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DETERMINATION OF SQ 27,519, THE ACTIVE PHOSPHINIC ACID—CARBOXYLIC ACID OF THE PRODRUG SQ 28,555, IN HUMAN SERUM BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN—PHOSPHORUS DETECTION AFTER A TWO-STEP DERIVATIZATION*

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SUMMARY

A method for the determination of SQ 27,519 (II), the active phosphinic acid—carboxylic acid of the prodrug SQ 28,555 (I), in human serum is presented. Compounds I and II are simultaneously extracted from acidified serum into ethyl acetate, and II is back-extracted into aqueous sodium bicarbonate. Compound I, in ethyl acetate, can be subsequently hydrolyzed and measured as II. The two acidic groups of II are selectively esterified, first by methylation of the carboxylic acid with methanolic hydrochloric acid and then by formation of the hexafluoroisopropyl ester of the phosphinic acid. The resulting product is measured by splitless-injection capillary gas chromatography with nitrogen—phosphorus detection. Linear standard curves were obtained for II with a detection limit of less than 10 ng/ml of serum. The method was successfully applied to the analysis of serum samples obtained from normal individuals after administration of I. In an ascending-dose study involving several human subjects the serum levels of II ranged from less than 10 to 7000 ng/ml of serum.

INTRODUCTION

SQ 28,555 (I, Fig. 1), a prodrug of SQ 27,519 (II, Fig. 1), is an inhibitor of angiotensin-converting enzyme [1], which is equally as potent as captopril [2—4] but has a longer duration of activity.

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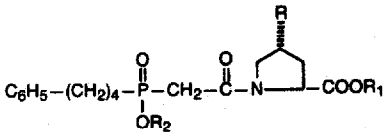
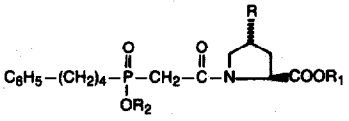


COMPOUND	R_1	R	R_2
SQ 28,555 (I)	Na	CYCLOHEXYL	$(CH_3)_2-CH-\underset{\displaystyle O}{\overset{\displaystyle \|}{CH}}-O-\overset{\displaystyle O}{\overset{\displaystyle \|}{C}}-C_2H_5$
SQ 27,519 (II)	H	CYCLOHEXYL	H
SQ 27,133 (III)	H	PHENYLTHIO	H

Fig. 1. Structures of the analytes SQ 28,555 (I), SQ 27,519 (II) and the internal standard, SQ 27,133 (III).



SCHEME	R_1	R_2	REAGENT
1	$-CH_3$	H	METHANOLIC HCl
2	$CF_3-\underset{\displaystyle H}{\overset{\displaystyle \|}{C}}-CF_3$	$CF_3-\underset{\displaystyle H}{\overset{\displaystyle \|}{C}}-CF_3$	HEXAFLUOROISOPROPANOL/TFAA
3	$-CH_3$	$-CH_3$	DIAZOMETHANE
4	$-Si(CH_3)_2t-Bu$	$Si(CH_3)_2t-Bu$	MTBSTFA
5	$-CH_3$	$CF_3-\underset{\displaystyle H}{\overset{\displaystyle \|}{C}}-CF_3$	a) METHANOLIC HCl b) HEXAFLUOROISOPROPANOL/TFAA

Fig. 2. Reaction schemes investigated for derivatization of II and the products formed.

The method developed for the determination of serum levels of II, or its parent after hydrolysis, relies on the intrinsic nitrogen—phosphorus properties of the compound for detection. Derivatization makes chromatography of the compound easier but not its detection, thus reducing interferences from co-extracted components or derivatizing agents. As a consequence, only a simple extraction scheme is required to isolate II and I. A quantitative separation of II from I is also effected. The carboxylic methyl phosphinic hexafluoroisopropyl (HFIP) ester derivative of II (compound IV, Fig. 2, scheme 5) is chromatographed by high-resolution capillary gas chromatography (GC) using splitless solvent trapping injection. Compound IV has sufficient nitrogen—phosphorus detection (NPD) response to produce a quantitative measurement from less than 50 pg injected. The limit of quantitation is less than 10 ng/ml of serum.

EXPERIMENTAL

Reagents and chemicals

Compounds I, II and III, the internal standard (Fig. 1), were characterized pharmaceutical-grade materials from Squibb & Sons, (Princeton, NJ, U.S.A.). Butyl acetate, glass-distilled, was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Sylon C.T. was obtained from Supelco (Bellefonte,

PA, U.S.A.). Methanolic hydrochloric acid was prepared as previously described [5]. Trifluoroacetic anhydride (TFAA) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were used as received from Aldrich (Milwaukee, WI, U.S.A.). Control serum was prepared from blood obtained commercially. Ethyl acetate, from Burdick & Jackson, was purified as described previously [5], to remove oxidizing substances such as peroxides.

Apparatus

A Hewlett-Packard 5840A gas chromatograph, equipped with a nitrogen—phosphorus detector and an autosampler, was used. The fused-silica capillary column (5 m × 0.32 mm I.D., 0.17 μm film thickness) was coated with cross-linked methyl silicone phase (Hewlett-Packard). The carrier gas was helium at an inlet pressure of 34 kPa and the helium make-up gas for the nitrogen—phosphorus detector was set at 30 ml/min. The voltage applied to the nitrogen—phosphorus detector was adjusted to obtain an offset of 70% full scale at an attenuation of 8. The air and hydrogen flow-rates to the nitrogen—phosphorus detector were 50 and 3.0 ml/min, respectively. The oven temperature was operated isothermally at 210°C for 2.0 min after injection, heated at a rate of 10°C/min to 260°C, and then held at the final temperature for 1.0 min. Injections were made by the splitless mode, with a split flow of 30 ml/min and a septum purge of 2.0 ml/min. The inlet purge was turned on 0.7 min after injection. The injector and detector temperatures were 270°C and 320°C, respectively.

Autosampler vials of 1 ml, with PTFE-lined seals, obtained from Supelco, were silylated with Sylon C.T. Each vial was filled with Sylon C.T., which was decanted after 1.0 min, followed by rinsing first with toluene and then twice with methanol and finally dried in an oven at 80°C. Glass scintillation vials of 20 ml, with cork-back tin foil liner (Fisher Scientific, Pittsburgh, PA, U.S.A.) were silylated in the same manner. A sample concentrator, Model SC-248, with a scintillation vial adapter (Brinkmann Instruments) and an SC-3 concentrator with DB-3 Dri-Block heating bath (Techne, Princeton, NJ, U.S.A.) were used.

Standard preparation

Stock solutions of I, II and III were prepared separately by accurately weighing approximately 25 mg of each and dissolving in 50 ml of methanol. Diluted methanolic solutions of I, II and III were stable for at least three months when stored in a refrigerator. Calibration standards were obtained by transferring 100 μl of the required number of appropriately diluted solutions of I and II and 100 μl of a diluted solution of III to culture tubes containing 1.0 ml of serum. A typical calibration set consisted of a 0 and six other values ranging from 10 to 1000 ng/ml I or II in serum, each containing 1000 ng of III.

Isolation and purification

Frozen serum samples were thawed at room temperature or kept in a refrigerator overnight. Serum (1 ml) was transferred to a culture tube (tube 1) containing 1000 ng of the internal standard, III. To each tube, 1.0 ml of 1 *M* hydrochloric acid was added followed by 10 ml of purified ethyl acetate,

the mixture was shaken for 5 min and the phases were separated by centrifugation. The upper ethyl acetate layer was quantitatively transferred to a culture tube (tube 2) containing 1.0 ml of 5% sodium bicarbonate solution, the mixture was shaken for 5 min and the phases were separated by centrifugation. The upper ethyl acetate layer, which contained I, was quantitatively transferred to a silylated scintillation vial containing 1000 ng of III, leaving II and III in the aqueous solution in tube 2. Ethyl acetate was evaporated at 50°C in the Brinkmann sample concentrator. After cooling to room temperature, 2.0 ml of 0.1 *M* sodium hydroxide were added to the scintillation vial, the solution was well mixed and allowed to stand for 1 h to hydrolyze I to II. This solution was then quantitatively transferred to a third culture tube (tube 3).

To each of tubes 2 and 3, 0.1 ml of 6 *M* phosphoric acid was added followed by 10 ml of purified ethyl acetate, the mixture was shaken for 5 min, and the phases were separated by centrifugation. The upper ethyl acetate layer from each tube was transferred to a separate silylated scintillation vial and the ethyl acetate was evaporated to ca. 0.2 ml at 50°C in the Brinkmann sample concentrator. After adding 0.5 ml of ethyl acetate to the scintillation vial and thoroughly mixing, the ethyl acetate solution was quantitatively transferred to a silylated autosampler vial. The scintillation vial was rinsed with a second 0.5 ml of ethyl acetate, which was then combined with the first solution in the autosampler vial. The ethyl acetate was removed by placing the autosampler vials in the Techne sample concentrator under a stream of nitrogen. The vials were then dried in a dessicator, under vacuum, for 15 min.

Derivatization and reconstitution

Derivatization of the dried extracts was performed in a two-step reaction. First, the extracts were methylated with methanolic hydrochloric acid [5]. After removing the reagents by evaporation under nitrogen at 50°C, the methylated dried residue was then reacted with 100 μl of HFIP and 10 μl of TFAA at 60°C for 1 h. After removing the reagents by evaporation under nitrogen at 50°C, the dried residue was reconstituted with 0.5 ml of *n*-tetradecane containing 10% isopropanol.

Method

The calibration curve was established at the beginning of the study. Daily, a control sample, which represented the calibration point of 500 ng/ml II and 1000 ng/ml III in serum, was processed together with the samples. The response of the control sample was used to correct the data for differences in response from the calibration slope [6].

The calibration curve was constructed by plotting area ratios of the analyte II to the internal standard III versus the amount ratios of II to III, the amount being the total nanograms of II or III in each calibration standard. A typical calibration curve gave a slope of 1.531, coefficient of correlation of 0.9983 and *y* intercept of −0.00841.

RESULTS AND DISCUSSION

Extraction

The simple liquid–liquid extraction quantitatively separates the drug (II)

from the prodrug (I), which can subsequently be hydrolyzed with sodium hydroxide to II. Among the solvents investigated for the extraction of I and II from acidified serum (Table I), ethyl acetate permits quantitative extraction and is readily evaporated. Compound I, quantitatively extracted from acidified serum into ethyl acetate, remains in the organic phase during the back-extraction step into 5% sodium bicarbonate. On the other hand, compound I could not be extracted quantitatively into ethyl acetate from 1.0 ml of serum to which 1.0 ml of 5% sodium bicarbonate had been added. Hydrolysis of I to II was incomplete when the ethyl acetate solution of I was shaken with 1.0 *M* sodium hydroxide (Table II), but complete hydrolysis was achieved when I was reacted with 0.1 *M* sodium hydroxide after removal of ethyl acetate (Table

TABLE I

SOLVENTS INVESTIGATED FOR THE EXTRACTION OF I AND II FROM ACIDIFIED SERUM

A 1-ml volume of serum and 10 ml of solvent were used.

Solvent	Extraction efficiency (%)	
	I	II
Cyclohexane	N.D.*	0
Pentane	N.D.	0
2,2,4-Trimethylpentane	N.D.	0
Toluene	N.D.	0
n-Butyl acetate	100	100
tert.-Butylmethyl ether	100	99
Diethyl ether	N.D.	86
Ethyl acetate	100	100
n-Butyl alcohol	N.D.	85
Methylene chloride	N.D.	51
Methyl ethyl ketone	100	100
Methyl isobutyl ketone	100	100

*N.D. = Not determined.

TABLE II

HYDROLYSIS OF I IN ETHYL ACETATE ON SHAKING WITH 1 *M* SODIUM HYDROXIDE

Ethyl acetate solution (10 ml) and 2.0 ml of 1.0 *M* sodium hydroxide were used.

Time of shaking (min)	Unhydrolyzed I (%)
0	100
5	37
10	28
15	24
20	23
30	22
45	22
60	22

TABLE III

HYDROLYSIS OF I AT ROOM TEMPERATURE (22°C) WITH 2.0 ml OF 0.1 *M* SODIUM HYDROXIDE

Hydrolysis time (min)	Hydrolysis (%)
15	87.3
30	92.1
45	99.5
60	100.1

III). For complete recovery of the analyte, the scintillation vials and the autosampler vials had to be silylated to prevent loss owing to adsorption of compounds during the evaporation steps.

Derivatization

Derivatization of II and III was performed only to make chromatography easier but not to enhance detection of the compounds by the nitrogen—phosphorus detector. Various reaction schemes were investigated to optimize the conditions for chromatographing the compounds (Fig. 2). The structures of the reacted products were confirmed by electron-impact (EI) GC—mass spectrometry. Typical EI spectra for the methyl-HFIP and the dimethyl esters (Fig. 2, schemes 5 and 3, respectively) are presented in Fig. 3 and the diagnostic ions are identified in Fig. 4.

In scheme 1 (Fig. 2), where methanolic hydrochloric acid is used for derivatization of II [5], the methylated carboxylic acid could not be chromatographed. In schemes 2, 3 and 4 (Fig. 2), where II was reacted with HFIP—TFAA mixture [7], diazomethane [8] and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [9], respectively, various diesters were formed that could be chromatographed. These procedures were not adopted, however, because of the problems associated with reproducibility, cleanliness of the chromatogram or loss of response in the low concentration region. Scheme 5 (Fig. 2), where the carboxylic acid of II is first methylated selectively followed by the HFIP ester formation of the phosphinic acid, was the method of choice.

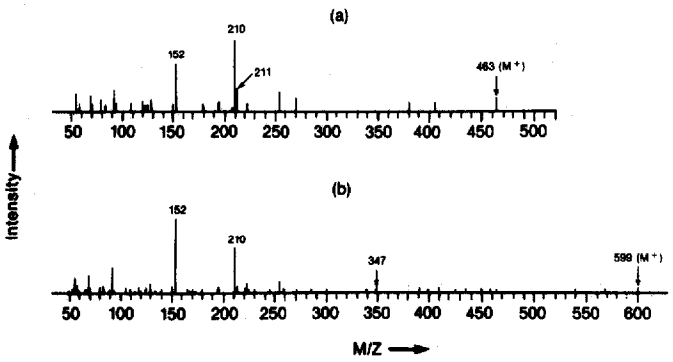
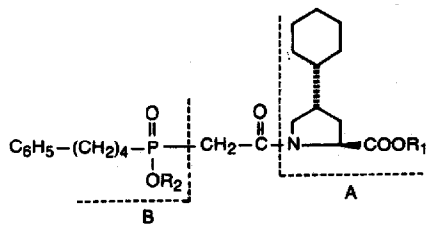


Fig. 3. Electron-impact mass spectra of II derivatives: (a) dimethyl ester (scheme 3 in Fig. 2); (b) methyl-hexafluoroisopropyl ester (scheme 5 in Fig. 2). A Hewlett-Packard 5970A mass-selective detector interfaced with a 5790A gas chromatograph was used.



R_1	R_2	M^+	A	B	COMMON**
CH_3	CH_3	463	210*	211	152
HFIP	HFIP	735	346	347	152*
CH_3	HFIP	599	210	347	152*
TBDMS	TBDMS	663	310	311	152*

*BASE PEAK

**C_4H_7NR, R = cyclohexyl

Fig. 4. Diagnostic ions from the electron-impact mass spectra of the diesters of II. HFIP = Hexafluoroisopropyl, TBDMS = *tert.*-butyldimethylsilyl. The ion at *m/z* 152, owing to protonated cyclohexyl proline, is common to all the esters. The TBDMS diester also gave a prominent peak (86%) at *m/z* 606 ($M^+ - 57$), a diagnostic ion for a TBDMS derivative.

Gas chromatography

Typical chromatograms are shown in Fig. 5. At adequately low column temperature, the methyl-HFIP esters of both II and III each give two peaks, as

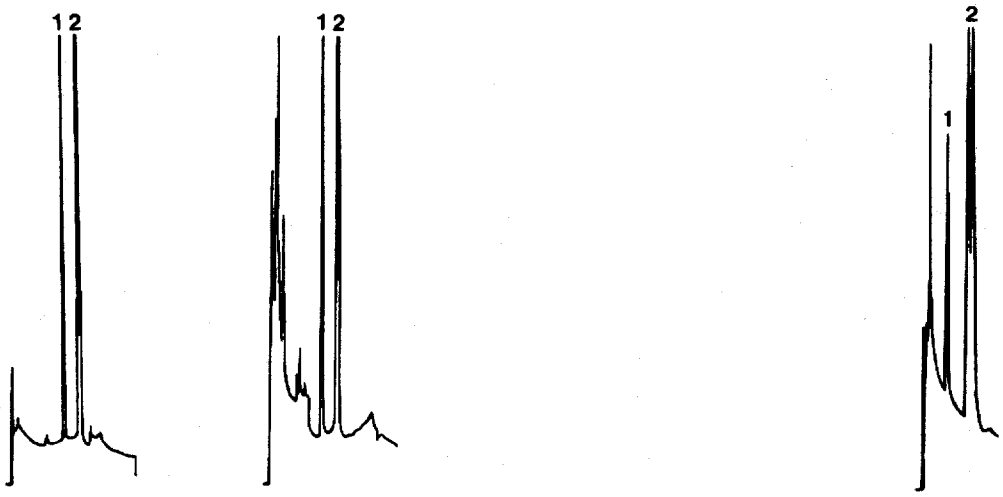


Fig. 5. Chromatograms of unextracted standard and control serum to which the standard was added. Left: unextracted standard, 800 ng of II and 2000 ng of the internal standard, III. Area ratio of peak 1 to peak 2 is 0.630. Right: control serum to which were added 800 ng of II and 2000 ng of III. Area ratio of peak 1 to peak 2 is 0.647. Peaks: 1 = methyl-HFIP ester of II, retention time 4.25 min; 2 = methyl-HFIP ester of III, retention times 5.33 and 5.49 min.

Fig. 6. Isothermal chromatogram of control serum to which II and III were added. Peaks: 1 = methyl-HFIP ester of II, retention time 2.24 min, 200 ng of II; 2 = methyl-HFIP ester of III, retention times 3.77 and 4.06 min, 2000 ng of III.

was the case with the dimethyl derivatives. The esterification of II or III at the phosphinic acid leads to a new asymmetric center resulting in a diastereomeric pair, which are resolved by high-resolution chromatography.

For the application of the splitless solvent effect mode of injection [10, 11] at a relatively high initial column temperature, a high-boiling hydrocarbon, *n*-tetradecane (boiling point of 254°C), with 10% isopropanol to ensure complete dissolution of the analyte, was used for reconstitution of injection solutions. Under the conditions, good chromatograms, with reasonably short retention times, could be obtained by programming the column temperature at a relatively moderate rate, avoiding the sharp baseline rise which was sometimes encountered with our nitrogen–phosphorus detector when a fast programming rate was used. With this solvent mixture, reasonably good chromatograms (Fig. 6) were obtained even with splitless isothermal mode, which is infrequently reported in the literature [12–14].

Clinical samples

The sensitivity of the method was adequate for the level of II in the serum samples assayed, which ranged from less than 10 to 7000 ng/ml of serum. The GC results correlated very well with the results independently obtained by radioimmunoassay (RIA), developed by Clinical Assay R & D Department of the Squibb Institute (Table IV).

TABLE IV

COMPARISON OF GC AND RIA RESULTS

Sample No.	Mean* (ng/ml)	Delta**	Delta/mean
1	20	−2	−0.10
2	25	−4	−0.16
3	41	+2	+0.05
4	43	+4	+0.09
5	43	−5	−0.12
6	48	−1	−0.02
7	58	−2	−0.03
8	59	+9	+0.15
9	59	+2	+0.03
10	74	+1	+0.01
11	86	−8	−0.09
12	91	0	0
13	98	−3	−0.03
14	229	−19	−0.08
15	406	+4	+0.01
16	1050	+15	+0.01
17	3010	−92	−0.03
18	6870	+115	+0.02

*Mean is the average of the RIA and GC results [(RIA + GC)/2].
**Delta is the difference between the mean and the GC results (mean − GC).

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