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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 urine

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ABSTRACT

A capillary gas chromatographic method using a sulphur-specific detector (Hall's electrolytic conductivity detector) was established to determine the thromboxane A_2 antagonist S-1452 and its metabolites in human urine. The target species were the free acid (+)-S-145 of the drug and its nine metabolites: the three hydroxyl forms of (+)-S-145 (I, II and III), bisnor-(+)-S-145 (IV) the hydroxylated forms of IV (V and VI), tetranor-(+)-S-145 (VII) and the hydroxylated forms of VII (VIII and IX). These ten compounds, which have the same sulphur-containing functional group in common, were determined simultaneously. Their conjugated forms, which were assumed to be glucuronides, were also assayed after hydrolysis. The first derivatization was esterification with diazomethane. The second, for the hydroxylated compounds, was trimethylsilylation with bis(trimethylsilyl)trifuloroacetamide. The ten analytes appeared as separate peaks without mutual interference during 5 min. Hall's detector distinguished the ten analytes selectively from the other urinary components, which removed the need for complex clean-up procedures and led to higher sensitivity with a lower noise level. The method is sensitive enough for the assay of substances present at more than 0.1 μg/ml of urine. All the compounds could be determined with a high level of precision and accuracy, with 2–5% relative standard deviation and within ±5% deviation from the actual value. Day-to-day measurements verified the reproducibility of the method. Recovered substances were quantified by following the time course, and the analytical data together with previously obtained plasma data clarified the metabolism pharmacokinetically.

INTRODUCTION

Previously we reported a gas chromatographic (GC) method using nitrogen–phosphorus detection (NPD) for the determination of a new drug, the thromboxane A_2 receptor antagonist S-1452, in human plasma [1]. The method can be used for pharmacokinetic studies of the plasma level of (+)-S-145 and its metabolites in humans after drug administration. In this study, we tried to establish a method of assaying these substances in human urine, to be followed by actual measurements after the drug had been administered to subjects. In rat urine, more than twelve metabolites, but not the conjugated forms, are produced [2–4]. Our results indicated that nine metabolites were formed in human urine, as shown in Fig. 1. Simultaneous determinations of all ten species, including (+)-S-145, required a more selective method than that for plasma. Thus, we chose a sulphur-specific detector (Hall's electrolytic conductivity detector, HECD) in conjunction with a gas chromatograph, because all the analytes contain sulphur, which is not abundant in the biological components of the urine. Hall's detector developed in 1978 [5] but few applications have been reported, especially in drug anal-

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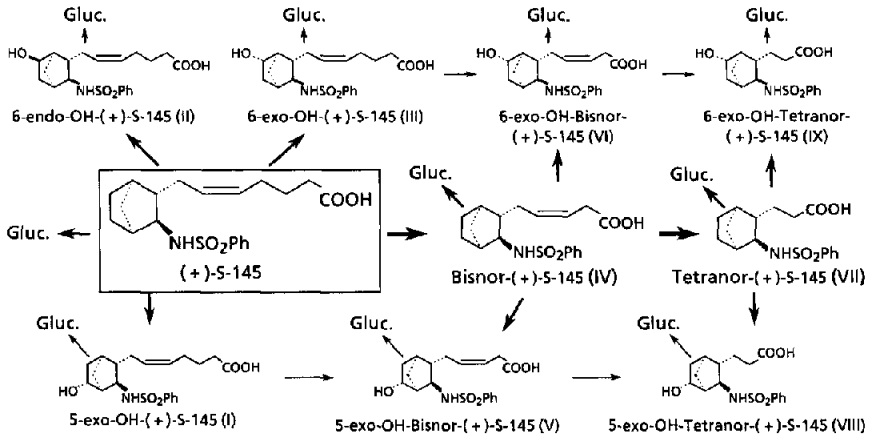

Fig. 1. Metabolic pathway of (+)-S-145 in humans.

ysis, except for examples of gas matrices, such as odorants or pesticides. The selectivity of the detector was only slightly affected by the urinary components, enabling simultaneous determinations of all ten analytes after a simple pretreatment procedure.

This paper describes the assay method and discusses the metabolism based on the analytical data of humans administered S-1452 in the Phase I test.

EXPERIMENTAL

Materials and reagents

Standard samples of nine metabolites of (+)-S-145, (*d*)-(1*R*,2*S*,3*S*,4*S*)-7-[3-*endo*-phenylsulphonylamino)bicyclo[2.2.1]hept-2-*exo*-yl]heptenoic acid, were prepared in our laboratories [6]. An internal standard used for chromatography, (*d*)-(1*R*,2*S*,3*S*,4*S*)-7-[3-*endo*-(phenylsulphonylamino)bicyclo[2.2.1]hept-2-*exo*-yl]pentanoic acid, was also synthesized (Fig. 2).

A silylation reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA), containing 1% trimethylchlorosilane (TMCS), was purchased from Pierce (Rockford, IL, USA). Ethyl acetate and toluene were of the grade for residual pesticide analysis.

A 0.25 *M* phosphate buffer solution (pH 2.3) was prepared by dissolving 69 g of sodium dihydrogenphosphate monohydrate and 190 g of trisodium phosphate 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.

An ether solution of diazomethane was prepared by the distillation method using N-nitrosomethyl urea and 20% NaOH solution.

Apparatus

A Hewlett-Packard (Avondale, PA, USA) 5890 Series II gas chromatograph, equipped with a Tremetric (Austin, TX, USA) Model 1000A electrolytic conductivity detector and a J&W (Folsom, CA, USA) 20 m × 0.25 mm I.D., DB-1 fused-silica capillary column treated with a 0.25-μm film of dimethyl silicone, was used. The oven was operated isothermally at 270°C. The injector temperature was 330°C. 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 10:1. The detector conditions (sulphur mode) were: reactor temperature, 820°C; detector base temperature, 300°C; reactor gas, air at a flow-rate of 30 ml/min; make-up gas, helium at a flow-rate of 15 ml/min. The electrolyte was pure methanol at a flow-rate of 0.5 ml/min through the cell.

For the pretreatment procedures, an Iwaki

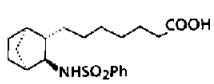

Fig. 2. Chemical structure of the internal standard.

(Tokyo, Japan) Model V-S shaker and a Kubota (Tokyo, Japan) Model KS-5000P centrifuge were used for the extraction. 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. Two centrifuge tubes of 10 and 12 ml were used. The diameter of the former tube was narrower at the bottom than at the centre. The latter tube was an ordinary test-tube. Both pyrex tubes were silanized before use.

Assay procedure

Method A: free forms (unconjugated form) of (+)-S-145 and nine metabolites. Pipette 1 ml of urine into a 12-ml centrifuge tube (adjust and reduce the sample volume from 1 to 0.25 ml). Add 0.25 ml of internal standard solution, 1 ml of 0.25 *M* phosphate buffer (pH 2.3) and 5 ml of ethyl acetate, successively. Shake the tube for 10 min and 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 evaporate the solution to dryness at 80°C for 5 min under a gentle stream of nitrogen gas. After complete drying, dissolve the residue with 75 μl of methanol. Add 0.25 ml of diazomethane solution and leave the mixture standing for 10 min at room temperature. Stop the reaction with 10 μl of glacial acetic acid, then evaporate the mixture to dryness at 80°C for 5 min under a gentle stream of nitrogen gas. Add 100 μl of acetonitrile and 50 μl of BSTFA. Heat on a dry block bath at 80°C for 20 min. Next, evaporate the solution to dryness at 80°C for 5 min under a gentle stream of nitrogen gas. Dissolve the residue in 50–200 μl of toluene. Adjust the volume of the toluene to a suitable concentration. Inject 1–3 μl of the solution into the GC system. Measure the peak area at the retention time of each analyte. Prepare a calibration curve by plotting the peak-area ratio (each analyte/internal standard) *versus* the concentration of the analyte (ten curves).

Method B: total forms (unconjugated and conjugated forms) of (+)-S-145 and nine metabolites. Pipette 1 ml of urine into a 12-ml centrifuge tube (adjust and reduce the sample volume from 1 to 0.25 ml). Add 0.25 ml of the internal standard solution and 0.4 ml of 5 *M* NaOH. After gentle shaking by hand, leave it standing 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 with ethyl acetate under the same conditions described in method A.

Standard solution. Weigh accurately the amount of (+)-S-145 and each standard metabolite as listed in Table I into a 50-ml volumetric flask. Dissolve in *ca.* 30 ml of methanol with a sonicator for 30 s and dilute with methanol to volume. Pipette 5 ml of the solution into a 50-ml volumetric flask and dilute with 25% methanolic water solution to volume (the concentrations of (+)-S-145 and each metabolite are in Table I).

Internal standard solution. Accurately weigh 10.0 mg of internal standard into a 50-ml volumetric flask. Dissolve in 25 ml of methanol with a sonicator for 30 s and dilute with methanol to volume. Pipette 5 ml of the solution into a 50-ml volumetric flask and dilute with 25% methanolic water to volume (concentration 20.0 μg/ml).

Calibration curves. Place 0.5 ml of human urine collected before the administration of S-1452 into four 12-ml centrifuge tubes. Add 0.05, 0.25, 0.5

TABLE I

SAMPLE SIZE AND CONCENTRATION OF STANDARD SOLUTION

Compound	Mass (mg)	Concentration (μg/ml)
(+)-S-145	1.5	3.0
I	1.5	3.0
II	1.5	3.0
III	1.5	3.0
IV	2.5	5.0
V	2.5	5.0
VI	2.5	5.0
VII	2.5	5.0
VIII	5.0	10.0
IX	5.0	10.0

TABLE II

CONCENTRATIONS OF STANDARD SOLUTIONS

Compound	Concentration (μg per tube)			
	Level 1	Level 2	Level 3	Level 4
(+)-S-145	0.15	0.75	1.50	2.25
I	0.15	0.75	1.50	2.25
II	0.15	0.75	1.50	2.25
III	0.15	0.75	1.50	2.25
IV	0.25	1.25	2.50	3.75
V	0.25	1.25	2.50	3.75
VI	0.25	1.25	2.50	3.75
VII	0.25	1.25	2.50	3.75
VIII	0.50	2.50	5.00	7.50
IX	0.50	2.50	5.00	7.50
Internal standard	5.00	5.00	5.00	5.00

and 0.75 ml each of the standard stock solution (for the concentrations of (+)-S-145 and each metabolite, see Table II). Add 0.25 ml of internal standard solution (5 μg per tube), 1 ml of 0.25 *M* phosphate buffer solution (pH 2.3) and 5 ml of ethyl acetate, shake the tubes for 10 min and centrifuge at 1100 *g* for 5 min. Proceed as for method A.

RESULTS AND DISCUSSION

The Hall detector was connected to the capillary gas chromatograph, which was operated at a reactor temperature of 820°C. The flow-rate of methanol in the reactor was 0.5 ml/min. These conditions were highly selective for (+)-S-145 and its metabolites, and the only pretreatment required was a single extraction with ethyl acetate.

Pretreatment

Unconjugated species. All the compounds to be analysed, together with the internal standard, were extracted completely by ethyl acetate from the urine. The glucuronides of these compounds were not extracted with ethyl acetate from the pH 2.3 buffer. Extracted analytes were needed for the derivatization. The carboxyl group contained in all the analytes was esterified with diazomethane. The metabolites with the hydroxyl group were also trimethylsilylated with BSTFA. The derivatizations gave satisfactory yields under the conditions generally used [6–8].

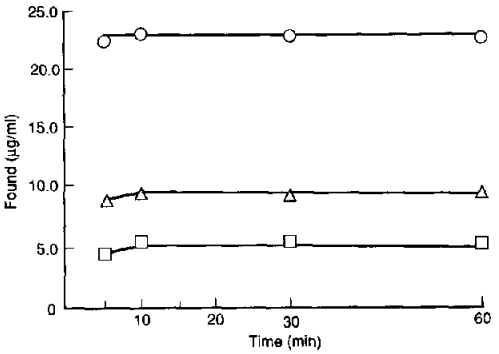

Fig. 3. Effect of hydrolysis time on the concentrations of tetranor-(+)-S-145 (VII) glucuronide, 5-*exo*-OH-tetranor-(+)-S-145 (VIII) glucuronide and 6-*exo*-OH-tetranor-(+)-S-145 (IX): (○) VII; (△) VIII; (□) IX.

Conjugated species. The main portion of (+)-S-145 and its nine metabolites as the free (unconjugated) forms conjugated with a glucuronic acid are excreted into urine. When the urine sample is hydrolysed and assayed by this method, the total amount of the unconjugated and the conjugated forms can be estimated. In the previous study, the glucuronides of IV and VII were hydrolysed in alkaline solution [1]. The glucuronides of (+)-S-145, IV and VII, formed with a hydroxyl substituent at the 5-*exo*- or 6-*exo*-position, will be hydrolysed under the same conditions. In general, such an ester-type glucuronide is hydrolysed with alkaline [9–13].

Fig. 3 shows the concentrations of the total glucuronides of VIII and IX against the time after the urine sample was hydrolysed: the sum of the original VII and its glucuronide. When hydrolysed in aqueous 2 *M* NaOH, the corresponding free form was produced for more than 10 min. Other compounds with the hydroxyl group (I, II, III, V and VI) were also hydrolysed, with similar time courses.

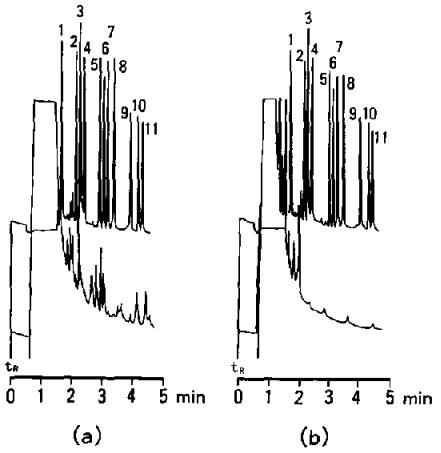

Fig. 4. Background of human control urine by capillary GC–NPD from (a) liquid-phase extraction method, (b) solid-phase extraction method (Certify II). Peaks: 1 = VII; 2 = VIII; 3 = IX; 4 = IV; 5 = V; 6 = VI; 7 = (+)-S-145; 8 = internal standard; 9 = II; 10 = I; 11 = III. Concentrations: VII = 1.8 μg/ml; VIII, IX and IV = 2.3 μg/ml each; V, VI, (+)-S-145, II, I and III = 3 μg/ml each.

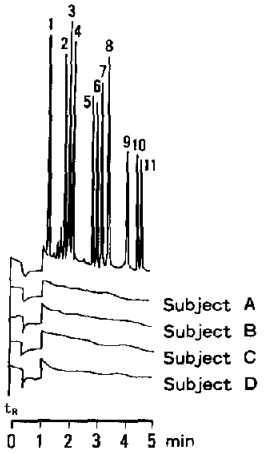

Fig. 5. Background of human control urine from capillary GC–HECD (n = 4). For peak identification, see Fig. 4.

Selectivity of GC–HECD

The Hall detector responses selectively to the sulphur atom in the analytes in the presence of other urinary components. In the previous work, we used NPD with a different fused-silica capillary column [1]. When the eleven compounds (the analytes and internal standard) were analysed by nitrogen detection using the GC–NPD method, they were eluted with retention times between 1.7 and 4.7 min (Fig. 4a). However, chromatography of the treated urine gave a few interfering peaks

TABLE III

LINEARITY AND PRECISION

Compound	Regression equation[a]	Within-day[b]		Between-day[c]	
		r	s^d	r	s^d
(+)-S-145	$y = 1.119x - 0.001$	0.9990	0.022	0.9987	0.036
I	$y = 0.963x + 0.002$	0.9991	0.031	0.9973	0.031
II	$y = 0.693x - 0.002$	0.9995	0.013	0.9968	0.025
III	$y = 0.924x - 0.004$	0.9995	0.011	0.9986	0.026
IV	$y = 1.216x - 0.003$	0.9998	0.020	0.9981	0.043
V	$y = 1.025x - 0.001$	0.9992	0.029	0.9976	0.056
VI	$y = 0.969x + 0.020$	0.9977	0.044	0.9976	0.052
VII	$y = 1.212x + 0.014$	0.9999	0.021	0.9993	0.056
VIII	$y = 1.077x + 0.011$	0.9995	0.036	0.9987	0.044
IX	$y = 0.865x + 0.033$	0.9957	0.032	0.9993	0.040

[a] x = concentration; y = peak-area ratio.
[b] n = 12.
[c] Three days; n = 36.
[d] Estimate of residual standard deviation.

from other urinary nitrogenous compounds, even when the extraction was performed twice. The solid-phase extraction by a Certify II column, which has been used for the analysis of the acidic compound Δ^9-tetrahydrocannabinol (THC) and its carboxy metabolites (THC-COOH) [14], led to little peaking in the background, especially in the faster retention time region, as shown in Fig. 4b.

A typical chromatogram of ten compounds from the GC–HECD analysis is shown in Fig. 5. The standard solutions containing our eleven compounds at concentrations of 3, 5 and 10 μg/ml were treated by the assay procedure, followed by chromatography. The uppermost chromatogram in Fig. 5 was from the standard solution and the lower four chromatograms were from human control urines. All the compounds were eluted within 5 min as well separated peaks. Few or no interfering peaks appeared from the urines of four subjects. The Hall detector detected the S-containing analytes selectively without interference from the urinary components. NPD detected both the analyte and the nitrogenous components in urine, with a selectivity that was less than that of HECD.

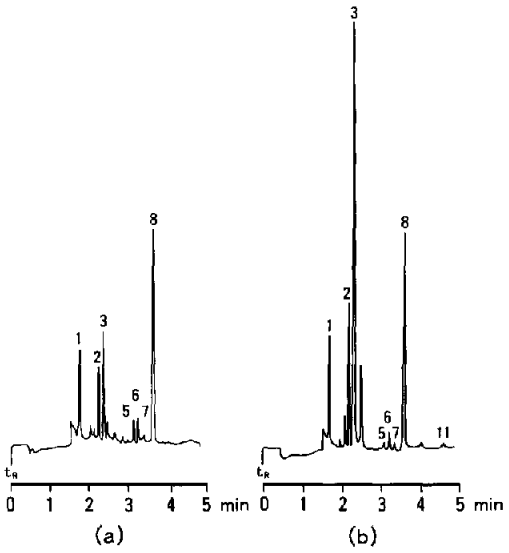

Fig. 6. Typical capillary gas chromatograms of (+)-S-145 and its metabolites in human urine following a single oral administration of 50 mg of S-1452. (a) Unconjugated form of (+)-S-145 and its metabolites; concentrations: VII = 2.1 μg/ml; VIII = 1.6 μg/ml; IX = 4.2 μg/ml; V = 0.8 μg/ml; VI = 0.3 μg/ml; (+)-S-145 = 0.3 μg/ml. (b) After alkaline hydrolysis of (+)-S-145 and its metabolites; concentrations: VII = 6.9 μg/ml; VIII = 8.7 μg/ml; IX = 41.2 μg/ml; V = 1.0 μg/ml; VI = 3.1 μg/ml; (+)-S-145 = 0.7 μg/ml; III = 0.4 μg/ml. For peak identification, see Fig. 4.

Quantitative analysis

The possibility of quantitation was examined by calibrating the output peak of each compound against its concentration. The spiked solutions of urines with (+)-S-145 and its metabolites (ten compounds) containing four different concentrations were assayed by GC–HECD. In all cases the peak-area ratio assayed was proportional to the concentration. A good linear relationship was obtained as a calibration curve, which is shown in Table III. The straight line passed the origin, and the linearity ranged from 0.1 to 7.5 μg/ml. The precision of the linearity was high, with regression coefficients of 0.99–0.999 and no significant day-to-day variation in the linearity. The dynamic range for the assay was extended: *ca.* 15 μg/ml of the analyte positioned at the extended line of the above calibration curve.

Urine samples were collected over 2 h after oral administration of 50 mg of S-1452 and assayed according to the method. Fig. 6 shows the analyses of the unconjugated and conjugated forms. In Fig. 6a, seven metabolites were detected clearly and analysed: greater amounts of the tetranor forms (VII, VIII and IX) were found than of the others, and the free acid (+)-S-145 was a minor component. Analysis of the same urine after hydrolysis is shown in Fig. 6b: the corresponding peak becomes larger with the amount of the glucuronide. More glucuronide was formed form VII, VIII and IX than from the others: IX ≫ VIII > VII. More of the glucuronide of the free acid (+)-S-145 was found, but less of its unconjugated form.

Urinary recovery of S-1452 and its metabolites

Recovery rate. In the Phase I tests for S-1452, the urinary recovery was monitored. The volunteers (n = 4) were described in the previous paper [15]. We obtained the urinary concentrations of

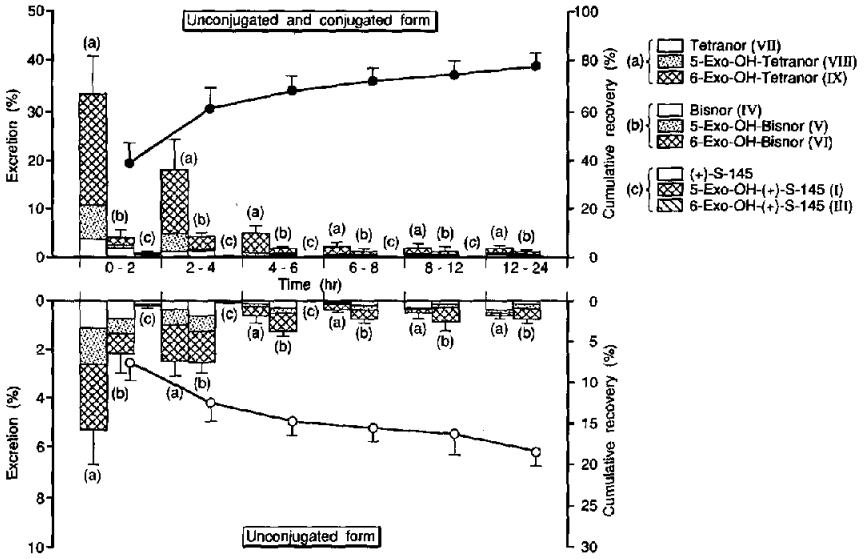

Fig. 7. Urinary excretion profile of (+)-S-145 and its metabolites in humans following a single oral administrations of 50 mg of S-1452.

(+)-S-145 and its nine metabolites from volunteers for whom the plasma levels were already known. Fig. 7 shows the cumulative excretion of the unconjugated and the conjugated forms. About 80% (±5%) of the drug and its metabolites was recovered within 24 h after administration, the conjugated forms accounting for 60% and the unconjugated forms 20%. The excretion was fast with *ca.* 60% being excreted in the first 4 h after administration.

Metabolites. As shown Fig. 7, the conjugated and unconjugated tetranor forms (VII, VIII and IX) accounted for most of the metabolites: *ca.* 55% during 0–4 h. Of these, the hydroxyl forms were predominant, the IX form being the most abundant, followed by the VIII form and the non-hydroxylated VII. The bisnor forms (IV, V and VI) accounted for *ca.* 10% of the total excretion.

Method validation

Precision and accuracy. In order to estimate the precision and accuracy, the assay was repeated using urine solutions spiked with (+)-S-145 and its nine metabolites. Four solutions of different concentrations prepared for each compound were assayed six times. The observed concentrations, the relative standard deviations (R.S.D.) and the deviation from the added value are shown in Tables IV and V. These data showed that all the analytes can be assayed with good accuracy to within ±2% and a precision of *ca.* 2–7%. A duplicate assay was done for each sample within one day (Table IV), and the averaged value was measured repeatedly for three days (Table V). The assayed value could be reproduced without lowering the accuracy, and its variation was in the range 3–11% of the R.S.D.

Sensitivity. The detection limit of each compound was 0.05 μg/ml, calculated as three times the signal-to-noise ratio. The lower limit of determination of each compound was 0.1 μg/ml, calculated for within 25% of the R.S.D. on replicate measurements (n = 6).

Reproducibility. The reproducibility of the method was confirmed by between-day assay of the ten compounds. Calibration curves of the compounds for each day were all linear with good reproducibility (see Table III). The assayed data for each compound were reproduced with little difference in accuracy and precision between days (Table V). The R.S.D. was within 3–6% in the day-to-day assay, and did not exceed 11% even in the cases of compound II with a little

TABLE IV

WITHIN-DAY PRECISION AND ACCURACY FOR (+)-S-145 AND ITS NINE METABOLITES IN URINE SAMPLES

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Precision[a] (%)	Accuracy[b] (%)
(+)-S-145	15	14.99 ± 0.47	3.1	−0.1
	7.5	7.41 ± 0.26	3.5	−1.2
	3	2.90 ± 0.11	3.8	−3.3
	1	1.01 ± 0.05	5.0	1.0
I	15	14.66 ± 0.57	3.9	−2.3
	7.5	7.47 ± 0.35	4.7	−0.4
	3	2.99 ± 0.14	4.7	−0.3
	1	1.00 ± 0.05	5.0	0
II	15	15.11 ± 0.86	5.7	0.7
	7.5	7.52 ± 0.45	6.0	0.3
	3	2.97 ± 0.15	5.1	−1.0
	1	1.01 ± 0.07	6.9	1.0
III	15	14.67 ± 0.45	3.1	−2.2
	7.5	7.47 ± 0.33	4.4	−0.4
	3	3.05 ± 0.11	3.6	1.7
	1	0.98 ± 0.06	6.1	−2.0
IV	25	24.83 ± 0.65	2.6	−0.7
	15	14.69 ± 0.39	2.7	−2.1
	8	7.94 ± 0.21	2.6	−0.8
	2.5	2.53 ± 0.08	3.2	1.2
V	20	19.68 ± 0.44	2.2	−1.6
	10	9.87 ± 0.28	2.8	−1.3
	5	4.98 ± 0.16	3.2	−0.4
	2	2.03 ± 0.17	8.4	1.5
VI	40	39.72 ± 1.12	2.8	−0.7
	25	24.18 ± 0.50	2.1	−3.3
	10	9.93 ± 0.28	2.8	−0.7
	2.5	2.52 ± 0.12	4.8	0.8
VII	25	24.68 ± 0.53	2.1	−1.3
	15	14.88 ± 0.48	3.2	−0.8
	8	7.85 ± 0.15	1.9	−1.9
	2.5	2.53 ± 0.12	4.7	1.2
VIII	25	24.93 ± 0.80	3.2	−0.3
	15	14.93 ± 0.43	2.9	−0.5
	8	7.94 ± 0.26	3.3	−0.8
	2.5	2.49 ± 0.10	4.0	−0.4
IX	100	98.30 ± 2.00	2.0	−1.7
	50	49.15 ± 1.20	2.4	−1.7
	25	24.85 ± 0.65	2.6	−0.6
	5	5.00 ± 0.18	3.6	0

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

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

TABLE V

BETWEEN-DAY PRECISION AND ACCURACY FOR (+)-S-145 AND ITS NINE METABOLITES IN URINE SAMPLES

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Precision[a] (%)	Accuracy[b] (%)
(+)-S-145	15	15.08 ± 0.60	4.0	0.5
	7.5	7.28 ± 0.28	3.8	−2.9
	3	2.95 ± 0.12	4.1	−1.7
	1	1.04 ± 0.05	4.8	4.0
I	15	14.96 ± 0.77	5.1	−0.3
	7.5	7.54 ± 0.49	6.5	0.5
	3	3.06 ± 0.14	4.6	2.0
	1	1.03 ± 0.07	6.8	3.0
II	15	15.23 ± 1.40	9.2	1.5
	7.5	7.64 ± 0.86	11.3	1.9
	3	3.02 ± 0.29	9.6	0.7
	1	1.08 ± 0.01	0.9	8.0
III	15	14.87 ± 0.74	5.0	−0.9
	7.5	7.46 ± 0.61	8.2	−0.5
	3	2.98 ± 0.11	3.7	−0.7
	1	1.01 ± 0.07	6.9	1.0
IV	25	24.78 ± 0.98	4.0	−0.9
	15	14.63 ± 0.59	4.0	−2.5
	8	8.04 ± 0.23	2.9	0.5
	2.5	2.49 ± 0.10	4.0	−0.4
V	20	19.52 ± 0.87	4.5	−2.4
	10	9.77 ± 0.41	4.2	−2.3
	5	5.06 ± 0.21	4.2	1.2
	2	2.09 ± 0.18	8.6	4.5
VI	40	39.20 ± 1.21	3.1	−2.0
	25	24.43 ± 0.65	2.7	−2.3
	10	10.06 ± 0.42	4.2	0.6
	2.5	2.56 ± 0.13	5.1	2.4
VII	25	24.58 ± 0.89	3.6	−1.7
	15	14.61 ± 0.78	5.3	−2.6
	8	7.91 ± 0.22	2.8	−1.1
	2.5	2.53 ± 0.20	7.9	1.2
VIII	25	24.88 ± 1.20	4.8	−0.5
	15	14.94 ± 0.62	4.1	−0.4
	8	8.06 ± 0.38	4.7	0.8
	2.5	2.44 ± 0.13	5.3	−2.4
IX	100	98.80 ± 3.01	3.0	−1.2
	50	48.75 ± 1.56	3.2	−2.5
	25	25.18 ± 0.68	2.7	0.7
	5	4.90 ± 0.14	2.9	−2.0

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

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

lower precision, indicating the usefulness of the method for long-term assay.

Stability. In the previous report [1], it was confirmed that S-1452, bisnor-(+)-S-145 (IV) and tetranor-(+)-S-145 (VII) were stable. The other seven metabolites, I, II, III, V, VI, VIII and IX, are also stable in methanol and in the buffer for at least two days. The derivatized metabolites are also stable in toluene solution. All the compounds remain stable when the urine samples are stored at −20°C or below.

Recovery. To evaluate the recovery rate of (+)-S-145 and its metabolites from the extraction process, we determined the ten compounds in the spiked sample using the corresponding calibration curve, which was constructed in the same manner as described in our previous report [1]. The assayed values of all the analytes coincided with those of the added amounts, indicating that the analytes were recovered almost quantitatively from the urine.

CONCLUSION

This study validated an established GC method using a sulphur-specific detector for the determination of a thromboxane A_2 receptor antagonist and its metabolites in urine. The data for Phase I test urine samples showed a high recovery rate of 80%. (+)-S-145 was shown to be a free acid of S-1452 that was absorbed and quickly metabolized to produce the hydroxyl (6-*exo*-position) tetranor forms (VII, VIII and IX) glucuronidated as the major metabolites. Such an oxidation is similar to a fatty acid metabolism, such as the β-oxidation from the bisnor to the tetranor form in living organisms [16,17].

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