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Capillary gas chromatographic method for the determination of the thromboxane A_2 receptor antagonist S-1452 and its metabolites in human plasma

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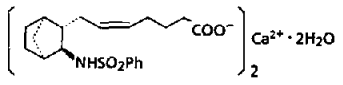
ABSTRACT

A capillary gas chromatographic method for the determination of a newly developed thromboxane A_2 antagonist, S-1452, and its metabolites in human plasma has been developed. This nitrogenous compound was detected as the free acid (+)-S-145 using a nitrogen–phosphorus detector. The two metabolites bisnor-(+)-S-145 and tetranor-(+)-145 were also assayed. The three compounds, which all possess carboxylic acid group, were converted into their hexafluoro-2-propyl esters with hexafluoropropan-2-ol–pentafluoropropionic anhydride as reagent. The esterification resulted in a decreased background response from the detector, and hence higher sensitivity and better precision. The glucuronides of the three compounds were also assayed after alkaline hydrolysis. The plasma concentration of the glucuronide of I was more than twice that of the free acid. This method allows determination of the three compounds at more than 2 ng/ml with a precision of 2–6% and an accuracy variation of less than ±5%. The analysis was performed within 2 min after injection to assay the sample rapidly from the Phase I test. The method is suitable for pharmacokinetic studies.

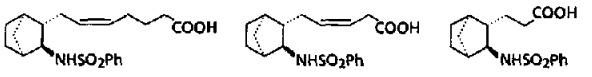
INTRODUCTION

S-1452 (Fig. 1) was developed in our laboratories as a new thromboxane A_2 receptor antagonist. It inhibits platelet aggregation, vasoconstriction and bronchoconstriction activities for potential application as a drug to treat human bronchial asthma, ischemic heart disease and cerebrovascular disorders. An assay method for S-1452 in human plasma was developed for pharmacokinetic studies. Capillary gas chromatography (GC) was applied to assay S-1452 [as a free acid (+)-S-145 (I)] and its two metabolites bisnor-(+)-S-145 (II) and tetranor-(+)-S-145 (III), present in more than nanogram amounts. Nitrogen–phosphorus detection (NPD) was used. GC–NPD was established as a practical means for the assay, with high sensitivity and precision at the nanogram level.

Capillary GC has been used for the analysis of prostaglandins and prostaglandin-like drugs,



S-1452



(+)-S-145 I | Bisnor-(+)-S-145 II | Tetranor-(+)-S-145 III

Fig. 1. Structures of S-1452, calcium (*d*)-(1*R*,2*S*,3*S*,4*S*)-7-[3-*endo*-(phenylsulphonylamino)bicyclo[2.2.1]hept-2-*exo*-yl]hep-tenoate hydrate, the free form of (+)-S-145 (I), and its two major metabolites bisnor-(+)-S-145 (II) and tetranor-(+)-S-145 (III).

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such as S-1452 [1]. In these methods, the mass spectrometer has been generally used as a detector [2–4]. However, a detector offering simpler operation and adequate sensitivity, not less than those of the mass spectrometer and the electron-capture detector, was desirable [5–7].

This paper describes a GC–NPD method for assaying I and its metabolites in human plasma, together with their glucuronides. We also discuss the practicability of the method on the basis of our analytical data.

EXPERIMENTAL

Materials and reagents

Standard samples of I, (*d*)-(1*R*,2*S*,3*S*,4*S*)-7-[3-*endo*-(phenylsulphonylamino)bicyclo[2.2.1]hept-2-*exo*-yl]heptenoic acid, II, (*d*)-(1*R*,2*S*,3*S*,4*S*)-7-[3-*endo*-(phenylsulphonylamino)bicyclo[2.2.1]-hept-2-*exo*-yl]penta-3′-enoic acid, and III, (*d*)-(1*R*,2*S*,3*S*,4*S*)-7-[3-*endo*-(phenylsulphonylamino)bicyclo[2.2.1]hept-2-*exo*-yl]propionic acid, of high purity were synthetized in our laboratories. An internal standard used for chromatography, (*d*)-(1*R*,2*S*,3*S*,4*S*)-7-[3-*endo*-(4-methylphenylsulphonylamino)bicyclo[2.2.1]hept-2-*exo*-yl]heptanoic acid, was also synthesized (Fig. 2).

Hexafluoropropan-2-ol (HFIP) was purchased from TCI (Tokyo, Japan). Pentafluoropropionic anhydride (PFPA) was obtained from Pierce (Rockford, IL, USA). Commercially available 1-chlorobutane of reagent grade (Wako, Osaka, Japan) was distilled before analysis. Acetone and toluene (residual pesticide analysis) were from Wako. Other chemicals were of JIS (Japanese Industrial Standard) special grade.

The HFIP–PFPA reagent solution was prepared by mixing 5 ml each of HFIP and PFPA in a 20-ml flask. It was stored at 5°C.

To prepare the 0.25 *M* phosphate buffer solution (pH 2.3), 69 g of sodium dihydrogenphosphate monohydrate and 190 g of trisodium phosphate were dissolved in 0.9 l of deionized water. The pH was adjusted to 2.3 with 1 *M* hydrochloric acid, to within ±0.02 units, and the solution was made up to 1 l with deionized water.

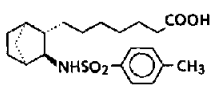

Fig. 2. Structure of the internal standard.

Apparatus

A Hewlett-Packard (Avondale, PA, USA) 5890 Series II gas chromatograph, equipped a nitrogen–phosphorus detector and a Model 3396 Series II integrator, was employed. The fused-silica capillary column (12.5 m × 0.31 mm I.D., HP Ultra 1) had a 0.53-μm thick layer of cross-linked methyl silicone. The oven was operated isothermally at 280°C. The injector and detector temperatures were 330 and 310°C, respectively. The carrier gas was helium at a flow-rate of 3.5 ml/min. The sample was introduced via split injection with a split ratio of 5:1 or 10:1.

An Iwaki (Tokyo, Japan) Model V-S shaker and a Kubota (Tokyo, Japan) Model KS-5000P centrifuge were used for the extraction procedure. Two types of evaporator were used: a Model S-10 from Tokyo Rikakikai (Tokyo, Japan) and a Model MG-2 (Dry Block Bath) from Jtorika (Tokyo, Japan). A Bransonic Model 521 sonicator from Yamamoto (Tokyo, Japan) was used to completely dissolve standard samples in aqueous methanol.

Assay procedure

Method A: free forms of I, II and III. Pipette 1.5 ml of plasma, after treating the blood with heparin, into a 12-ml centrifuge tube. Add 1.5 ml of acetone and shake the tube by hand two or three times. Centrifuge at 2300 *g* for 5 min. Transfer 1 ml of the supernatant solution into a 12-ml centrifuge tube. Add 100 μl of internal standard solution. Add 1 ml of 0.25 *M* phosphate buffer (pH 2.3) and 5 ml of 1-chlorobutane, successively. Shake the tube for 10 min and centrifuge at 1100 *g* for 5 min. Transfer the entire upper layer into a 12-ml centrifuge tube. Add 2 ml of 1 *M* NaOH. Shake the tube for 10 min and centrifuge at 1100 *g* for 5 min. After discarding the upper layer, wash the aqueous layer twice with 0.5 ml of 1-chlorobutane. Add 0.4 ml of 5 *M*

HCl, 0.5 ml of 0.25 *M* phosphate buffer solution (pH 2.3) and 5 ml of 1-chlorobutane, successively. Shake the tube for 10 min. Centrifuge at 1100 *g* for 5 min. Transfer the entire upper layer into a 10-ml centrifuge tube, and leave it to evaporate at room temperature under reduced pressure. Place the tube on a dry block bath and allow the solution to evaporate to dryness at 75°C for 5 min under a gentle stream of nitrogen gas. Add 150 μl of HFIP–PFPA. Heat on a dry block bath at 75°C for 15 min, then evaporate the solution to dryness at 75°C under a gentle stream of nitrogen gas. Dissolve the residue with 25–50 μl of toluene. Adjust the volume of the toluene within a suitable sample concentration range.

Method B: total forms of I, II and III. Add 100 μl of the internal standard solution and 0.4 ml of 5 *M* NaOH to the deproteinized supernatant solution obtained by method A. Gently shake the mixture by hand then allow it to stand for 15 min at room temperature. Add 0.4 ml of 5 *M* HCl and then 1 ml of 0.25 *M* phosphate buffer solution. Extract the solvent with 1-chlorobutane according to the conditions described in method A.

Standard solution. Accurately weigh 2.5 mg of I, 2.5 mg of II and 2.5 mg of III into a 250-ml volumetric flask. Dissolve in 50 ml of methanol with a sonicator for 30 s and dilute with deionized water to volume. Pipette 1 ml of the solution into a 10-ml volumetric flask and dilute with deionized water to volume (final concentration of each substance 1 ng/μl).

Internal standard solution. Accurately weigh 2.5 mg of internal standard into a 250-ml volumetric flask. Dissolve in 50 ml of methanol with a sonicator for 30 s and dilute with deionized water to volume. Pipette 5 ml of the solution into a 50-ml volumetric flask and dilute with deionized water (final concentration 1 ng/μl).

Calibration curves. Place 3 ml of human control plasma into a 10-ml centrifuge tube. Add 3 ml of acetone. Shake the tube by hand two or three times and centrifuge at 2300 *g* for 10 min. Transfer 1 ml of the deproteinized supernatant solution into four 12-ml centrifuge tubes.

Add 5, 25, 75 and 125 μl each of the standard solution (each standard, I, II and III, respectively). After addition of 100 μl of the internal standard solution (100 ng per tube), add 1 ml of 0.25 *M* phosphate buffer (pH 2.3) and 5 ml of 1-chlorobutane. Proceed as for method A.

RESULTS AND DISCUSSION

Pretreatment

Plasma was deproteinized with acetone. 1-Chlorobutane could be used for complete extraction of I, II and III from the deproteinized supernatant. Less of the plasma components was extracted than with other solvents, such as ethyl acetate and dichloromethane, causing less interference for the chromatographic analysis. The internal standard was extracted to the same extent as the three compounds. No glucuronides of the three compounds were extracted with 1-chlorobutane, which means that analysis of free unconjugated forms alone is possible. The solid extraction method for the pretreatment, which has been generally used for the analysis of prostaglandins [8,9], was unsatisfactory for separating the unconjugated forms because of poor selectivity for separating unconjugated and conjugated forms on the pretreatment C_{18} column. The range of pH in the aqueous phase needed to extract the three compounds was 2.3–3.0. Compound I is an acid of pK_a 4.92, and the two metabolites will be acids of similar strength. The pH was set at 2.3 for easy extraction of the undissociated forms.

Derivatization conditions

Pentafluorobenzyl bromide (PFB-Br) reagent. The GC analysis of prostaglandins derivatized with PFB-Br has been reported [10,11]. However, this reagent caused higher background on the chromatogram due to its presence as a plasma component, complex procedures were needed to remove any excess, and its retention time of 10 min was long. Therefore, this reagent was not used in this analysis.

Diazomethane. This reagent is a popular one, but when the derivatized methyl esters of the compounds were detected using NPD, interfering peaks appeared, as when PFB-Br was used. The interference could not be removed, even after the

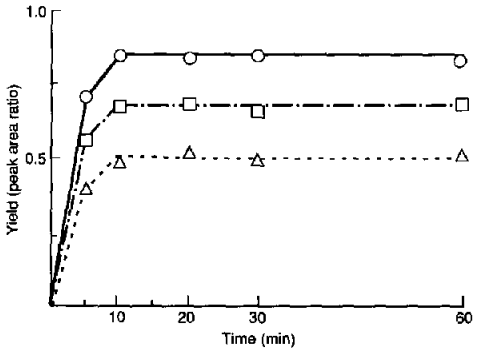

Fig. 3. Effect of reaction time on peak-area ratio at 75°C: (○) I; (□) II; (△) III.

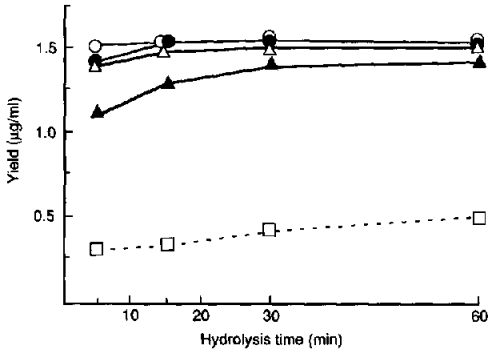

Fig. 4. Hydrolysis of the glucuronide of I in plasma samples with various reagents at room temperature: (○) 3 *M* NaOH; (●) 2 *M* NaOH; (△) 1 *M* NaOH; (▲) 0.5 *M* NaOH; (□) 3 *M* HCl.

first extract was transferred to the alkaline aqueous phase, followed by reextraction with 1-chlorobutane.

HFIP–PFPA reagent solution. A mixture of HFIP and PFPA (1:1) has been widely used as a reagent for the derivatization of amino acids in GC analysis [12–14]. This reagent causes simultaneous esterification by HFIP and acetylation by PFPA. PFPA also acts as a dehydrating reagent to cause the esterification to proceed. The two reagent compounds are volatile and easily removed by evaporation. There are few reports about the derivatization of general carboxylic acids such as prostaglandins. When HFIP–PFPA was used to esterify compounds I–III, the background was better than that obtained with the other reagents. The effect of reaction time on the esterification rate was examined. As shown in Fig. 3, with a reaction time of more than 10 min, the yields (peak areas) for the three compounds and the internal standard became constant. The conditions for use were set at 75°C and 15 min.

Hydrolysis conditions of glucuronide

Glucuronide formation when the racemate S-145 was administered to humans was studied. The three glucuronides of S-145 and the corresponding bisnor-S-145 and tetranor-S-145 forms were isolated, and their structures were identified [15]. Because we expected the glucuronides to appear in both plasma and urine, the hydrolysis conditions of the glucuronides were examined using the urine sample collected after administration. Because glucuronides, such as the ester type, can be easily hydrolysed with alkali [16], the artificial human plasma sample spiked with urinary extract by ethyl acetate was hydrolysed with alkali. The three free forms increased on hydrolysis, as the peaks appeared at the same retention times as the original ones. Fig. 4 shows the time course of the hydrolysis with various concentrations of NaOH. Hydrolysis was rapid when 2 or 3 *M* NaOH was used to achieve a constant yield for more than 15 min. Lower-yields were obtained with acid hydrolysis: only 30% yield was obtained even after hydrolysis for 60 min with 3 *M* HCl.

Capillary gas chromatography

Fig. 5 shows chromatograms obtained by analysis of two types of human plasma: control plasma from four volunteers collected before drug administration and plasma spiked with the three compounds. The analytes peaked within 2 min with good separation (Fig. 5b), being eluted in the order III, II, I, and the internal standard. Little interference around the compounds from the four volunteers (before and after meal). The chromatogram obtained with diazomethane (Fig. 5c) shows interfering peaks. Esterification with HFIP–PFPA after the first extraction alone led to insufficient clean-up (Fig. 5a).

A typical chromatogram obtained from a plasma sample collected 0.5 h after the oral adminis-

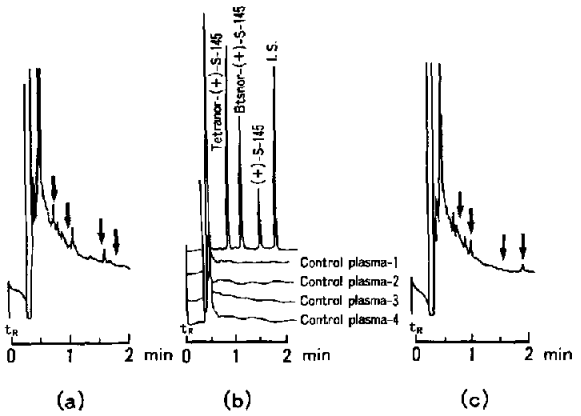

Fig. 5. Background of human control plasma derivative with HFIP–PFPA after first extraction (a), with HFIP–PFPA after back extraction (b), and with diazomethane after back extraction (c). Attenuation × 1.

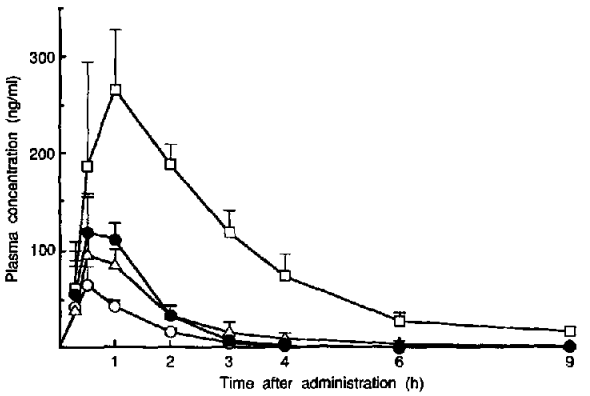

Fig. 7. Plasma concentration–time curves of (+)-S-145 and its metabolites in humans following single oral administration of S-1452 of 50 mg (mean ± S.D., n = 4): (○) I; (●) I-glucuronide; (△) total II; (□) total III.

tration of S-1452 (50 mg) is shown in Fig. 6, together with that obtained after hydrolysis.

Plasma levels of I and its metabolites

This assay method was applied to the simultaneous determination of I and the two metabolites in human plasma. Fig. 7 shows a plasma level–time profile obtained following single oral administration of S-1452 at 50 mg to healthy male volunteers (n = 4) in the Phase I test. Compound I was absorbed well and quickly reached the maximum value at nearly 40 min (C_{max}, mean ± S.D.: 73 ± 43 ng/ml), then decreased over 4–6 h, which indicated a relationship with the decreasing platelet aggregation activity [17]. The glucuronidation rates to the metabolites were *ca.* 40–50% for II and *ca.* 50–60% for III.

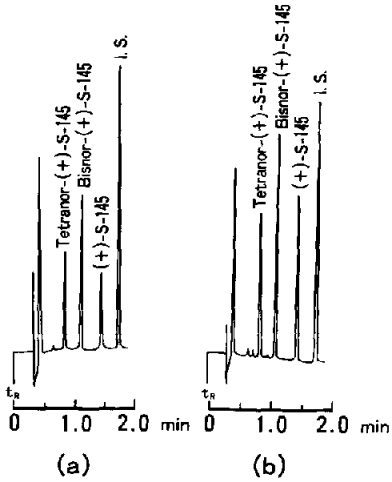

Fig. 6. Typical capillary gas chromatograms of I and its two metabolites. (a) Plasma sample treated by method A for 0.5 h following single oral administration of S-1452 of 50 mg; concentrations: I = 37.1 ng/ml; II = 58.6 ng/ml; III = 19.6 ng/ml. (b) Plasma sample treated by method B for 0.5 h following single oral administration of S-1452 of 50 mg; concentrations: I = 83.6 ng/ml; II = 96.7 ng/ml; III = 41.5 ng/ml. Atttenuation × 1.

Assessment of the assay method.

Spiked samples of the three compounds were assayed repeatedly. The method proved to be practical for pharmacokinetic studies, as can be seen from the following analytical data.

Linearity of the calibration graphs

The calibration graphs constructed for each of the three compounds showed good linearity. The regression coefficients calculated from the replicated measurements for the compounds were all more than 0.9996. Table I shows the reproducibility of calibration within and between days.

Precision and accuracy

Tables II and III shows the assay values for the three compounds in spiked plasma (four concentration levels). The precision of the method is *ca.*

TABLE I

LINEARITY AND PRECISION

Compound	Regression equation[a]	Within-day[b]		Between-day[c]	
		r	s^d	r	s^d
I	$y = 0.8809x - 0.0054$	0.9999	0.025	0.9991	0.037
II	$y = 0.9307x - 0.0103$	0.9997	0.014	0.9997	0.019
III	$y = 1.1042x + 0.0178$	0.9996	0.022	0.9991	0.024

[a] x = concentration; y = peak-area ratio.
[b] $n = 8$.
[c] Three days; $n = 24$.
[d] Estimate of residual standard deviation [18].

5% relative standard deviation (R.S.D.) and does not exceed 10% deviation from the real amount. Five replicate assays were conducted within a day (Table II) and between days (Table III, three days). Good reproducibility was validated with significant difference in variation in both within-day and between-day data.

Sensitivity

The method detects each analyte at a concentration of 1 ng/ml in plasma. The detection limit was defined as the value of three times the signal-to-noise ratio. The lower limit of determination was 2 ng/ml, at which each analyte was assayable within 25% (R.S.D.).

Stability

It is known that the drug S-1452 is very stable in both the solid state and in solutions, such as methanol and the buffer. Stability tests of the free form I and its two metabolites in methanol and human plasma resulted in little or no deteriora-

TABLE II

WITHIN-DAY PRECISION AND ACCURACY FOR I AND ITS TWO METABOLITES IN PLASMA SAMPLES

Compound	Concentration added (ng/ml)	Concentration found[a] (mean ± S.D.) (ng/ml)	Precision[b] (%)	Accuracy[c] (%)
I	5	4.5 ± 0.5	11.1	−10.0
	10	10.5 ± 0.4	3.8	5.0
	100	105.7 ± 5.5	5.2	5.7
	250	252.3 ± 12.0	4.8	0.9
II	5	5.5 ± 0.3	5.5	10.0
	10	10.3 ± 0.4	3.9	3.0
	100	95.4 ± 4.7	4.9	− 4.6
	250	239.5 ± 13.0	5.4	− 4.2
III	5	4.5 ± 0.3	6.7	−10.0
	10	9.6 ± 0.6	6.3	− 4.0
	100	95.4 ± 4.7	4.9	− 4.6
	250	239.5 ± 13.0	5.4	− 4.2

[a] $n = 5$.
[b] Coefficient of variation = S.D./mean × 100.
[c] Percentage deviation = (found − added)/added × 100.

TABLE III

BETWEEN-DAY PRECISION AND ACCURACY FOR I AND ITS TWO METABOLITES IN PLASMA SAMPLES

Compound	Concentration added (ng/ml)	Concentration found[a] (mean ± S.D.) (ng/ml)	Precision[b] (%)	Accuracy[c] (%)
I	5	5.1 ± 0.5	9.8	2.0
	10	9.7 ± 0.7	7.2	−3.0
	100	95.7 ± 5.2	5.4	−4.3
	250	247.3 ± 11.3	4.6	−1.1
II	5	5.4 ± 0.4	7.4	8.0
	10	9.8 ± 0.7	7.1	−2.0
	100	98.9 ± 5.7	5.8	−1.1
	250	258.8 ± 10.3	4.0	3.5
III	5	4.6 ± 0.4	8.7	−8.0
	10	10.7 ± 0.6	5.6	7.0
	100	99.1 ± 5.6	5.7	−0.9
	250	257.5 ± 9.8	3.8	3.0

[a] $n = 5$.

[b] Coefficient of variation = S.D./mean × 100.

[c] Percentage deviation = (found − added) /added × 100.

tion of their quality compared with the initial value. When plasma samples stored at −20°C were assayed repeatedly over a long term, each analyte was recovered at a constant value, indiating the stability of the corresponding glucuronide in the frozen plasma. The final derivatives of the analytes were stable in toluene solution for at least three days. It was confirmed that all the analytes and their derivatives existed in a stable state during the assay procedure. It is possible to store samples at −20°C or below for more than six months.

Recovery

To evaluate the extraction recovery of the sample and the internal standard, a calibration curve was established by direct derivatization. Methanolic solutions of the standard and the internal standard were evaporated and the reagent was added, followed by the analytical procedure. When the calibration curve was compared with that constructed by the original method, little difference was observed between the two linear lines. Also, the peak areas corresponding to all three analytes (I, II and III) showed the same degree of counts between the two methods. These results indicate that the analytes were recovered almost quantitatively from the plasma.

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