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SIMULTANEOUS DETERMINATION OF THE PRODRUG ZOFENOPRIL AND ITS ACTIVE DRUG IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY-MASS-SELECTIVE DETECTION

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SUMMARY

After oral administration of zofenopril, the active sulfhydryl angiotensin-converting enzyme inhibitor is released Zofenopril is currently under clinical investigation as an antihypertensive. Blood samples are reacted with N-ethylmaleimide, immediately after collection, processed into plasma and stored frozen for subsequent analysis. After addition of two internal reference standards, one each for the prodrug and the active compound, the plasma samples are purified by a combination of liquid-liquid and solid-phase extractions. The dried methylated extracts are reconstituted with tetramethylbenzene and chromatographed by automated splitless injection on a fused-silica capillary column, connected to a mass-selective detector. The analytes and the internal reference standards are chromatographically resolved and a common fragment ion is monitored for the analytes. A limit of quantitation of approximately 1 ng/ml of plasma is achieved

INTRODUCTION

Zofenopril (compound I, Fig. 1) is an investigational prodrug related to captopril, the first orally active inhibitor of the angiotensin-converting enzyme used for the treatment of hypertension [1,2]. A method was required for the simultaneous measurement of the prodrug and the active sulfhydryl drug (compound II, Fig. 1), released from the orally administered prodrug, in plasma.

Various methods developed for the measurement of captopril in plasma [3–6] rely upon the conversion of the thiol to the succinimide derivative to prevent the oxidation of the sulfhydryl group to disulfides. In a similar manner, blood samples collected for the measurement of I and II were reacted, immediately after collec-

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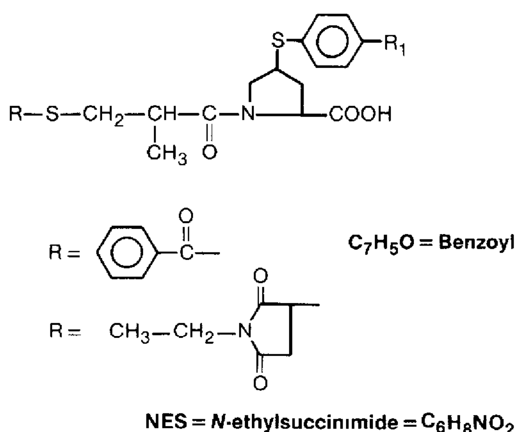

Fig 1. Structure of compounds.

IS the calcium salt of I, IIS is the arginine salt of II, IVS is the dicyclohexylamine salt of IV and VS is the arginine salt of V Squibb-designated names for I, IS, II, IIS, IVS and VS are zofenopril, zofenopril calcium, SQ 26,333, SQ 26,703, SQ 28,618 and SQ 26,830, respectively.

Compound	R	R_1
Zofenopril (I)	C_7H_5O	H
(II)	H	H
(III)	$C_6H_8NO_2$	H
IV	C_7H_5O	F
V	H	F
VI	$C_6H_8NO_2$	F

tion, with N-ethylmaleimide (NEM) to convert II to the succinimide derivative (compound III, Fig. 1), processed into plasma and then stored in a frozen state until analysis. After addition of two internal reference standards (IV and VI, Fig. 1), one for each of the prodrug and the active compound, I and III were isolated from plasma by liquid–liquid extraction followed by solid-phase purification. The methyl esters of I and III were measured by capillary gas chromatography–mass-selective detection (GC–MSD). The fused-silica capillary column resolved the methyl esters of I and III, which allowed the use of a common electron-impact (EI) mass fragment ion for quantitative measurement of the analytes.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 5890 gas chromatograph equipped with a split/splitless capillary inlet system was used. The fused-silica capillary column was DB-17 (J&W Scientific, Folsom, CA, U.S.A.), 6 m$\times$0.25 mm I.D., 0.15 μm film thickness. The carrier gas was helium at an inlet pressure of 34 kPa (5 p.s.i.g.). The oven temperature was operated isothermally at 210°C for 0.8 min after injection, heated at a rate of 30°C/min to 300°C, and then held at the final temperature of

300 °C for 5.0 min. Injections were made in the splitless mode, with a split flow of 50 ml/min and a septum purge of 2.0 ml/min. The inlet purge was turned on 0.8 min after injection. The injection temperature was maintained at 280 °C.

A Hewlett-Packard 7673 autosampler was attached to the gas chromatograph. Injection was made from 1-ml vials with 5×31 mm inserts (American Scientific Products, McGaw Park, IL, U.S.A.). The autosampler vials were sealed with PTFE liners. Usually, 1-μl aliquots of the 50-μl sample solution were injected.

A Hewlett-Packard 5970 mass-selective detector was interfaced with the 5890 gas chromatograph, with the capillary column inserted directly into the ion source. The GC–MSD interface was maintained at 300 °C. The detector was first calibrated with the Autotune® program at the beginning of each day using perfluorotributylamine (PFTBA) as the calibration compound. Following the Autotune, the detector was "user-tuned" using the *m/z* 219, 264 and 414 ions of PFTBA. Depending upon the sample concentrations, the electron multiplier voltage was set 200–800 V above the user-tuned value. The mass peak width was set to 0.6 Dalton at one half the amplitude and the other user-tuned parameters were used without change. The peak dwell time was set at 100 ms. The detector was turned on from 2.8 to 4.8 min after injection. The mass ions monitored were *m/z* 338 for the two analytes and *m/z* 356 for the internal reference standards.

A Hewlett-Packard 59970C computer was used for the control of the GC–MSD system and for the reduction of data. The entire processing, from injection of the first standard to the printing of the results of the last sample, was fully automated providing unattended operation.

Disposable solid-phase extraction columns and a ten-place vacuum manifold, used for holding the columns, were obtained from Analytichem International (Harbor City, CA, U.S.A.). A model SC248 sample concentrator (Brinkmann Instrument, Westbury, NY, U.S.A.), connected to nitrogen and house vacuum, was equipped with an adaptor for 20-ml scintillation vials.

Reagents and chemicals

Characterized pharmaceutical products IS and IIS (Fig. 1), the calcium and arginine salts of I and II, respectively, were materials obtained from the Department of Organic Chemistry (E.R. Squibb & Sons, U.S.A.). Compounds IVS and VS (Fig. 1), the dicyclohexylamine and arginine salts of the fluoro analogues of I and II, respectively, were used as internal reference standards.

Phosphate buffer, pH 7.0, was prepared by dissolving 40 g of dibasic sodium phosphate heptahydrate in 1800 ml of distilled water, adjusting the pH to 7.0 with phosphoric acid and diluting to 2000 ml with distilled water. Tetrabutylammonium hydrogen sulfate (TBAHS)–phosphoric acid solution was prepared by dissolving 69 ml of 85% phosphoric acid and 10 g of TBAHS in 400 ml of distilled water and diluting to 500 ml. A 25 mg/ml solution of NEM (Aldrich, Milwaukee, WI, U.S.A.) in pH 7.0 phosphate buffer was freshly prepared. A methanolic hydrogen chloride solution, approximately 3% (w/w), was prepared by reacting 5

ml of anhydrous methanol with 0.25 ml of acetyl chloride (Applied Science Labs., State College, PA, U.S.A.). RBS-35 was from Pierce (Rockford, IL, U.S.A.).

Standard preparation

Stock solutions of I and IV were prepared separately by dissolving accurately weighed amounts of IS and IVS in methanol. A stock solution of III was prepared by treating 40 mg of IIS with 10 ml of NEM solution for 1 h, adding three 25-ml portions of acetone, with thorough mixing after each addition, diluting to 100 ml with acetone and filtering through a fine-porosity sintered-glass filter. A stock solution of VI was similarly prepared by reacting VS with NEM solution.

Isolation and purification

Frozen plasma samples were thawed at room temperature on the day of extraction. A 1-ml volume of each plasma sample was transferred to a 16×125 mm screw-cap test tube. To each tube containing sample, 10 ml of toluene, 0.1 ml of internal standard solution and 1 ml of TBAHS–phosphoric acid solution were added. After shaking the tubes on a shaker for 5 min, the phases were separated by centrifugation for 5 min at 500 *g*. By means of a 5-ml glass pipet, the upper toluene layer was transferred to a 10-ml plastic syringe attached to a C_{18} extraction column, which had been activated by rinsing successively with 5 ml of 0.03% (v/v) monoethanolamine in methanol and 5 ml of toluene. The toluene extract was passed through the column, dropwise, and the column was then rinsed with 5 ml of toluene. The compounds I, III, IV and VI were then eluted with 5 ml of methanol into a 20-ml scintillation vial. The methanol was removed by placing the scintillation vial in a Brinkmann sample concentrator set at 60°C. The dried residue was dissolved in 1.0 ml of methanol and the solution was transferred to a 1.0-ml autosampler vial by means of a Pasteur pipet. The methanol was removed under nitrogen, at 50°C, to obtain a clean, dry residue.

Methylation and reconstitution

To the dried extract in a 1-ml vial, 0.1 ml of methanolic hydrogen chloride was added. The vial was crimp-sealed with a PTFE-lined cap, vortex-mixed and heated in a dry block heater, set at 60°C, for 10 min. The methanolic hydrogen chloride was removed by placing the vial in a desiccator with vacuum. The dried residue was dissolved with 0.5 ml of methanol and then dried under nitrogen, at 50°C. The dried residue was again dissolved in 0.1 ml of methanol, which was then completely removed with a vacuum, in a desiccator. For chromatography, the dried methylated residue was reconstituted in 50 µl of 1,2,3,4-tetramethylbenzene. By means of a Pasteur pipet, the solution was transferred to a micro vial contained in a 1-ml autosampler vial. The vial was then crimp-sealed with a PTFE-lined cap.

Method

The calibration curve was determined at the beginning of the study to demonstrate linearity. Daily, four control samples, representing the calibration points of 0, 25, 125 and 250 ng of I and III per ml of plasma, each containing 125 and 250 ng of IV and VI, were processed together with the samples. The calibration graph was constructed by plotting peak-area ratios of the analyte to the respective internal reference versus the weight (ng) ratios of analyte to internal reference. A typical calibration plot for I gave a correlation coefficient of 0.998, a y-intercept of 0.004 and a slope of 1.0. The corresponding values for III were 0.998, 0.008 and 1.5, respectively.

RESULTS AND DISCUSSION

The analytical method presented here utilizes stabilization of the active compound (II) in plasma samples by reacting it with NEM to form the succinimide derivative (III). Zofenopril, compound III and the added internal reference standards are extracted into toluene and further purified using a C_{18} solid-phase extraction cartridge. The isolates are then derivatized by a simple methylation reaction and analysed by GC–MSD. The capillary column used resolves the two analytes and internal reference standards, allowing for the use of only two analytical mass ions, one for the analytes and the other for the internal references, for quantification of the analytes. A typical selected ion chromatogram of a plasma extract is shown in Fig. 2. Under the specified conditions, the limit of quantification was estimated to be approximately 1.0 ng/ml of plasma.

The purification and extraction scheme from plasma with toluene and passage of the organic extract through a C_{18} extraction column produces a clean and reproducible product. Isolation of I and III with C_1, C_2, C_4, C_6, C_{18}, phenyl, cyclohexyl, cyanopropyl and diol solid-phase extraction columns from acidic, neutral and alkaline diluted plasma samples was incomplete and not reproducible. This was probably due to the adsorption of the compounds onto plasma proteins.

Because incomplete extraction was obtained with toluene from plasma acidified with phosphoric acid, ion-pair extraction with toluene as a function of pH was investigated. For these experiments, 1.0 ml of 2% TBAHS was added to an aqueous solution of I and III and the pH was adjusted with sodium hydroxide. Both I and III were extracted quantitatively below pH 6.0. When only 2% TBAHS was used for acidification, cloudy toluene extracts were obtained. This was rectified by the addition of phosphoric acid. Among the solvents investigated, only toluene, methyl isobutylketone, dichloromethane and ethyl acetate gave quantitative extraction for both I and III (Table I). Toluene produced the cleanest extract. Complete retention of I from toluene on the C_{18} column could be achieved by conditioning the column with organic bases, triethylamine, triethanolamine and monoethanolamine.

Typical EI mass spectra of the methyl ester of the analytes (VII and VIII, Fig.

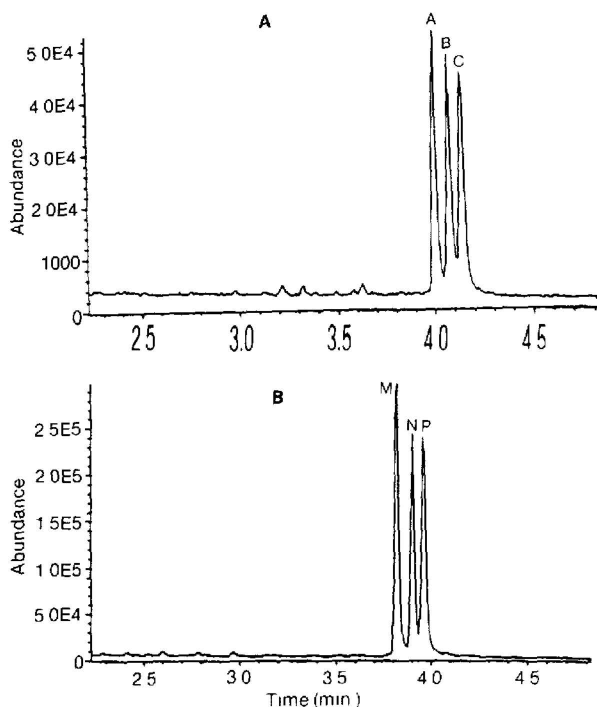

Fig. 2. Selected ion chromatograms from control plasma spiked with 25, 25, 125 and 250 ng of compounds I, II, IV and V, respectively, per ml of plasma. (A) m/z 338; (B) m/z 356. Peaks (retention times in parentheses): A=VII (4.00 min); B=VIII (4.08 min); C=VIII (4.14 min); M=IX (3.81 min); N=X (3.90 min); P=X (3.96 min). See Fig. 3 for identification of compounds VII, VIII, IX and X.

TABLE I

EXTRACTION OF I AND III FROM ACIDIFIED PLASMA AS A FUNCTION OF SOLVENT

Solvent	Extraction (%)	
	I	III
Toluene	93	100
Isooctane	0	59
Methyl ethyl ketone	39	51
Methyl isobutyl ketone	107	101
Dichloromethane	99	100
Hexane	0	70
Cyclohexane	0	33
Heptane	0	68
Ethyl acetate	100	99

3) and the internal reference standards (IX and X, Fig. 3) are shown in Figs. 4 and 5, respectively. The important fragment ions are depicted in Fig. 3. The most intense ion for thiosuccinimide compounds VIII and X is from fragment B (m/z 338 and 356, respectively). For the thiobenzoyl compounds VII and IX, the most intense ion is fragment C, m/z 105, while fragment B is the second most intense

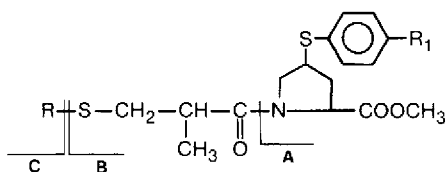

Fig. 3. Fragment ions of the methyl ester derivatives.

Compound	R	R_1	A	B	C	M^+
VII	C_7H_5O	H	236	338	105	443
VIII	$C_6H_8NO_2$	H	236	338	126	464
IX	C_7H_5O	F	254	356	105	461
X	$C_6H_8NO_2$	F	254	356	126	482

ion. The ions from fragment B, *m/z* 338 and 356, were chosen as the analytical ions. With the choice of these relatively high-mass-ions, the MSD could be user-tuned to optimize for a high mass, using the *m/z* 414 ion of PFTBA. Ocassionally, the *m/z* 105 ion was also monitored, simultaneously with the *m/z* 338 ion, to confirm the presence of zofenopril. Surprisingly, there was little interference by other compounds in the plasma extracts when this lower-mass ion was monitored.

Since a common analytical mass ion was utilized for the two analytes, it was necessary to chromatographically separate the two analytes. The low-polarity HP-1, HP-5, DB-1, DB-5, CP sil 5CB or CP sil 8CB columns did not separate VII from VIII. The moderately polar columns CP sil 19CB and DB-17 separated the two analytes as two peaks for VIII and one peak for VII. The reaction of the optically pure compound II with NEM, in a non-stereospecific manner, leads to the formation of a new asymmetric center resulting in a diastereomeric pair of III. The resolving power of these capillary columns was adequate to resolve the two methyl ester diastereomers. The separation of the diastereomeric pair from VII was better with CP sil 19CB than with DB-17. The DB-17 column was adopted for the method because it consistently gave well shaped peaks whereas certain batches of CP sil 19CB produced two peaks of VIII which were merged or deformed.

Because the thiobenzoyl group potentially may hydrolyze during methylation of the proline acid, the methylation reaction with methanolic hydrogen chloride was studied. Control plasma extracts were spiked with the non-methylated analytes, I and III, and then reacted with methanol containing varying amounts of hydrogen chloride. The samples were then spiked with methyl esters of the internal reference standards, IX and X. Low hydrogen chloride concentrations gave incomplete methylation for both I and III (Table II). A 3% (w/w) methanolic hydrogen chloride solution was adopted for the method. Methylation via methanolic hydrogen chloride [3% (w/w)] was also compared with methylation by diazomethane. There was no significant difference in the degree of methylation for both compounds I and III, indicating that the thiobenzoyl ester I was not hydrolyzed during the methanolic hydrogen chloride reaction.

The solvent used for injection had a tremendous effect on the response obtained from VII or VIII. The response of VII or VIII in acetone, toluene, N,N-

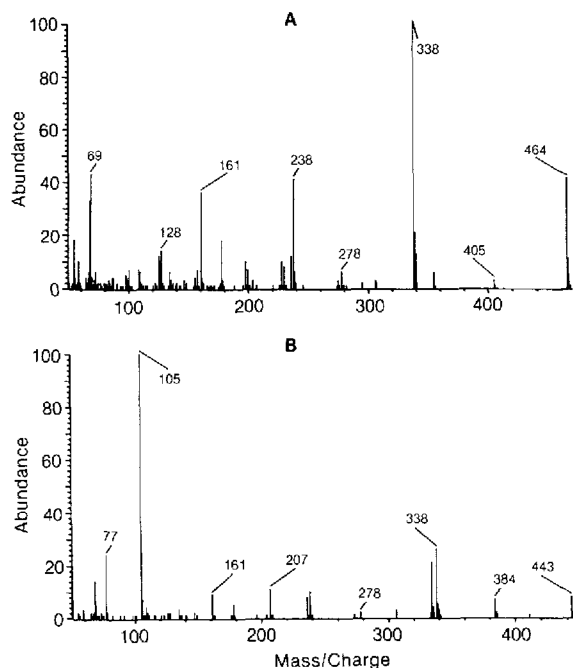

Fig. 4. Electron impact mass spectra of (A) methyl ester of III (compound VIII, Fig 3) and (B) zofenopril methyl ester (compound VII, Fig. 3).

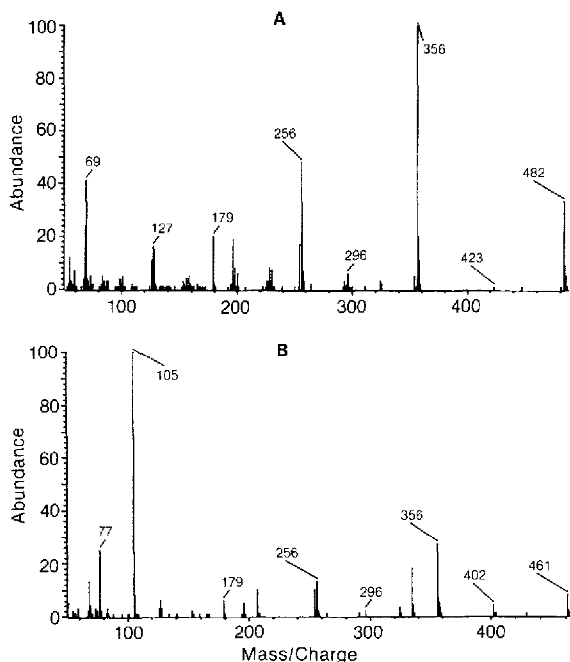

Fig. 5. Electron impact mass spectra of (A) methyl ester of fluoro analogue of III (compound X, Fig. 3) and (B) methyl ester of fluoro analogue of zofenopril (compound IX, Fig. 3).

TABLE II

METHYLATION WITH VARYING CONCENTRATIONS OF HYDROGEN CHLORIDE IN METHANOL

HCl (%, w/w)	Area ratio*	
	III	I
3	0.70	0.58
1	0.68	0.60
0.3	0.28	0.24
0 1	0.12	0.09

*Area ratio = area of analyte divided by area of internal reference.

dimethylformamide and N,N-dimethylacetamide was approximately 0.5, 2.0, 20, 25% of that obtained in 1-nonanol or 1,2,3,4-tetramethylbenzene. The presence of plasma extract components affected the relative response values in the different solvents. Considering the various factors such as the effect on response and ease of use, due to lower viscosity, 1,2,3,4-tetramethylbenzene was adopted as the solvent of choice for injection.

Proper treatment of the quartz splitless injection insert was essential to obtain good area counts because the compounds were lost when injected into poorly treated inserts. The quartz inserts were soaked first in a 50% (v/v) solution of nitric acid, followed by soaking in a 50% solution of RBS-35 in distilled water at 70°C for at least 24 h. The inserts were then washed thoroughly with distilled water, rinsed with acetone and dried in an oven at 175°C. The inserts were then soaked in Sylon CT at 70°C for at least 24 h, after which they were rinsed thoroughly with toluene and soaked in methanol for about 1 h. After pouring off the methanol, the inserts were dried in an oven set at 175°C. Cleaned silanized inserts were stored in a desiccator until used.

The DB-17 capillary column was of a narrow internal diameter (0.25 mm) and a thin stationary phase (0.15 μm). With a short column length (6 m), a relatively high column head pressure and a high oven temperature, the compounds were eluted in approximately 4.3 min. The 6 m column length, under 34 kPa pressure, is close to the shortest length usable because of the limited pumping capacity of the mass-selective detector. Starting the temperature programming as low as 40°C below or as high as 40°C above the boiling point of 1,2,3,4-tetramethylbenzene (204°C) did not cause significant differences in area counts or in the value of height/area, which is indicative of the sharpness of the peak. Changing injection temperatures from 250 to 300°C did not cause significant differences whereas at the lower 230°C injection temperature, the area counts were lower.

Recovery of I and II from plasma was studied by spiking control plasma with varying amounts of I and III, before or after extraction, and then adding the methyl ester of the two internal references to the methylated plasma extracts. The area ratios of the samples spiked after and before extraction were used to measure absolute recovery, which was typically over 90%. The area ratio of a control plasma spiked with analytes and internal references prior to extraction

was equal to the area ratio of control plasma spiked similarly after extraction showing that the internal references and the analytes have identical extraction behavior. Extracted methylated samples, reconstituted in tetramethylbenzene, were stable at room temperature for at least six days. Stability studies of zofenopril in plasma as a function of time and temperature showed that the compound is stable at 5°C for at least seven days and at −20°C for over 40 days.

The accuracy and precision of the method was checked by analyzing blank plasma specimens which had been spiked with the analytes. The results of samples spiked at four different levels of I and II are summarized in Table III. For each level of I and II, there were five samples, resulting in a total of twenty spiked samples. Each sample was injected four times in a span of about 24 h. A calibration set, injected four times, twice at the beginning and twice in the middle of the study, was used to determine the concentration of the sample from each injection. The value shown in Table III for each level of spiking is the mean of the concentrations obtained from all injections ($n=5\times4=20$). The experimentally obtained mean value is within 10% of the expected value and the coefficient of variation (C.V.) for the twenty data points for each level of spiking is less than 10%.

The method has been used for the analysis of over 3000 human plasma samples from studies conducted to demonstrate dose proportionality, bioavailability and bioequivalence. The bioavailability of zofenopril (I) and its active drug (II) for a typical subject is presented in Table IV.

The alternative silylation reaction was studied with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), at 60°C. The reaction products obtained after heating I and III for 30 min are summarized in Fig. 6. Compound I gave, in addition to the expected product XI, compound XII, as a result of the displacement of the benzoyl (C_7H_5O) group by *tert.*-butyldimethylsilyl (TBDMS) group. The EI mass spectrum of XII showed a base peak at m/z 496, $[M-57]^+$, whereas the corresponding peak in XI, m/z 486, was significantly lower than the m/z 105 C_7H_5O base peak. Compound III also gave XII in addition to XIII, where the $[M-57]^+$ ion is significantly lower than the m/z 69 base ion. Whereas XII

TABLE III

ACCURACY AND PRECISION OF QUANTIFICATION OF I AND II

Compound	Amount added (ng)	Amount found (ng)	Coefficient of variation (%)
I	10	9.1	7.9
	50	51.4	4.9
	100	101	4.7
	200	202	3.5
II	10	10.3	7.2
	50	51.0	4.0
	100	102	3.9
	200	203	2.7

TABLE IV

HUMAN PLASMA SAMPLES FROM A SUBJECT COLLECTED AT VARIOUS TIMES AFTER DOSING WITH A 15-mg ZOFENOPRIL CALCIUM TABLET

Time (h)	Amount found in plasma (ng/ml)	
	I	II
0	0	0
0.5	11	5
1.0	31	53
1.5	40	119
2.0	22	59
3.0	13	39
4.0	5	25
6.0	1	5
8.0	0	3
12.0	0	2
24.0	0	1

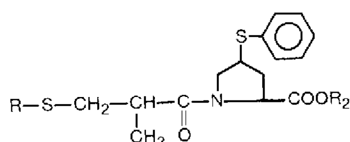

Fig. 6. Reaction products of zofenopril (I), III and their methyl esters with MTBSTFA.

Compound	R	R_2	M^+	M^+-57
XI	C_7H_5O	TBDMS	543	486
XII	TBDMS	TBDMS	553	496
XIII	$C_6H_8NO_2$	TBDMS	564	507
XIV	TBDMS	CH_3	453	396

gave a single chromatographic peak, XIII gave two chromatographic peaks, like the corresponding methyl ester, VIII. Displacement of the succinimide $C_6H_8NO_2$ group by the TBDMS was also seen when VIII was reacted with MTBSTFA, resulting in XIV with a base peak of $[M-57]^+$. Unlike VIII, this compound gave a single chromatographic peak. Although the reaction of MTBSTFA was interesting, it was not adopted for method development because the reaction gave a mixture of products and, more importantly, the two analytes gave a common reaction product, XII, which made individual measurement of the two plasma components impossible.

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