4—7.5. HSA was dissolved in isotonic phosphate buffer solution (pH 7.4). One milliliter of 4.2% HSA solution containing GLZ-NH_4 (0.24—7.29 mM) and IBU, WAR, SAL, or DCA (1.0 or 4.0 mM each) was applied to a filtration membrane (Amicon Micropartition system, MPS-1, Danvers, MA) after incubation at 37°C for 5 min. Ultrafiltration of samples was accomplished by centrifugation (1000 *g*) at 37°C for a period (approximately 10 min) sufficient to produce an ultrafiltrate volume of approximately 20% of the initial sample. (2) HSA bindings of IBU (0.5—4.0 mM), WAR, SAL (0.3—4.0 mM each), and DCA (0.2—4.0 mM) in the absence and in the presence of GLZ (1.0 or 4.0 mM) were determined by the above method. (3) HSA bindings of IBU (0.5—4.0 mM) and DCA (2.0—4.0 mM) in each other's presence (4.0 mM) were also determined by this method. The applied HSA solutions (50 μl each) and their filtrates (200 μl each) were used for the determination of each drug. Adsorption of the drugs on the membrane and the leakage of macromolecules (HSA) were negligible.

Analytical Method The extraction procedure and high-performance liquid chromatographic (HPLC) method for GLZ in each sample were the same as those described previously,[10] as were those for IBU, WAR, and SAL in each sample, except that the volume ratio of the mobile phase, MeOH–0.05 M phosphate buffer solution (pH 2.1), and the ultraviolet wavelength for detection were modified as follows: 3 : 1 and 220 nm for IBU, 2 : 1 and 282 nm for WAR, and 1 : 1 and 304 nm for SAL. DCA was determined using a commercial kit.

Data Analysis The binding data of GLZ, WAR, and DCA and of IBU and SAL were subjected to curve fitting based on Eqs. 1 and 2, respectively, using a digital computer.[11] Data were weighted with the reciprocals of the molar ratio (r) of the bound drug to the binding protein, HSA, assuming a molecular weight of 69000.

$$r = \frac{n_s K_s C_f}{1 + K_s C_f} + \phi C_f \quad (1)$$

where K_s is the association constant corresponding to n_s, the number of specific binding sites, C_f is the free drug concentration, and ϕ is the linear binding coefficient.

$$r = \frac{n_1 K_1 C_f}{1 + K_1 C_f} + \frac{n_2 K_2 C_f}{1 + K_2 C_f} \quad (2)$$

where K_1 and K_2 are the association constants corresponding to n_1 and n_2, the numbers of primary and secondary binding sites, respectively. The binding parameters were calculated using a nonlinear iterative least squares method without parameter constraints.[11] The Scatchard plots from the binding data were resolved into two segments to obtain initial estimates of the binding parameters.[12]

Results

The GLZ binding site on HSA was detected through competitive displacement of GLZ with IBU, WAR, SAL, or DCA by means of an ultrafiltration technique. Figure 1 shows Scatchard plots of HSA binding data of GLZ in the absence and in the presence of IBU, WAR, SAL, or DCA at a concentration of 1 or 4 mM. The plot of GLZ alone was based on the binding data in the previous paper.[1] The specific GLZ binding was reduced by each of the

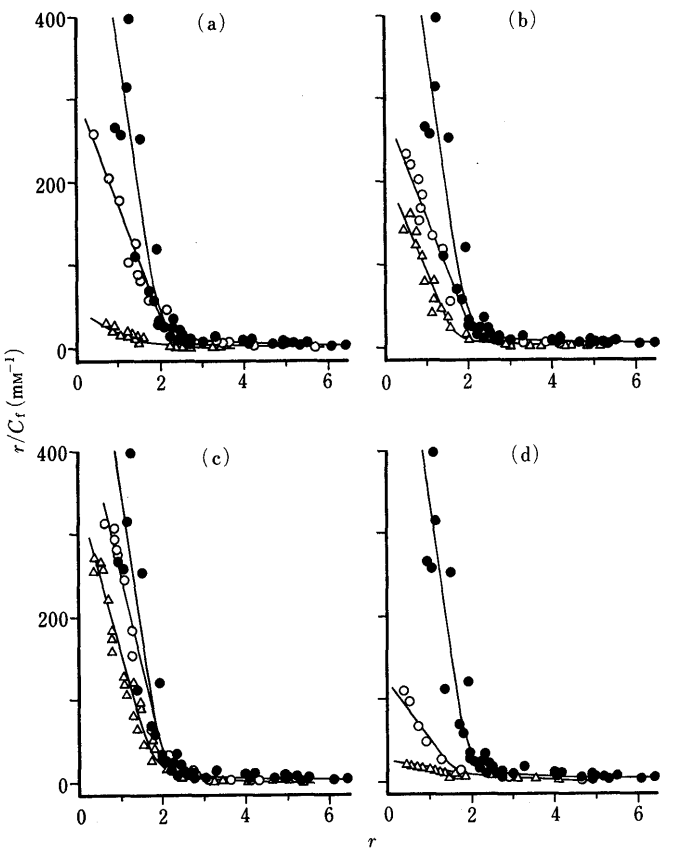

Fig. 1. Scatchard Plots for HSA Binding of GLZ in the Absence and in the Presence of IBU (a), WAR (b), SAL (c), and DCA (d)

●, GLZ alone[1]; ○, 1 mM inhibitors; △, 4 mM inhibitors. r, the molar ratio of the bound drug to 0.609 mM (4.2%) HSA; C_f, free drug concentration. The points represent the experimental values determined by an ultrafiltration technique. The solid lines are the curves simulated by the least squares method using a digital computer.[11]

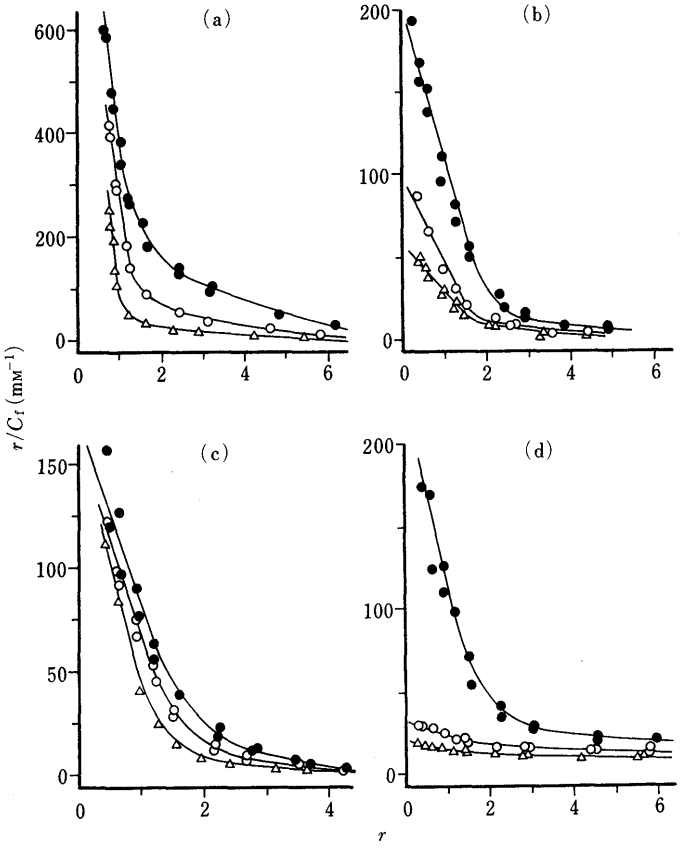

Fig. 2. Scatchard Plots for HSA Binding of IBU (a), WAR (b), SAL (c), and DCA (d) in the Absence and in the Presence of GLZ

●, drug alone; ○, 1 mM GLZ; △, 4 mM GLZ. For details of abbreviations, data points, and solid lines, see Fig. 1 caption.

TABLE I. Binding Parameters of GLZ to HSA in the Absence and in the Presence of Inhibitors Determined by an Ultrafiltration Technique

Drug	Inhibitor conc. (mM)	n_s[a]	$K_s \times 10^{-5}$[b] (M^{-1})	$\phi \times 10^{-3}$[c] (M^{-1})
GLZ alone[d]		1.95±0.01	3.87±0.94	3.09±0.24
+IBU	1.0	2.23±0.32	1.36±0.38	1.02±0.33
	4.0	1.68±0.69	0.25±0.20	0.46±0.29
+WAR	1.0	2.21±0.11	1.31±0.11	0.63±0.09
	4.0	1.82±0.20	1.26±0.33	0.58±0.08
+SAL	1.0	2.19±0.13	2.14±0.24	0.79±0.11
	4.0	1.95±0.23	1.92±0.51	0.81±0.09
+DCA	1.0	1.73±0.18	0.70±0.18	1.61±0.20
	4.0	2.29±0.38	0.11±0.03	1.17±0.28

The parameters (±S.D.) were calculated according to Eq. 1 by the least squares method.[11] *a*) The number of specific binding sites. *b*) The association constant corresponding to n_s. *c*) The linear binding coefficient. *d*) Reported values.[1]

other drugs. Fitting of the binding data with Eq. 1 gave converged parameters in all cases. The calculated association constant (K_s), the number of binding sites (n_s), and the linear binding coefficient (ϕ) are listed in Table I, together with those for GLZ alone.[1] Similar, n_s values were observed in all cases, but the K_s value was reduced in all cases by the presence of an inhibitor, indicating that the specific GLZ binding was subject to competitive inhibition by the drug examined. The decreases of K_s value by IBU and DCA (*ca.* 3 and 15 times by IBU and *ca.* 6 and 35 times by DCA at 1 and 4 mM, respectively) were larger than those by WAR and SAL (*ca.* 3 and 2 times at both concentrations). The ϕ value decreased in the presence of all inhibitors. Figure 2 shows Scatchard plots of the binding data of IBU, WAR, SAL, and DCA in the adsence and in the presence of 1 or 4 mM GLZ. The bindings of IBU, WAR, SAL, and DCA were reduced in all cases by the presence of GLZ. Fitting the binding data of WAR and DCA with Eq. 1 and those of IBU and SAL with Eq. 2 gave converged parameters. The calculated values of n_s, K_s, and ϕ, the numbers of primary (n_1) and secondary (n_2) binding sites and the association constants K_1 and K_2 corresponding to n_1 and n_2, respectively, are listed in Table II. The n_s values of WAR and DCA alone were almost identical with those in the presence of GLZ, respectively. The K_s value was reduced in all cases by GLZ, but showed a much greater decrease by DCA (*ca.* 11—20 times) than WAR (*ca.* 2—3 times). The ϕ values tended to decrease in the presence of inhibitors. The values of n_1, n_2, and K_1 of IBU and SAL were almost identical with those in the presence of GLZ, respectively, but the K_2 values were decreased by GLZ (*ca.* 3—9 times and 2.3 times for IBU and SAL, respectively). The results indicate that GLZ competitively inhibits the specific bindings of both WAR and DCA and the low-affinity bindings of both IBU and SAL. As IBU and DCA showed clear inhibitory effects on GLZ binding and their bindings were decreased by GLZ, the bindings of IBU and DCA

TABLE II. Binding Parameters of WAR, DCA, IBU, and SAL to HSA in the Absence and in the Presence of GLZ Determined by an Ultrafiltration Technique

Drug	GLZ conc. (mM)	n_s	$K_s \times 10^{-5}$ (M^{-1})	$\phi \times 10^{-3}$ (M^{-1})	Drug	GLZ conc. (mM)	n_1 [a]	$K_1 \times 10^{-5}$ [b] (M^{-1})	n_2 [c]	$K_2 \times 10^{-3}$ [d] (M^{-1})
WAR	0	2.13±0.11	0.94±0.08	3.20±0.35	IBU	0	0.91±0.05	14.15±0.51	6.80±0.22	17.33±1.26
	1.0	1.81±0.20	0.55±0.12	2.34±0.38		1.0	1.01±0.03	11.70±0.54	6.88±0.16	5.41±0.40
	4.0	1.72±0.12	0.35±0.05	2.02±0.19		4.0	0.95±0.03	11.96±0.30	7.12±0.27	1.91±0.11
DCA	0	1.59±0.13	1.40±0.24	14.70±0.72	SAL	0	1.58±0.34	1.06±0.35	3.18±0.32	2.90±1.32
	1.0	1.58±0.06	0.13±0.01	10.38±0.27		1.0	1.56±0.09	1.03±0.09	3.88±0.73	1.29±0.47
	4.0	1.48±0.09	0.07±0.01	7.69±0.31		4.0	1.21±0.03	1.41±0.12	3.90±0.13	1.20±0.11

The parameters (±S.D.) for WAR and DCA, and IBU and SAR were calculated according to Eqs. 1 and 2 by the least squares method,[11] respectively. *a*) and *c*) are the numbers of primary and secondary binding sites, respectively. *b*) and *d*) are the association constants corresponding to n_1 and n_2, respectively. For other abbreviations, see Table I.

TABLE III. Binding Parameters of DCA and IBU to HSA in the Absence and in the Presence of One Another Determined by an Ultrafiltration Technique

Drug	n_s	$K_s \times 10^{-5}$ (M^{-1})	$\phi \times 10^{-3}$ (M^{-1})	Drug	n_1	$K_1 \times 10^{-5}$ (M^{-1})	n_2	$K_2 \times 10^{-3}$ (M^{-1})
DCA alone[a]	1.59±0.13	1.40±0.24	14.70±0.72	IBU alone[a]	0.91±0.05	14.15±0.51	6.80±0.22	17.33±1.26
+IBU (4.0 mM)	1.70±0.12	0.26±0.02	4.12±0.23	+DCA (4.0 mM)	0.95±0.03	11.57±0.64	6.43±0.25	3.92±0.30

The parameters (±S.D.) for DCA and IBU were calculated according to Eqs. 1 and 2 by the least squares method,[11] respectively. *a*) The values are the same as in Table II. For abbreviations, see Tables I and II.

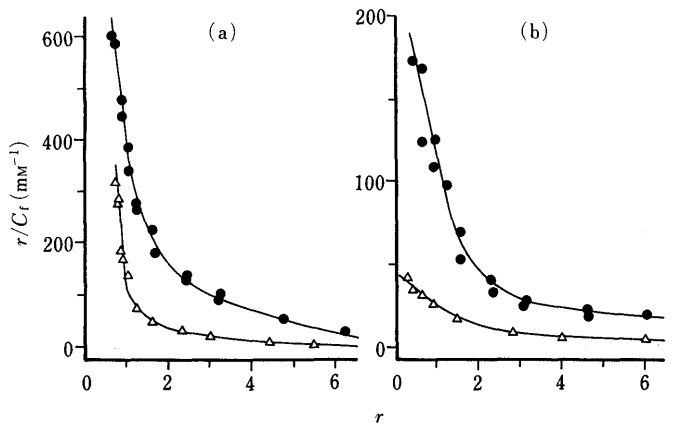

Fig. 3. Scatchard Plots for HSA Binding of IBU (a) and DCA (b) in the Absence and in the Presence of the Other

●, drug alone (the same data as in Fig. 2); △, 4 mM IBU or DCA. For details of abbreviations, data points, and solid lines, see Fig. 1 caption.

in each other's presence were examined. Figure 3 shows Scatchard plots of the binding data thus obtained (4 mM), together with the plots for each drug alone (Fig. 2). Both drugs showed decreased binding in the other's presence. Table III shows the binding parameters calculated from Eqs. 1 and 2 for DCA data and IBU data, respectively. The values of n_s of DCA and n_1, n_2, and K_1 of IBU alone were almost identical with those in the presence of the other drug. But the K_2 value of IBU and the K_s value of DCA decreased in the presence of the other drug, with almost identical extents of decrease (*ca.* 4.4 times for IBU and *ca.* 5.4 times for DCA). The results suggest that the low-affinity IBU binding site and specific DCA binding site may be located in an overlapping binding area on HSA. Thus, it was concluded that the specific GLZ binding site on HSA is located largely within the secondary IBU binding site area (probably the same as the specific DCA binding site area) and partially within the specific WAR binding site and secondary SAL binding site area.

Discussion

The binding parameters of IBU, WAR, and SAL (Table II) were in reasonable agreement with those reported.[13-15] The existence of high- and low-affinity DCA binding sites in HSA has been reported.[16] In this study, DCA binding to HSA showed specific and nonspecific bindings at lower and higher concentrations than approximately 1.5 mM, respectively, but the n_s and K_s values agreed reasonably well with the reported n_1 and K_1 values,[16] respectively. As shown in Tables I and II, the K_s and K_2 values of each drug alone were in the order of: GLZ > WAR > IBU > SAL. Therefore, the decrease of K_s of GLZ by these inhibitors ought to be in the order of WAR > IBU > SAL; however, the actual extent of the decrease was in the order of IBU > WAR ≒ SAL. The decrease of bindings of IBU, WAR, and SAL by GLZ was in the same order. In addition, an overlapping binding area for IBU and DCA was also suggested. Therefore, the specific GLZ binding site area may be largely in common with the low-affinity IBU binding site area in HSA. The value of ϕ decreased in all cases in the presence of inhibitor (Tables I and II). This is likely the result of drug-induced conformation change of the albumin molecule.[8]

It has been reported that the secondary binding site of SAL on HSA may be common with the high-affinity thyroxine site,[17] which has been classified in the diazepam site group.[18,19] The primary IBU binding site is also common with the diazepam site.[2,3] However, GLZ inhibited the low-affinity binding of both IBU and SAL (Table II). Why GLZ did not inhibit the high-affinity IBU binding is not clear.

GLZ has frequently been used in the treatment of chronic hepatitis.[20] An increase of bile acid concentration (maximum 84.3 μM) in the serum of patients with chronic hepatitis has been reported.[21] GLZ and DCA show mutual competitive inhibition of their binding to specific

sites. Therefore, C_f of GLZ and DCA in the presence of one another was calculated using Eqs. 3 and 4.

$$C_b = \frac{n_s(p)K_sC_f}{1+K_sC_f+K_{s,I}C_{f,I}} + \phi(p)C_f \quad (3)$$

$$C_{f,I} = \frac{-X+\sqrt{X^2+4YZ}}{2Y} \quad (4)$$

$$X = n_{s,I}(p)K_{s,I} + \phi_I(p) - C_{tot,I}K_{s,I} + \phi_I(p)K_sC_f + K_sC_f + 1$$

$$Y = K_{s,I} + \phi_I(p)K_{s,I}$$

$$Z = -C_{tot,I} - C_{tot,I}K_sC_f$$

where subscript I represents an inhibitor (GLZ or DCA); (p) is HSA concentration (0.609 mM, 4.2%); C_{tot} is total drug concentration. When C_b is $C_{b,I}$, Eq. 4 is obtained from Eq. 3. C_f was determined by the simulation method of Hanano *et al.*[22] using a digital computer (NEC PC-9801). Using the parameters for GLZ alone and DCA alone (Tables I and II), the values of C_f at 60 μg/ml (72.9 μM) GLZ after 200 mg/kg i.v. dosing[23] and at 84.3 μM DCA, each in the presence of the other drug (0—100 μM), were calculated, and found to be in the ranges of 0.168—0.187 and 0.627—0.684 μM, respectively. The incrase of C_f was small, approximately 10% for both drugs, but could be significant in clinical treatment, considering the very high level of binding of GLZ to HSA.

In conclusion, the specific binding site of GLZ on HSA is located mostly within the low-affinity IBU binding site area (probably the same as the specific DCA binding site area), and also takes in parts of the specific WAR binding site area and the low-affinity SAL binding site area.

References and Notes

1) S. Ishida, Y. Sakiya, T. Ichikawa, M. Kinoshita, and S. Awazu, *Chem. Pharm. Bull.*, **37**, 226 (1989).
2) G. Sudlow, D. J. Birkett, and D. N. Wade, *Mol. Pharmacol.*, **12**, 1052 (1976).
3) I. Sjöholm, B. Ekman, A. Kober, I. Ljungstedt-Påhlman, B. Seiving, and T. Sjödin, *Mol. Pharmacol.*, **16**, 767 (1979).
4) K. J. Fehske, W. E. Müller, U. Wollert, and L. M. Velden, *Mol. Pharmacol.*, **16**, 778 (1979).
5) K. J. Fehske, W. E. Müller, and U. Wollert, *Biochem. Pharmacol.*, **30**, 687 (1981).
6) K. J. Fehske, U. Schläfer, U. Wollert, and W. E. Müller, *Biochem. Pharmacol.*, **21**, 387 (1982).
7) S. Goya, A. Takadate, H. Fujino, M. Otagiri, and K. Uekama, *Clhem. Pharm. Bull.*, **30**, 1363 (1982).
8) U. Kragh-Hansen, *Biochem. J.*, **225**, 629 (1985).
9) H. Takikawa, Y. Sugiyama, M. Hanano, M. Kurita, H. Yoshida, and T. Sugimoto, *Biochim. Biophys. Acta*, **926**, 145 (1987).
10) T. Ichikawa, S. Ishida, and Y. Sakiya, *Chem. Pharm. Bull.*, **33**, 3031 (1985).
11) K. Yamaoka, Y. Tanigawara, T. Nakagawa, and T. Uno, *J. Pharmacobio-Dyn.*, **4**, 879 (1981).
12) A. K. Thakur and C. Delisi, *Biopolymers,* **17**, 1075 (1978).
13) J. B. Whitlam, M. J. Crooks, K. F. Brown, and P. V. Pedersen, *Biochem. Pharmacol.*, **28**, 675 (1979).
14) Z. Taira and H. Terada, *Biochem. Pharmacol.*, **34**, 1999 (1985).
15) K. F. Brown and M. J. Crooks, *Biochem. Pharmacol.*, **25**, 1175 (1976).
16) A. Roda, G. Cappelleri, R. Aldini, E. Roda, and L. Barbara, *J. Lipid Res.*, **23**, 490 (1982).
17) M. Tabachnick, *Arch. Biochem. Biophys.*, **106**, 415 (1964).
18) G. L. Tritsch and N. E. Tritsch, *J. Biol. Chem.*, **238**, 138 (1963).
19) U. Kragh-Hansen, *Biochem. J.*, **209**, 135 (1983).
20) K. Hino, H. Miyakawa, Y. Miyahara, S. Fujikura, M. Iwasaki, and K. Takahashi, *Minophagen Med. Rev.*, **26**, 270 (1981).
21) A. Mangione, T. E. Imhoff, R. V. Lee, L. Y. Shum, and W. J. Jusko, *Chest*, **73**, 616 (1978).
22) M. Hanano, Y. Sugiyama, Y. Koda, and Y. Sawada, "Introduction to Pharmacokinetics," Nanzando, Tokyo, 1987, pp. 216—221.
23) H. Kato, K. Nakanishi, and M. Kanaoka, *Minophagen Med. Rev.*, Supplement, **15**, 5 (1984).