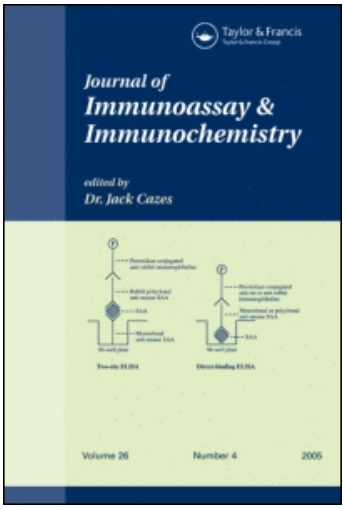

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ENZYME-LINKED IMMUNOSORBENT ASSAY OF PRAVASTATIN, A HMG-CoA REDUCTASE INHIBITOR, IN HUMAN PLASMA

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed for sensitive and specific determination of pravastatin (PS) sodium, a HMG-CoA reductase inhibitor. Preparation of immunogens to obtain antisera was carried out using chemically modified PS; β-alanine derivative of PS (for ELISA-1) and 5-deoxy- PS (for ELISA-2) were linked to bovine serum albumin via its terminal carboxylic acid by the N-succinimidyl ester method, to avoid intramolecular lactonization of PS. Enzyme-labeled antigens were prepared similarly by coupling with horseradish peroxidase, and were used by homogeneous combination of antisera. The enzymic activity was determined using a microtiter plate coated with second antibody and tetramethylbenzidine as a chromogenic substrate.

Both of the ELISA systems enabled the determination of PS in a range of 5 to 500 pg/well, with an IC_{50} of 36 to 130 pg/well. Cross-reactivities with main metabolites in plasma, which differed from PS in decaline moiety, were less than a few percent. When ELISA-1 was applied to the determination of PS in human plasma directly after dilution with the ELISA buffer, the detection limit and the intra-assay coefficient (5 ng/ml of PS) were 500 pg/ml and 4.5%, respectively. Further, ELISA-1 was validated by gas chromatography-mass spectrometry with the determination of PS in human plasma after oral administration at a dose of 10 mg/body.

(KEY WORDS: enzyme-linked immunosorbent assay, pravastatin sodium, a HMG-CoA reductase inhibitor, N-succinimidyl ester method, human plasma)

INTRODUCTION

Pravastatin (PS) sodium is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and it is used as an anti-hypercholesterolemic drug in

clinical fields (1). Since the plasma concentration of PS in rats and humans is quite low, due to specific uptake into the liver, the development of a highly sensitive and specific assay in biological matrices has been required to study the pharmacokinetics of PS. High-performance liquid chromatography with ultraviolet detection was not sensitive enough to reliably measure the low level of PS (quantification needed for biological samples). A sensitive method based on gas chromatography-mass spectrometry (GC-MS) with electron ionization or negative ion chemical ionization have been useful for the determination of PS in biological fluids (2, 3). However, these methods are not simple, owing to the need for solid-phase extraction and derivatization of PS prior to analysis.

Enzyme immunoassay has general advantages of sensitivity and simplicity, and it is suitable to determine a drug in such biological fluids as plasma. When immunoassay is applied to determine PS, it is necessary to avoid cross-reactivities with the metabolites, which have been found in plasma after giving PS to animals or humans, as shown in Fig. 1 (1).

To establish an enzyme-linked immunosorbent assay (ELISA) to determine PS selectively, preparation of an immunogen by linking PS to a carrier protein via 1-carboxylic acid has been thought to be reasonable. However, a suitable immunogen has not been obtained, owing to intramolecular lactonization of PS under the reaction condition.

In this study, two antibodies for ELISAs were prepared using chemically modified PS as immunogens; PS was linked to bovine serum albumin (BSA) via β-alanine introduced into 1-carboxylic acid (for ELISA-1), and 5-deoxy-PS was linked to the protein via 1-carboxylic acid directly (for ELISA-2). To confirm the validity of the ELISAs, the human plasma concentration of PS measured by ELISAs was compared to that measured by GC-MS.

MATERIALS AND METHODS

Materials

BSA, human serum albumin (HSA), and Freund's complete adjuvant were from Sigma (St. Louis, MO). Goat immunoglobulin G against rabbit immunoglobulin G was purchased from Cappel (West Chester, PA), and microtiter plates for ELISA were from Sumitomo Bakelite (Tokyo, Japan). Horseradish peroxidase (EC

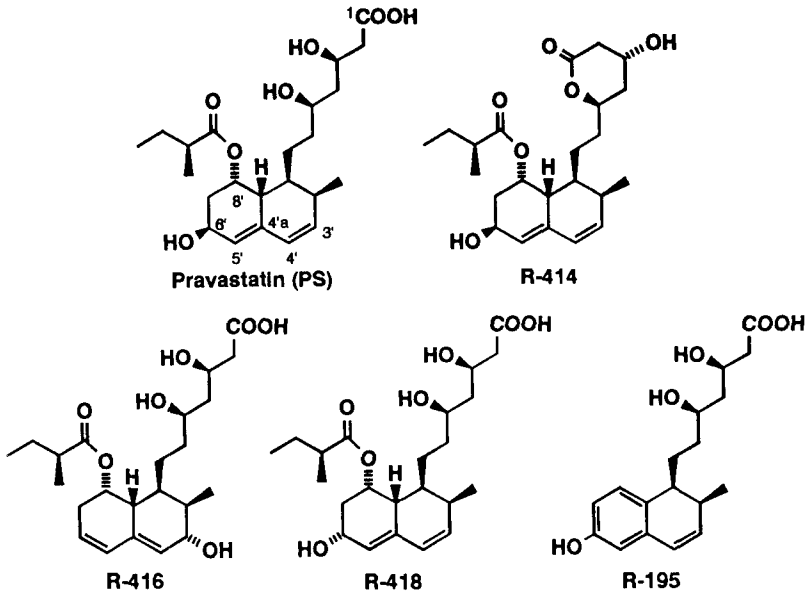

FIGURE 1 Structures of PS and its metabolites.

1.11.1.7, grade I-C, 260 units/mg) was obtained from Toyobo (Osaka, Japan), and 3,3',5,5'-tetramethylbenzidine was from Tokyo Kasei Kogyo (Tokyo, Japan). The standard samples of PS sodium and its metabolites: R-414, R-416, R-418, and R-195, were synthesized in the Fermentation Research Laboratories and Analytical and Metabolic Research Laboratories of Sankyo. Other solvents and chemicals, purchased from Wako (Osaka, Japan), were of analytical grade and were used without further purification.

Syntheses of Hapten-1 and -2 ([*1*] and [*4*], Fig. 2)

1. Synthesis of N-PS-β-alanine ([*1*], Hapten-1): To a solution of PS sodium (4.47 g) and β-alanine ethyl ester hydrochloride (1.54 g) in DMF (20 ml), were added diethyl phosphorocyanidate (1.82 ml) and triethylamine (1.67 ml), and the mixture was stirred at room temperature for 30 min. The reaction mixture was diluted with ethyl acetate, washed successively, with each of H_2O, 5% HCl, and H_2O, and dried over Na_2SO_4. After removal of the solvent in vacuo, the crude

product was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH= 90/10), to give 3.27 g of PS-β-alanine ethyl ester. To a solution of PS-β-alanine ethyl ester (3.27 g) in MeOH (20 ml), was added 1 M NaOH aqueous solution (7.5 ml), and the mixture was stirred at room temperature for 1.5 hr. After removal of the MeOH in vacuo, the residue was acidified by 1 M HCl and extracted with ethyl acetate. The extracts were washed with H_2O, and dried over Na_2SO_4. After removal of the solvent in vacuo, the crude product was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH/acetic acid= 85/15/5), to give 1.59 g of compound [*1*]. ^{1}H NMR date of compound [*1*] in DMSO-d6 (TMS) : 0.82(3H, t, J=7.3 Hz, 4"-CH_3), 0.83(3H, d, J=5.9 Hz, 2'-CH_3), 1.03(3H, d, J=6.8 Hz, 2"-CH_3), 5.19(1H, br, 8'-CH-), 5.48(1H, br, 5'-CH=), 5.83(1H, dd, J=5.9, 8.3 Hz, 3-CH=), 5.90(1H, d, J=9.8 Hz, 4-CH=), 7.84(1H, t, J=5.4 Hz, -CONH-).

2. Synthesis of 5-deoxy-PS ([*4*], Hapten-2): To a solution of R-414 (10 g) in DMF (15 ml), were added imidazol (4.0 g) and a solution of t-butyldimethylsilyl chloride (9.27 g) in DMF (15 ml) under cooling by ice, and the mixture was stirred at room temperature for 5 hr. The reaction mixture was diluted with ethyl acetate, washed with H_2O, and dried over Na_2SO_4. After removal of the solvent in vacuo, the crude product was purified by silica gel column chromatography (eluent: hexane/ ethyl acetate=90/10-70/30), to give 15.6 g of the silyl ether. To a solution of the silyl ether (15.6 g) in CH_3CN (100 ml), was added 1 M NaOH aqueous solution (25.8 ml), and the mixture was stirred at room temperature for 1 hr. After removal of the MeOH in vacuo, the residue was acidified by 1 M HCl, and the mixture was extracted with ethyl acetate. The extracts were washed with H_2O, and dried over Na_2SO_4. To the extracts were added diazomethane in ether, and the solvent was removed in vacuo. The crude product was purified by silica gel column chromatography (eluent: benzene/ethyl acetate=97/3-90/10), to give 14.7 g of the methyl ester.

To a solution of the methyl ester (18.0 g) in pyridine (70 ml), was added methanesulfonyl chloride (4.18 ml) under cooling by ice, and the mixture was stirred at room temperature for 1 hr. After removal of pyridine, the residue was diluted with ethyl acetate, washed with H_2O, and dried over Na_2SO_4. After removal of the solvent in vacuo, the crude product was purified by silica gel column chromatography (eluent: benzene/ethyl acetate=95/5-80/20), to give 12.1 g of the methanesulfonate. To a solution of the methanesulfonate (10.1 g) in DMF (80.8 ml) and hexamethylphosphoramide (20.2 ml), was added LiCl (10.1 g), and the mixture was stirred at room temperature for 5 hr. The reaction mixture was diluted with ethyl acetate, washed with H_2O, and dried over Na_2SO_4. After removal of the solvent in

vacuo, the crude product was purified by silica gel column chromatography (eluent: hexane/ethyl acetate=95/5-85/15), to give 6.48 g of the chloride. To a solution of the chloride (2.0 g) in benzene (15 ml) were added α,α-azobisisobutyronitrile (95.9 mg) and tributyltinhydride (1.57 ml), and the mixture was refluxed for 1 hr. After removal of the solvent in vacuo, the crude product was purified by silica gel column chromatography (eluent: benzene/ethyl acetate=98/2-90/10), to give 1.77 g of the deoxy-PS-silyl ether. To a solution of the deoxy-PS-silyl ether (2.0 g) in tetrahydrofuran (THF, 71.4 ml), were added 1 M tetrabutylammoniumfluoride in THF solution (30.7 ml) and acetic acid (3.6 ml) and the mixture was stirred at 50 ℃ for 30 min. The reaction mixture was diluted with ethyl acetate, washed successively, with each of H_2O, 5% $NaHCO_3$ aqueous solution and H_2O, and dried over Na_2SO_4. After removal of the solvent in vacuo, the crude product was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH=99/1-94/6), to give 610 mg of the deoxy-PS methyl ester. To a solution of the deoxy-PS methyl ester (100 mg) in CH_3CN (5 ml) was added 1 M NaOH aqueous solution (0.36 ml), and the mixture was stirred at room temperature for 1 hr. After removal of the solvent in vacuo, the crude product was purified by CHP-20P (Mitsubishi Kasei, Tokyo, Japan) column chromatography (eluent: H_2O/acetone=100/0-80/20), to give 95mg of compound [*4*]. ^1H NMR data of compound [*4*] in $CDCl_3$ (TMS) : 0.68(3H, t, J=7.7 Hz, 4"-CH_3), 0.70(3H, d, J=7.0 Hz, 2'-CH_3), 0.93(3H, d, J=7.0 Hz, 2"-CH_3), 2.10(1H, dd, J=7.7, 16.2 Hz, 2-CH), 2.17(1H, dd, J=6.2, 16.2 Hz, 2-CH), 3.72-3.83(1H, m, 3-CH), 4.18-4.28(1H, m, 6'-CH), 5.24(1H, br, 8'-CH), 5.39(1H, br, 5'-CH=), 5.84(1H, dd, J=5.9, 9.9 Hz, 3-CH=), 5.90(1H, d, J=5.9 Hz, 4'-CH=).

Syntheses of Antigen-1 and -2 ([*3*] and [*6*], Fig. 2)

1. Synthesis of N-PS-β-alanine N-succinimidyl ester [*2*]: To a solution of [*1*] (248 mg) in CH_3CN (5 ml) was added N,N'-disuccinimidyl carbonate (128 mg) under cooling by ice, and the mixture was stirred at room temperature for 1.5 hr. The reaction mixture was diluted with ethyl acetate, washed successively, with each of H_2O, 5% Na_2CO_3 aqueous solution and H_2O, and dried over Na_2SO_4. After removal of the solvent in vacuo, the crude product was purified by silica gel column chromatography (eluent: CH_2Cl_2/MeOH=90/10), to give 150 mg of compound [*2*].

2. Synthesis of 5-deoxy-PS-N-succinimidyl ester [*5*]: Starting from 99 mg of compound [*4*], 92.7 mg of compound [*5*] was obtained according to the same procedures described in the synthesis of compound [*2*].

3. Preparation of antigens ([3] and [6]): The PS derivatives were conjugated with BSA by the N-succinimidyl ester method (4). Briefly, N-succinimidyl ester ([2]: 17.8 mg or [5]: 15.2 mg), dissolved in pyridine (3.0 ml), was added to a solution of BSA (67 mg) in 50 mM phosphate buffer (pH 7.3, 3.0 ml). The mixtures were gently stirred at room temperature for 7 hr, followed by dialysis, successively, against DMF/H_2O (40/60), H_2O, and saline.

Preparations of Anti- PS Antisera

The obtained antigens, [3] and [6], were diluted to 2 mg protein/ml with saline and emulsified with equal volumes of Freund's complete adjuvant. Three rabbits for each antigen were immunized with 1 ml of immunogen, by several intradermal injections every two weeks. Four months after the first immunization, blood was taken from each rabbit, by cardiac puncture, and allowed to stand at 4 ℃ for 16 hr. The antisera were obtained by centrifugation at 3,000xg for 20 min.

Preparation of Enzyme-Labeled Antigens

Peroxidase-labeled antigens were prepared according to the method described by Hosoda et al. (5). Briefly, N-succinimidyl ester ([2]: 890 μg or [5]: 760 μg), dissolved in dioxane (0.2 ml), was added to a solution of horseradish peroxidase (2 mg) in 50 mM phosphate buffer (pH 7.3, 0.4 ml) at 0 ℃. The mixture was gently stirred at 4 ℃ for 4 hr, followed by dialysis, successively, against phosphate-buffered saline (pH 7.4, PBS)/DMF (50/50), PBS/DMF (70/30), and PBS, at 4 ℃. The dialysate was diluted with PBS containing 0.1% gelatin, to a concentration of 500 mg peroxidase/ml, and stored at 4 ℃.

ELISA

The microtiter plates, were coated with goat immunoglobulin G against rabbit immunoglobulin G and post-coated with HSA, washed 3 times with PBS containing 0.05% Tween 20 (buffer A), just before use. Fifty μl of antiserum, appropriately diluted with PBS containing 0.1% HSA (buffer B), was placed in each well. Peroxidase-labeled antigen (50 μl), diluted to 200-1000 ng/ml (for ELISA-1) or 100-500 ng/ml (for ELISA-2) with buffer B, and the standard or unknown solution (50 μl), diluted with buffer B, were added to the wells. The plates were incubated at 4 ℃ for 20-24 hr, then washed with buffer A (350 μl x 3). The peroxidase activity retained on the plates was measured according to the method described previously

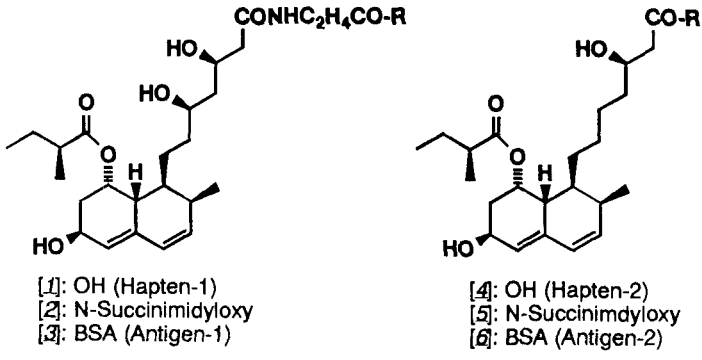

FIGURE 2 Structures of Haptens and Antigens

with slight modification (6). Briefly, 200 μl of 0.01% tetramethylbenzidine in 50 mM acetate-citric acid buffer (pH 5.5) containing 3% DMSO and 0.002% H_2O_2, was added to the wells, and the plates were incubated at room temperature for 30 min. The reaction was terminated by adding 0.5 M H_2SO_4 (50 ml), and the absorbance of the sample was measured at 450 nm using an MTP-100 microplate reader (Corona-denki, Ibaragi, Japan).

PS Administration to Humans

Six healthy male volunteers took one 10-mg tablet of PS after an overnight fast. Blood samples were taken at 0.5, 1, 2, 3, 4, 6, and 8 hr after the administration, to determine the plasma concentrations of PS.

RESULTS

ELISA of PS

At first, appropriate dilutions of anti-PS antisera for ELISAs were determined by construction of antiserum dilution curves. The result, obtained with homogeneous combination of antiserum and labeled antigen (20 ng), is shown in Fig. 3. The binding ability was expressed, for convenience, as a percentage of that obtained with 1:10,000 dilution. The dilutions showing 50% binding were observed between 1:100,000 and 1,000,000 in both of the antisera obtained by different immunogens.

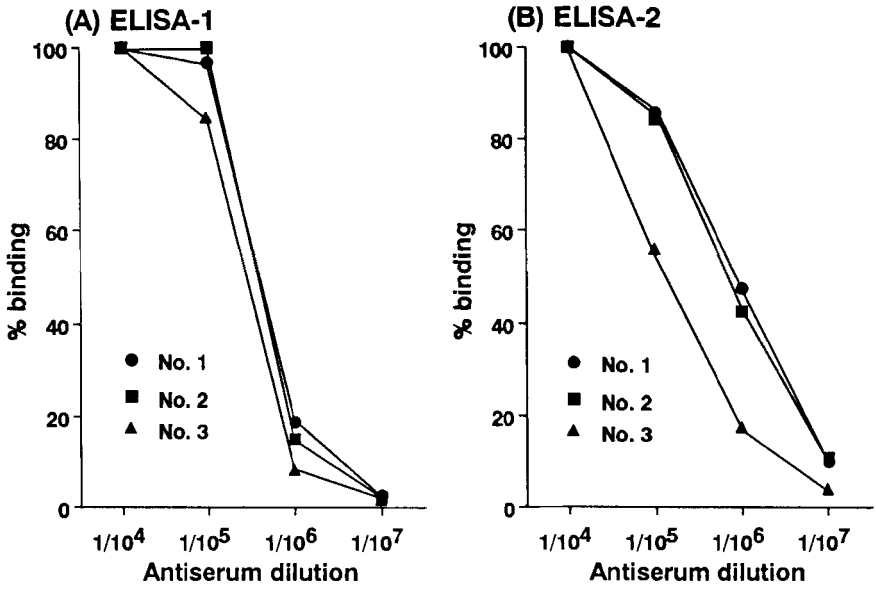

FIGURE 3 Antiserum dilution curves with the HRP-labeled antigens.

Sensitivities obtainable with the assay systems were tested by examining the inhibition of enzymatic activity caused by the addition of 200 pg of PS (Table 1).

To develop a highly sensitive ELISA, the combination of both amounts of labeled antigen and antiserum was examined, in consideration of the above dilution curves. The optimal combination of sensitivity (% inhibition) and precision (% background) was clarified in each antiserum of both of the ELISAs. Dose-response curves of both of the ELISAs, under consideration of the optimal combination enabled the determination of PS over the range of 5 to 500 pg/well (ca. 90 and 10% of maximum binding, respectively).

The amounts of unlabeled PS required to displace 50% of the maximal binding of peroxidase-labeled antigen (IC_{50}) were 36-130 pg/well (Table 2). The representative dose-response curves of ELISA-1 (antiserum No.1) and -2 (antiserum No.2) are shown in Fig. 4.

The cross-reactivities with metabolites of PS in the two ELISAs were calculated from the amount of IC_{50} caused by PS or metabolites, as shown in Table 2.

TABLE 1
Inhibition of bound enzymic activity
of HRP-labeled antigens by 200 pg of PS

ELISA system (Antiserum No.)		Amount of label (ng)	Antiserum dilution	Inhibition (%)	NSB* (%)
1	(No.1)	10	1 : 5 x 10^5	60	5
		20	1 : 10 x 10^5	46	8
		50	1 : 20 x 10^5	44	17
	(No.2)	10	1 : 5 x 10^5	56	7
		20	1 : 10 x 10^5	57	10
		50	1 : 20 x 10^5	33	21
	(No.3)	10	1 : 5 x 10^5	59	3
		20	1 : 10 x 10^5	46	8
		50	1 : 20 x 10^5	39	15
2	(No.1)	5	1 : 5 x 10^5	59	4
		10	1 : 10 x 10^5	48	4
		20	1 : 20 x 10^5	38	7
	(No.2)	5	1 : 5 x 10^5	68	8
		10	1 : 10 x 10^5	52	8
		20	1 : 20 x 10^5	38	16
	(No.3)	5	1 : 2 x 10^5	62	5
		10	1 : 5 x 10^5	51	8
		20	1 : 10 x 10^5	33	14

* Non-specific binding (background).

TABLE 2
Sensitivity and selectivity of two ELISA systems for PS

ELISA system (Antiserum No.)		IC_{50} (pg/well)	Cross-reactivity (%)			
			R-414	R-416	R-418	R-195
1	(No.1)	74	308	6.2	0.4	0.1
	(No.2)	130	650	6.2	0.4	0.2
	(No.3)	96	367	6.4	0.6	0.1
2	(No.1)	50	2632	7.1	1.0	1.3
	(No.2)	36	1059	6.7	1.1	0.7
	(No.3)	74	1850	4.1	0.4	0.6

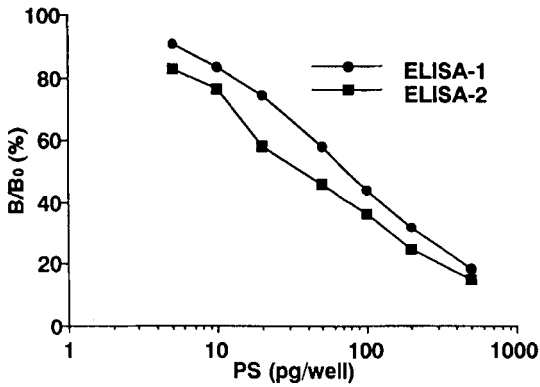

FIGURE 4 Dose-response curves of two ELISA systems.

Structural differences in decaline moiety and 8'-side chain from PS were significantly recognized by antisera used in both of the ELISAs. It was noteworthy that cross-reactivities with R-418, which is 6'-epimer of PS, were less than 0.6% in ELISA-1. But all antisera showed a higher affinity to R-414 than PS, and in particular, antisera used in ELISA-2 showed more then 10-fold higher affinity compared to an original immunogen, PS. That is to say, the antisera could not recognize the moiety around the bridge to a carrier protein.

Validity of ELISA Applied to Determine PS in Human Plasma

Various amounts of human plasma, were mixed with authentic PS and directly diluted with ELISA buffer, were subjected to ELISA-1 and -2, to examine if plasma interferes with the interaction of anti-PS antiserum and PS (Fig. 5). When 4 ng/ml of PS in control plasma was determined using 2.5-50 μl of the original volume of plasma, good linearity between both volume of plasma and PS measured by ELISA-1 was observed. ELISA-2, however, showed a slightly steep slope and non-linearity, owing to interference by plasma.

When ELISA-1 using antiserum No.1 was applied to the determination of PS in human plasma directly diluted 5-fold with the buffer, the intra-assay coefficient of variation at a PS level of 5 ng/ml was 4.5% (n=10). The detection limit based on 2 S.D. of maximum binding was 500 pg/ml.

Plasma concentrations of PS in healthy volunteers after oral administration of one 10-mg tablet were measured by ELISA-1 and -2 using a direct-dilution method for samples. And, the value measured was compared to that by GC-MS (7,8), as shown in Fig. 6. There was good correlation between the values measured by GC-MS and ELISA-1 using antiserum No.1, as shown in Fig. 6A ($y = 1.03x + 0.30$, $r = 0.906$). In the case of ELISA-2 using antiserum No.2, ca. 2-fold value was resulted comparing to that by GC-MS (Fig. 6B).

In addition, the average values with S.E. of plasma concentrations of PS measured by ELISA-1 are shown in Fig. 7. A plasma AUC of 29.96 ± 5.25 ng · hr/ml, and decreasing of the level with $t_{1/2}$ of 2.12 ± 0.17 hr, were observed after oral administration of PS sodium to humans at a dose of 10 mg/body .

DISCUSSION

As there is a principle that anti-hapten antibody cannot recognize the moiety around the bridge to a carrier protein, several trials to get a specific antibody against

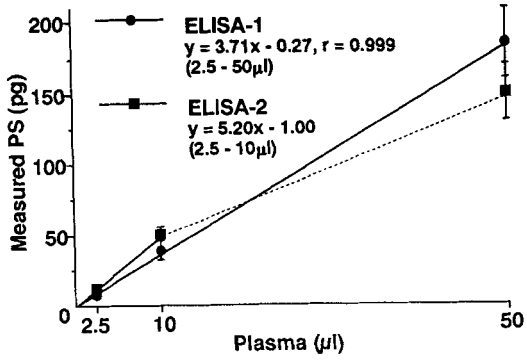

FIGURE 5 Influence of the plasma volume on the determination of PS by ELISA.

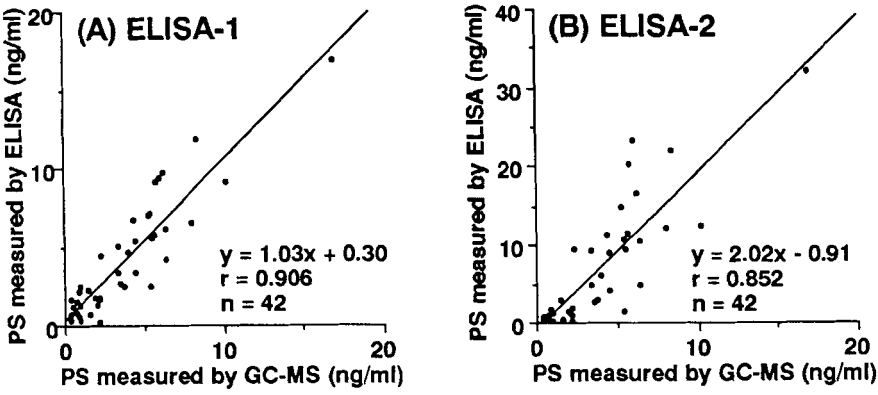

FIGURE 6 Correlation analysis between two ELISA systems and GC-MS for PS levels in human plasma.

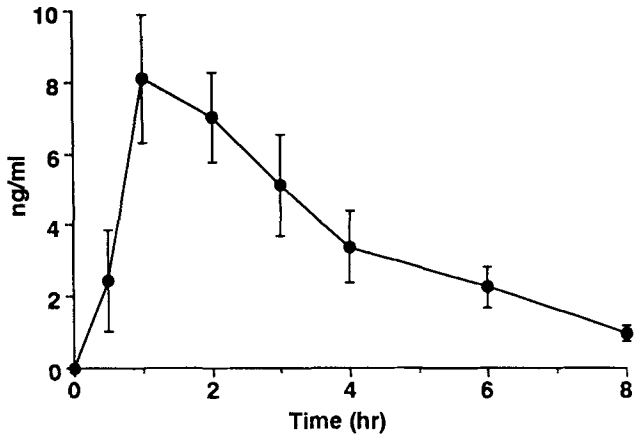

FIGURE 7 Plasma concentrations of PS after oral administration of PS sodium at a dose of 10mg/body to humans.

decaline moiety of PS had been carried out in our laboratories. But, intramolecular lactonization of PS under the reaction condition prevented utilizing 1-carboxylic acid as a bridge site for preparation of an immunogen. In this paper, we investigated chemical modification of PS, in order to overcome this difficulty, and we were able to develop specific ELISAs for PS. One is the introduction of β-alanine to 1-carboxylic acid, and the other is the elimination of 5-hydroxy moiety, which is necessary for lactonization. These modifications enabled obtaining reasonable immunogens, and resulted in specific ELISAs for decaline moiety of PS.

Tsay et al. (9) reported the synthesis of PS-6'-carboxymethyl oxime and preparation of an immunogen. Their investigation resulted in a specific radioimmunoassay that recognized PS separately from R-414 and R-416, but not R-418. In our ELISAs, equally low cross-reactivities with R-416, and almost negligible cross-reactivities with R-418, were observed. The contrast between these results concerning cross-reactivities with metabolite, except for R-414, is understandable when the structures of immunogens are considered.

An antiserum used in ELISA-1 was judged to have a high affinity to PS, because it enabled highly sensitive and precise determination of PS in plasma directly diluted with the ELISA buffer. At the same time, the validity of ELISA-1 was confirmed by

GC-MS using plasma samples containing metabolites. It is suggested that a higher affinity to R-414 than PS observed in ELISA-1 has no influence on assay precision, even if plasma samples after dosing of PS are measured. But, ELISA-2 showed ca. 2-fold overestimation of PS compared to the value by GC-MS, probably, owing to co-measurement of plasma components and R-414.

The ELISA-1 established in this paper can also be applied to determine PS in urine and other body fluids. Additionally, further investigations of monoclonal antibody against the derivatives of PS will be useful to clarify the clinical effects of PS by immunological methods more extensively.

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REFERENCES

1. Arai, M., Serizawa, N., Terahara, A. et al. Pravastatin sodium (CS-514), a novel cholesterol-lowering agent which inhibits HMG-CoA reductase. Annu. Rep. Sankyo Res. Lab. 1988; 40: 1-38.

2. Funke, P.T., Ivashkiv, E., Arnold, M.E. and Cohen, A.I. Determination of pravastatin sodium and its major metabolites in human serum / plasma by capillary gas chromatography / negative ion chemical ionization mass spectrometry. Biomed. Environ. Mass Spectrom. 1989; 18: 904-9.

3. Morris, M.J., Gilbert, J.D., Hsieh, J.Y.-K., Matuszewski, B.K., Ramjit, H.G. and Bayne, W.F. Determination of the HMG-CoA reductase inhibitors simvastatin, lovastatin, and pravastatin in plasma by gas chromatography/ chemical ionization mass spectrometry. Biol. Mass Spectrom. 1993; 22: 1-8.

4. Hosoda, H., Yoshida, H., Sakai, Y., Miyairi, S., Ishii, K. and Nambara, T. The preparation of steroid N-hydroxysuccinimide esters and their reactivities with bovine serum albumin. Chem. Pharm. Bull. 1979; 27: 742-6.

5. Hosoda, H., Karube, T., Kobayashi, N. and Nambara, T. Enzyme labeling of steroids by the N-succinimidyl ester method: Preparation of horseradish peroxidase-labeled antigen for use in enzyme immunoassay. Chem. Pharm. Bull. 1985; 33: 249-55.

6. Hosoda, H., Takasaki, W., Oe, T., Tsukamoto, R. and Nambara, T. A comparison of chromogenic substrates for horseradish peroxidase as a label in steroid enzyme immunoassay. Chem. Pharm. Bull. 1986; 34: 4177-82.

7. Sasahara, K., Kawabata, K., Nakaya, N. and Goto, Y. Phase I study of CS-514, an inhibitor of HMG-CoA reductase (II) : Pharmacokinetics of CS-514 in helthy volunteers. Rinsho Iyaku 1998; 4: 45-65

8. Kawabata, K., Hoshiyama, K., Suzuki, S., Nitanai, T. and Sasahara, K. Quantitation methods of pravastatin and its metabolites in human biological fluids using automated gas chromatographic / mass spectrometry and their application to pharmacokinetic studies in volunteers and patients. Proc. Jpn. Soc. Biomed. Mass Spectrom. 1991; 16: 149-52.

9. Tsay, H., Reiss, A., Stouffer, B. et al. A specific radioimmunoassay for the measurement of pravastatin in human serum. Clin. Chem. 1989; 35: 1189.