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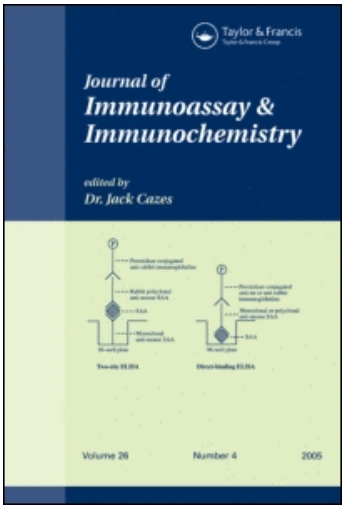

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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Enzyme-Linked Immunosorbent Assay of CS-518, a Thromboxane Synthetase Inhibitor, in Rabbit Plasma and Platelets

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To cite this Article Takasaki, Wataru , Nakagawa, Akihiko , Shigeta, Akemi and Tanaka, Yorihisa(1992) 'Enzyme-Linked Immunosorbent Assay of CS-518, a Thromboxane Synthetase Inhibitor, in Rabbit Plasma and Platelets', Journal of Immunoassay and Immunochemistry, 13: 4, 495 — 508
To link to this Article: DOI: 10.1080/15321819208019831
URL: http://dx.doi.org/10.1080/15321819208019831

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JOURNAL OF IMMUNOASSAY, 13(4), 495-508 (1992)

ENZYME-LINKED IMMUNOSORBENT ASSAY OF CS-518, A THROMBOXANE SYNTHETASE INHIBITOR, IN RABBIT PLASMA AND PLATELETS

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ABSTRACT

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for determination of CS-518, a novel thromboxane synthetase inhibitor. Antisera against CS-518 were obtained from rabbits immunized with bovine serum albumin linked to CS-518 *via* carboxylic acid introduced into the imidazolyl ring (for ELISA-1) or *via* 6-carboxylic acid directly (for ELISA-2). Each of two CS-518 derivatives was conjugated to horseradish peroxidase by a *N*-succinimidyl ester method, and it was used as a labeled-antigen in homogeneous combination with antisera. In ELISA-1, CS-518 was detectable in a range of 5pg - 1ng, and all cross-reactivities with main metabolites were less than 5 %, in contrast to high affinity to the taurine and glucuronic acid conjugates of CS-518 in ELISA-2. Validity of ELISA-1 was confirmed by a high-performance liquid chromatography and ELISA-1 enabled specific determination of CS-518 in plasma samples deproteinized by methanol. When ELISA-1 was applied to determine CS-518 in platelets after oral administration to rabbits, CS-518 uptake up to maximum capacity in platelets (4.2 - 5.4 x 10^{-6} M) and slow elimination of CS-518 from platelets ($T_{1/2}$ = 36 - 41 hr) were observed independent of CS-518 doses. These results confirm that CS-518 binds to thromboxane synthetase in platelets with high affinity.

(KEY WORDS: enzyme-linked immunosorbent assay, CS-518, a thromboxane synthetase inhibitor, *N*-succinimidyl ester method, rabbit platelets)

INTRODUCTION

CS-518, sodium 6-[2-[1-imidazolyl]methyl-4,5-dihydrobenzo[*b*]thiophene]-carboxylate, is a novel thromboxane synthetase inhibitor with a potent and extended duration of action (1-3). To evaluate this drug, its pharmacokinetic behavior should be considered not only in plasma but also in platelets, which contain thromboxane

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synthetase. High-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry were used to determine CS-518 in plasma in our laboratories, but neither method could be applied to assay the drug in platelets because of poor sensitivity.

Enzyme immunoassay has general advantages of sensitivity and simplicity, and it is suitable for the determination of a drug in such a small organ as platelets. When immunoassay is applied to determine CS-518, it is necessary to avoid cross-reactivities with the metabolites, which have been found in plasma after giving CS-518 to animals, as shown in Fig. 1 (4).

In this study, two antibodies were raised to CS-518 in hapten linkage with bovine serum albumin using either a carboxylic acid bridge to the imidazolyl ring of CS-518 or a direct link to the 6-carboxyl group of CS-518 (Fig. 2). An enzyme-linked immunosorbent assay (ELISA) using the former antibody was applied to the determination of CS-518 in platelets of rabbits.

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MATERIALS AND METHODS

Materials

Bovine serum albumin, human serum albumin, 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 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 CS-518 and its metabolites: R-5206, 5512, and 5652, were synthesized in the Medicinal Chemistry Research Laboratories of Sankyo. Glucuronide of CS-518 was purified from the bile of a rat given CS-518 orally. Other solvents and chemicals, purchased from Wako (Osaka, Japan), were of analytical grade and were used without further purification.

Synthesis of *N*-Succinimidyl Esters of CS-518 (Fig. 2)

6-[4,5-Dihydro-2-[1-(1H)-[4(5)-[1-[4-(*N*-succinimidyloxy)-1,4-dioxobutyl]-oyl]methyl]imidazolyl]methylbenzo[*b*]thiophene]carboxylate [*6*] : 0.625 g of 55 %

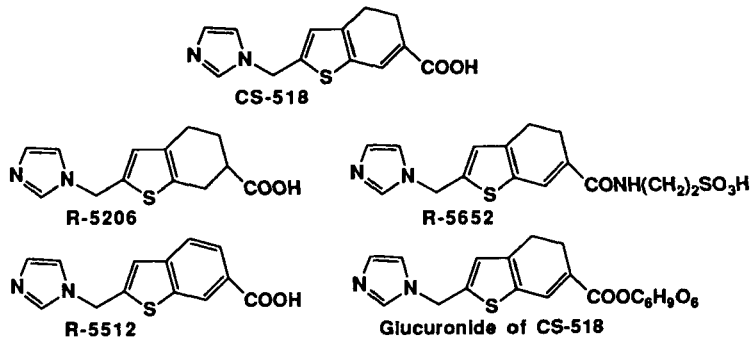

FIGURE 1 Structures of CS-518 and its metabolites.
R-5652 and glucuronide of CS-518 are identified metabolites.
R-5206 and 5512 are possible metabolites.

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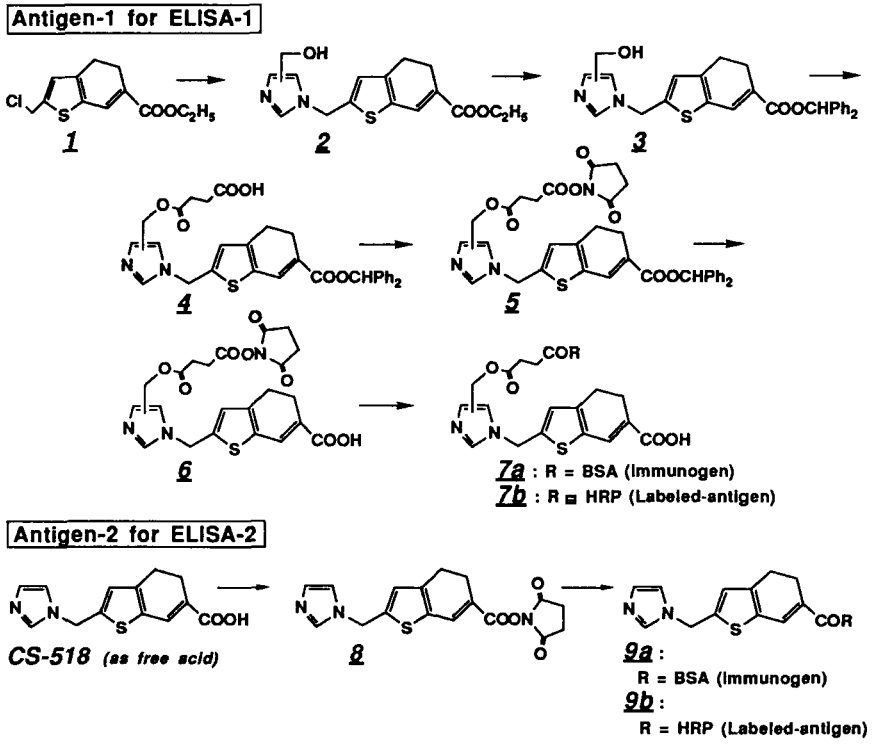

FIGURE 2 Preparation of antigens for ELISA.

sodium hydride was added to a DMF solution of 0.964 g of 4-hydroxymethyl-imidazole hydrochloride in an ice-water bath. After 30 min, the reaction residue was mixed with DMF solution of ethyl 2-chloromethyl-4,5-dihydrobenzo[*b*]thiophene-6-carboxylate [*1*] in an ice-water bath with continuous stirring at room temperature for 5 hr. The reaction mixture was extracted with ethyl acetate and dried. The residue was chromatographed on SiO_2 to give 1.74 g of ethyl 2-[1-[4(5)-hydroxymethyl]imidazolyl]methyl-4,5-dihydrobenzo[*b*]thiophene-6-carboxylate[*2*]. 0.33 g of t-Butyldimethylsilylchloride was added to a solution of 0.35 g of [*2*] in pyridine and kept at room temperature for 2 hr. The reaction mixture was extracted with ethyl acetate followed by washing with saturated $NaHCO_3$ and NaCl. The obtained residue after evaporation was chromatographed on SiO_2, to give 0.44 g of t-butyldimethylsilyl ether of [*2*]. Then, hydrolysis of compound [*2*] by alkaline treatment gave the carboxylic acid derivative, which was in turn treated with Ph_2CN_2 in DMSO, followed by treatment with tetrabuthylammoniumfluoride in THF, to give compound [*3*]. 79 mg of Succinic anhydride was added to a solution of 0.18 g of [*3*] in pyridine and kept at 60 °C for 5 hr, to give 0.22 g of the acylated compound [*4*]. A solution of 0.22 g of [*4*] and 0.22 g of disuccinimidylcarbonate in dioxane/acetonitrile 5/1 was mixed with 0.12 ml of triethylamine at room temperature and kept for 2 hr. The reaction mixture was extracted with ethyl acetate, followed by washing with saturated $NaHCO_3$ and NaCl, and then the organic layer was evaporated. The resultant succinimidyl ester derivative [*5*] was dissolved in anisole/TFA 1/5 and kept at room temperature for 10 min. The reaction mixture was evaporated and the residue was triturated in Et_2O, to give 0.19 g of [*6*] as TFA salt amorphous. ^1H NMR data of compound [*5*] in $CDCl_3$ (TMS) : d 3.05 - 2.50 (m, 12H), 5.09 and 5.20 (s, each 2H), 6.72 and 6.80 (s, 1H in total), 7.00 (s, 1H), 7.37 (s, 10H), 7.60 - 7.40 (m, 3H).

N-succinimidyl 6-[4,5-dihydro-2-[1-(1H)-imidazolyl]methylbenzo[*b*]thiophene]-carboxylate [*8*] : A solution of 1.00 g of CS-518 (as free acid) and 2.17 g of disuccinimidylcarbonate in dioxane was mixed with 1.18 ml of triethylamine at room temperature for 30 min. The reaction mixture was extracted with ethyl acetate, followed by washing with saturated $NaHCO_3$ and NaCl. The organic layer was dried on Na_2SO_4 and evaporated, to give 1.08 g of [*8*].

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Preparation of Anti- CS-518 Antisera

The CS-518 derivatives were conjugated with bovine serum albumin by the *N*-succinimidyl ester method (5) shown in Fig. 2. Briefly, [*6*] (25.9 mg) or [*8*] (16.2 mg) dissolved in pyridine (1.2 ml) was added to a solution of bovine serum albumin (50 mg) in phosphate buffer 50 mmol/L (pH 7.3, 1.2 ml). The mixtures were gently stirred at 4 °C for 24 hr, followed by dialysis successively against DMF/H_2O 50/50, DMF/H_2O 20/80, and saline. The obtained antigens, [*7a*] and [*9a*], 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 °C for 16 hr. The antisera were obtained by centrifugation at 3,000 g for 20 min.

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Preparation of Enzyme-Labeled Antigens

Peroxidase-labeled antigens were prepared according to the method described by Hosoda *et al.* (6). Briefly, *N*-succinimidyl ester ([*6*] : 855 μg or [*8*] : 536 μg) dissolved in dioxane (0.2 ml) was added to a solution of horseradish peroxidase (2 mg) in phosphate buffer 50 mmol/L (pH 7.3, 0.4 ml) at 0 °C. The mixture was gently stirred at 4 °C for 4 hr, followed by dialysis successively against phosphate-buffered saline (pH 7.4, PBS)/DMF 60/40, PBS/DMF 80/20, and PBS, at 4 °C. The dialysate was diluted with PBS containing 0.1 % gelatin to a concentration of 500 μg peroxidase/ml and stored at 4 °C.

ELISA

The microtiter plates, which were coated with goat immunoglobulin G against rabbit immunoglobulin G and post-coated with human serum albumin, were washed 3 times with PBS containing 0.05 % Tween 20 (buffer A) just before use. 50 μl of Antiserum, diluted 50,000- (anti-[*7a*] for ELISA-1) or 20,000-fold (anti-[*9a*] for ELISA-2) with PBS containing 0.1 % human serum albumin (buffer B), was placed in each well. Peroxidase-labeled antigen (50 μl), diluted to 400 ng/ml ([*7b*] for ELISA-1) or 4 μg/ml ([*9b*] for ELISA-2) with buffer B, and the standard or

unknown solution (50 μl) were added to the wells. The plates were incubated for 20-24 hr at 4 °C and washed with buffer A (350 μl x 3). The peroxidase activity retained on the plates was measured according to the method described previously with slight modification (7). Briefly, 200 μl of 0.01 % tetramethylbenzidine in acetate-citric acid buffer 50 mmol/L (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 H_2SO_4 500 mmol/L (50 μl), and the absorbance of the sample was measured at 450 nm using an MTP-100 microplate reader (Corona-denki, Ibaragi, Japan).

Preparation of Samples

Blood (3 ml) was collected from rabbits from an ear artery using a syringe containing a solution of sodium citrate at a final concentration of 0.38 %. The blood, obtained at scheduled times after the oral administration of CS-518, was immediately centrifuged at room temperature (170 g, 10 min), to obtain platelet-rich plasma. After the platelet-rich plasma was divided into two portions, one (0.2 ml) was used for the counting of platelets using a K-1000 (Sysmex, Tokyo, Japan), and the other (0.5 ml) was subjected to further centrifugation at room temperature (3,000 g, 3 min), to separate the plasma and platelets. The mean volume of platelets (5.52 ± 0.05 fl), which was calculated from data obtained in this investigation, and the number of platelets in each sample were used to express the concentrations of CS-518 in platelets.

The pellets of platelets were washed with acid-citrate-dextrose solution (0.4 ml x 2) and re-suspended with PBS (0.4 ml), followed by heating (100 °C, 2 min) to release the drug from platelets. After centrifugation, the supernatant was subjected to ELISA at a 3-fold dilution with buffer B.

The plasma proteins were precipitated by methanol (a 10-fold volume of plasma), and the supernatant was evaporated and subjected to ELISA after being dissolved in buffer B. The residue from plasma was also subjected to an HPLC, which was constructed with Cosmosil $5C_{18}$-P as a column and acetonitrile/H_2O/ acetic acid 15/84/1 as a mobile phase.

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TABLE 1

Comparison of Two ELISA Systems for CS-518 in Sensitivity and Selectivity

ELISA system (Antiserum No.)		B/B_0=0.5 (pg/well)	Cross-reactivity (%) R-5206	R-5512	R-5652*	Glucuronide*
1	(No.1)	52	4	0.5	5	0.1
	(No.2)	74	3	0.6	4	0.3
	(No.3)	82	3	0.1	5	0.2
2	(No.1)	26	84	10	68	96
	(No.2)	82	88	4	99	99
	(No.3)	34	77	8	44	68

* CS-518 equivalent

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CS-518 Administration to Rabbits

Japanese white male rabbits were obtained from Shiraishi (Tokyo, Japan) and maintained in a 12-hr light/dark cycle at constant room temperature and humidity. Various doses (0.1, 1, and 10 mg/kg) of CS-518 dissolved in saline were given orally to rabbits that had been fasted for 16 hr.

RESULTS

ELISA of CS-518

The cross-reactivities with metabolites of CS-518 in the two ELISAs were calculated from the amount of unlabeled CS-518 or metabolites required to displace 50 % of the maximal binding of peroxidase-labeled CS-518; these cross-reactivities are shown in Table 1. Much lower cross-reactivities were observed with all metabolites in ELISA-1. In particular, cross-reactivity with the major metabolite, the glucuronide of CS-518, was 0.1 % by ELISA-1 using antiserum No.1, in contrast to 68 - 99 % by ELISA-2.

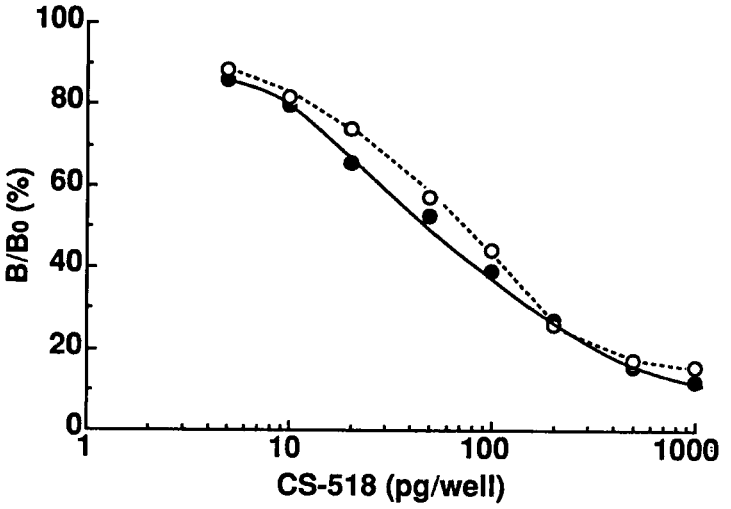

FIGURE 3 Dose-response curves of two ELISA systems. (●) is a dose-response curve for ELISA-1 and (○) is for ELISA-2. B: bound enzymic activity at various concentrations of CS-518 and Bo: bound enzymic activity at zero concentration.

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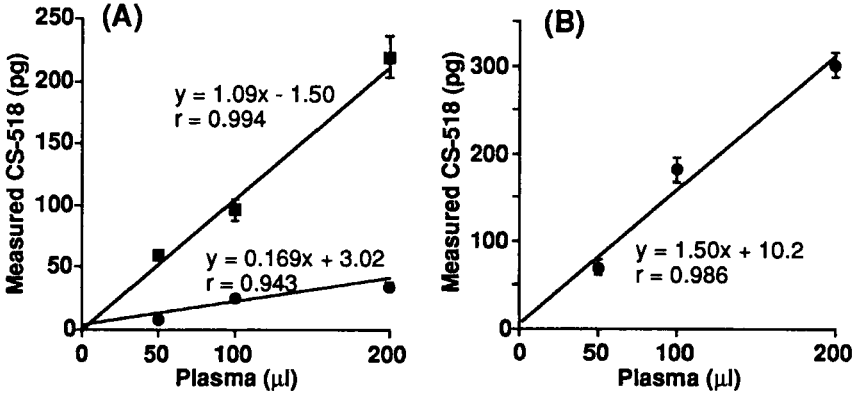

FIGURE 4 Influence of the plasma volume on the determination of CS-518 by ELISA-1.

(A) Control plasma of rabbits mixed with 200 pg/ml (●) and 1 ng/ml (■) of CS-518, and (B) rabbit plasma collected 24 hr after oral administration of CS-518 at a 10 mg/kg dose. The amount of the methanol extract is presented in terms of the original volume of rabbit plasma.

The representative dose-response curves of ELISA-1 (antiserum No.1) and -2 (antiserum No.2) are shown in Fig. 3. CS-518 could be determined over the range of 5 to 1000 pg/well (*ca.* 90 and 10 % of maximum binding, respectively) in both ELISAs. The intraassay coefficients of variation at a CS-518 level of 100 pg/well were of 13.5 and 19.7 %, respectively for ELISA-1 and -2 (n = 10). The interassay coefficients of variation in ELISA-1 and -2 were 10.4 and 12.5 %, respectively (n = 5) over 3 months. The detection limits based on 2 S.D. of maximum binding, were 5 pg/well for ELISA-1 and 10 pg/well for ELISA-2.

Validity of ELISA Applied to Determine CS-518 in Rabbit Plasma

Various amounts of the methanol extract from rabbit plasma, which was mixed with standards or was collected after CS-518 dosing, were subjected to ELISA-1 (Fig. 4). When 200 pg/ml and 1 ng/ml of CS-518 in control plasma were determined using 50 - 200 µl of the original volume of plasma, the extract by methanol deproteinization hardly interfered with ELISA-1. Also, the linearity between the volume of plasma sample collected after oral administration of CS-518 and the value measured by ELISA-1 was observed despite the coexistence of metabolites in plasma.

When the plasma concentrations of CS-518 in rabbits after oral administration at a 10 mg/kg dose were measured by ELISA-1 and -2, there was a difference between the area under the curve (AUC, 0 - 24 hr) obtained by ELISA-1 (7.7 ± 1.2 µg.hr/ml) and that by ELISA-2 (10.0 ± 0.8 µg.hr/ml). A similar difference was also observed with the other doses of CS-518 (0.1 and 1 mg/kg). Also, the plasma samples (30 min - 4 hr) after oral administration of CS-518 at the 10 mg/kg dose were deproteinized by methanol and subjected to an HPLC method. There was good correlation between the values by HPLC and ELISA-1, as shown in Fig. 5(A) ($y = 0.949x - 0.349$, $r = 0.960$, $s_{x,y} = 3.68$). The same samples were subjected to HPLC after alkaline treatment to hydrolyze the glucuronide of CS-518 and ELISA-2 without alkaline treatment. Though a certain linearity was observed as shown in Fig. 5(B), it was not as good as the result obtained in Fig. 5(A).

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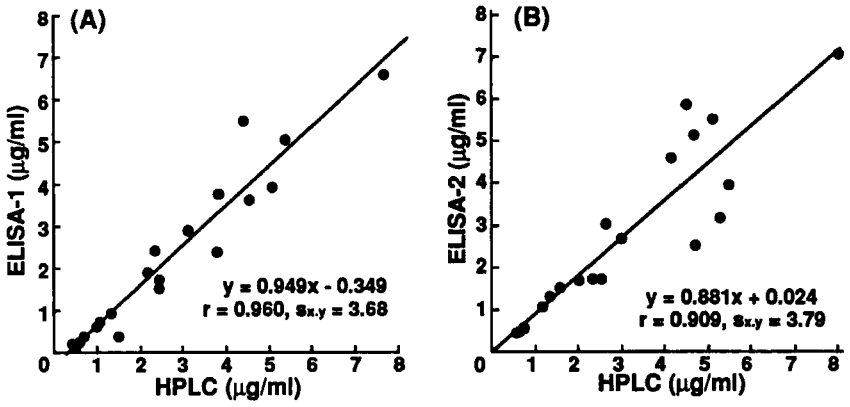

FIGURE 5 Correlation analysis between two ELISA systems and HPLC for CS-518 levels in rabbit plasma.
(A) ELISA-1 vs. HPLC and (B) ELISA-2 vs. HPLC. HPLC values in (B) expressed the total amount of free and glucuronide of CS-518 that was hydrolyzed by alkaline treatment.

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Concentrations of CS-518 in Rabbit Platelets

The efficiency of release of CS-518 from platelets was examined using rabbit platelets saturated with ^{14}C-CS-518 *in vitro*. More than 80 % of radioactivity appeared in the supernatant by the heating (100 °C, 2 min), which is thought to be a more simple and suitable clean-up method than the deproteinization applied to plasma samples, without decomposition of CS-518.

The amounts of CS-518 in rabbit platelets after oral administration at 0.1, 1, and 10 mg/kg doses were measured by ELISA-1, and are expressed as a concentration (ng/μl), calculated from the mean volume and numbers of platelets, in Fig. 6. T_{max} of CS-518 in platelets (2 - 8 hr) was later than in plasma (30 min), and C_{max} was 0.6 - 0.8 pg/10^5 platelets, corresponding to 1.1 - 1.4 ng/μl, *i.e.* 4.2 - 5.4 x10^{-6}M, and these parameters were almost constant at various doses. The elimination of CS-518 from platelets ($T_{1/2}$ = 36 - 41 hr) was *ca.* 30 times slower than from plasma.

Relationships between doses of CS-518 and AUC measured by ELISA-1 in plasma and platelets of rabbits are shown in Fig. 7. Good linearity was observed between AUC values in plasma and doses of CS-518, but AUC values in platelets were almost constant (*ca.* 75 mg.hr/ml) and were higher than those in plasma.

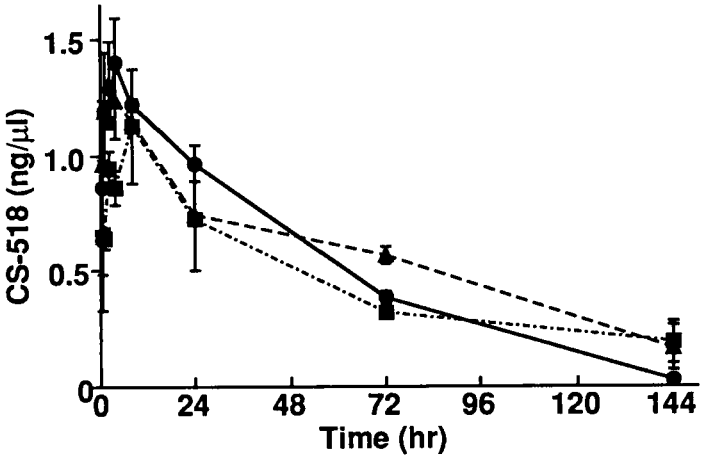

FIGURE 6 Platelet levels of CS-518 measured by ELISA-1 after oral administration of CS-518 to rabbits.

Various doses of CS-518 were examined at 0.1 (●), 1 (■), and 10 mg/kg (▲). One μl of platelets was considered to be obtained from about 500 μl of blood.

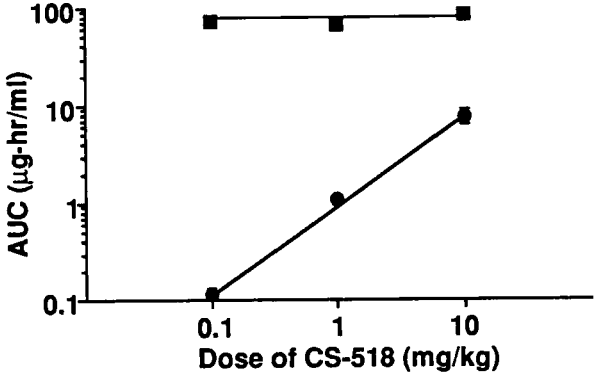

FIGURE 7 Relationship between dose of CS-518 and AUC measured by ELISA-1 in plasma (●) and platelets (■) of rabbits.

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DISCUSSION

We investigated the development of ELISAs for CS-518 using two derivatives, which were linked to proteins to prepare immunogen and labeled-antigen. The use of the 6-carboxylic acid of the dihydrobenzothiophene moiety as a bridge linked to a carrier protein was easy, because there was no need for further modification of the hapten. But the antisera obtained showed a high affinity to all metabolites of CS-518, with the exception of R-5512, which is further oxidized on the dihydrobenzothiophene moiety and has a difference in aromaticity from CS-518. These results, observed in ELISA-2, conformed to the principle that anti-hapten antibody cannot recognize the moiety around the bridge to a carrier protein. In contrast, ELISA-1, which was constructed with antigen linked to proteins *via* carboxylic acid introduced into the imidazolyl ring, resulted in less than 5 % of cross-reactivities with all known metabolites in plasma. That is to say, metabolites modified around the dihydrobenzothiophene moiety were recognized by the antibody, because the linkage position to protein was kept at a distance from the metabolic site.

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Also, antibody used in ELISA-1 was judged to have a high affinity to CS-518, because it was highly sensitive and precise, and it was applicable to plasma samples deproteinized by only methanol. At the same time, the validity of ELISA-1 was confirmed by HPLC using plasma samples containing metabolites. On the other hand, the usefulness of ELISA-2 or its antibody used might be expected from the viewpoints of 1) determination of the total amount of CS-518 containing conjugate, *e.g.* glucuronide, and 2) broad specific extraction with immobilized antibody as a clean-up for ELISA-1. Similar applications are shown in the reports by Doucet *et al.* (8) and Meyer *et al.* (9), respectively. In paticular, ELISA-1 combined with the immobilized antibody used in ELISA-2 could concentrate plasma samples (data not shown). This combination of an immunoassay and an immobilized antibody column may be useful for improvement of not only sensitivity but also specificity, if a compound has more than two metabolic sites that are kept some distance from each other.

ELISA-1 could be applied to the determination of CS-518 in not only plasma but also platelets, only one μl of which can be obtained from about 500 μl of blood, due to its high sensitivity and specificity. CS-518 uptake up to maximum capacity in platelets (*ca.* 5 x 10^{-6} M) and the slow elimination of CS-518 from platelets ($T_{1/2}$: *ca.* 40 hr) were similar to the phenomena reported for Y-20811 (10), a thromboxane synthetase inhibitor, in platelets of dogs. Additionally, in our study, platelet levels of CS-518 were measured after oral administration to rabbits at various doses of CS-518, while minimizing the volume of blood taken to obtain platelets, and independence of these phenomena from doses of the drug was clarified. From these results, it was confirmed that a drug having strong thromboxane synthetase inhibitory activity can bind to the enzyme in platelets with high affinity.

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ACKNOWLEDGMENTS

We wish to thank Dr. M. Tanaka, the director of our laboratories, for his helpful discussions and encouragement during this work. We also thank Dr. A. Terada and Dr. Y. Amemiya for their advice on the synthesis of haptens, and Dr. T. Ikeda and Dr. F. Asai for the preparation of platelets, and Mr. H. Shioya and Ms. M. Soma for the analysis by HPLC.

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