Simultaneous Determination of Captopril and S-Benzoyl Captopril in Human Blood by Capillary Gas Chromatography–Mass Selective Detection

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Using a new table-top electron-impact mass selective detector, interfaced with a narrow bore, fused silica capillary gas chromatograph, a method has been developed for the simultaneous determination of captopril and S-benzoyl captopril. With a capillary column the two components could be chromatographically resolved, thus permitting the use of electron-impact ionization for quantitative measurements.

INTRODUCTION

Captopril (**1**, Fig. 1), an orally active inhibitor of the angiotensin-coverting enzyme,[1,2] is currently marketed for the treatment of both renovascular and essential hypertension and also shows great promise in congestive heart failure.[3,4] S-Benzoyl captopril is a new investigational drug.

Several packed column gas chromatography/mass spectrometry (GC/MS) methods have been published[5–7] for the determination of captopril in plasma or whole blood, which relied on the conversion of captopril to the succinimide derivative (**2**, Fig. 1) by the addition of *N*-ethylmaleimide (NEM). For the simultaneous determination of S-benzoyl captopril (**3**) and **1** in whole blood, as a result of the administration of **3**, the use of an electron-impact (EI) mass selective detector (MSD) interfaced with a capillary gas chromatograph was investigated. Blood, previously treated with NEM,[5] was further treated with acetone to prevent the hydrolysis of **3**. Sample purification was essentially by the method described previously for the captopril determination.[7]

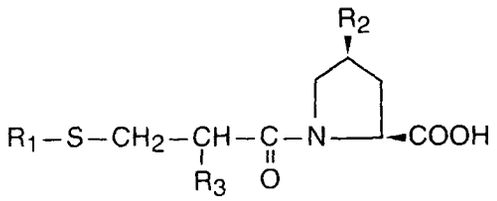

Figure 1. Structures of captopril and related compounds.

Compound	R_1	R_2	R_3
Captopril (**1**)	H	H	CH_3
Captopril-NES (**2**)	$C_6H_8NO_2$	H	CH_3
S-Benzoyl captopril (**3**)	C_7H_5O	H	CH_3
4-Fluorocaptopril (**4**)	H	F	CH_3
4-Fluorocaptopril-NES (**5**)	$C_6H_8NO_2$	F	CH_3
4-Fluoro-S-benzoyl captopril (**6**)	C_7H_5O	F	CH_3
Desmethylated captopril (**11**)	H	H	H

NES = *N*-ethylsuccinimide = $C_6H_8NO_2$
C_7H_5O = Benzoyl

Two internal standards (**5** and **6**, Fig. 1) were used, one for each of the analytes.

High-resolution chromatography of the methyl esters of **2** and **3** permitted the use of the common EI fragment ion for quantitative measurements, which was not achievable with the packed column GC/MS methods.[5–7] An added benefit is that this detector is ten times more sensitive than Hewlett-Packard 5985B, configurated for capillary GC/MS.

EXPERIMENTAL

Reagents and chemicals

Purified XAD-2 resin (Applied Science Lab, Inc.) was conditioned as previously described.[5] Ethyl acetate was purified immediately before use by passing 600 ml through a neutral alumina 2.5 × 4.0 cm column to remove oxidizing substances.[5] Preparation of the phosphate buffer (pH 7.0) and methanolic hydrochloric acid have been previously described.[5] Acetone, hydrochloric acid, phosphoric acid, *N*-ethylmaleimide (NEM), dibasic sodium phosphate, sodium chloride and sodium bicarbonate were reagent grade. Control blood, obtained commercially, was treated with 5 mg of NEM per ml of blood and stored in a freezer.

Compounds **1**, **3**, **4** and **6**, the internal standard for **3** (Fig. 1) were characterized pharmaceutical materials (E. R. Squibb & Sons). Compounds **2** and **5**, the internal standard for **2**, were prepared in solution from **1** and **4**, respectively, as described under standard preparation.

Apparatus

A Hewlett-Packard 5790A gas chromatograph equipped with a capillary inlet system was used. The fused silica capillary column (15 m, 0.22 mm, i.d., 0.20 μm film thickness) was coated with bonded cyanopropylphenyl methylpolysiloxane phase, CP sil 19 CB (Chrompak). The carrier gas was helium with an inlet pressure of 48 kpa (7 psig). The oven temperature was operated isothermally at 190 °C for 1.0 min after injection and

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then heated at a rate of 25 °C min^{-1} to 285 °C, and then held at the final temperature for 2.5 min. Injections were made by the splitless cold trapping mode, with a split flow of 30 ml min^{-1} and a septum purge of 2.0 ml min^{-1}. The inlet purge was turned on 0.7 min after injection. The injector temperature was maintained at 250 °C.

A Hewlett-Packard 5970A MSD was interfaced with the 5790A gas chromatograph, with the capillary column inserted directly into the ion source. The GC/MSD interface was maintained at 290 °C. The vacuum in the MSD, which changed with the flow rate in the column, remained in the region of $4\text{–}6\times10^{-5}$ torr during the column temperature programming. The MSD was calibrated with the Autotune® program at the beginning of each day to the mass of 502 Daltons using perfluorotributylamine (PFTBA) as the calibration compound. While the peakfinder mode was used for qualitative work, the selected ion monitoring (SIM) mode was used for the quantitative measurements. Depending on sample concentrations, the electron multiplier voltage applied was equal to or up to 400 V above the Autotune® value. Except for setting the peak width to 0.9 Dalton at one-half amplitude, all the other Autotune® parameters were used without change. The peak dwell time was 50 ms at a window of 0.1 Dalton. The detector was turned on from 4.8 to 6.7 min after injection. The mass ions monitored were m/z 230 for compounds **7** and **8** (the analytes) and m/z 248 for compounds **9** and **10** (the internal standards), as shown in Fig. 2. Each day, after tuning, the precise mass of each ion was determined by creating a window of monitored ions around the nominal values of m/z 230 and 248.

Standard preparation

A stock solution of **2** was prepared by treating 50 mg of **1** with 250 mg of NEM in 10 ml, PH 7.0, buffer for 15 min, diluting to 100 ml with acetone, and filtering through a fine-porosity sintered-glass filter. A stock solution of **5** was similarly prepared by reacting 50 mg of **4**. Stock solutions of **3** and **6** were prepared separately by accurately weighing approximately 25 mg of each and dissolving in 100 ml of acetone. Diluted solutions of **2**, **3**, **5** and **6**, prepared by dilution with acetone, were stable for at least 3 months when stored in a refrigerator. Calibration standards were obtained by transferring 100 µl of the required number of appropriately diluted solutions of **2** and **3** and 100 µl of a diluted solution of **5** and **6** to culture tubes and 3.0 ml of control blood. A typical calibration set consisted of a 0 and 8 other values ranging from 8 to 1000 ng of **1** and 3 to 333 ng of **3** per ml of blood, each containing 500 ng of **4** and 167 ng of **6** per ml of blood.

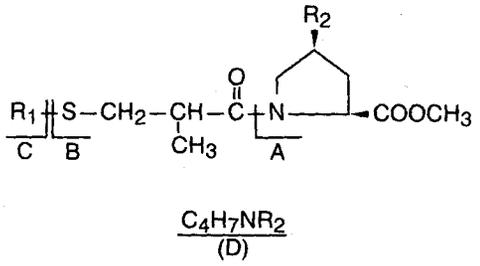

Figure 2. Fragment ions of captopril and related compounds.

Compound	R_1	R_2	A	B	m/z C	M^+	D
7	$C_6H_8NO_2$	H	128	230	126	356	70
8	C_7H_5O	H	128	230	105	335	70
9	$C_6H_8NO_2$	F	146	248	126	374	88
10	C_7H_5O	F	146	248	105	353	88

Blood collection and stabilization

From 12 to 15 ml of blood was drawn with a vacutainer (Becton-Dickinson) and processed as previously described[5] by the addition of 75 mg of NEM to each sample. In less than 72 h after collection, the blood samples, which had been stored in a freezer, were thawed at room temperature and exactly 3.0 ml of each well mixed sample was transferred to a culture tube containing 100 µl of an acetone solution of **5** and **6** (internal standards). Next, while mixing each sample tube on a vortex mixer, 5 ml of acetone was added dropwise from a 25 ml buret and immediately followed by a rapid addition of 10 ml of acetone. The tubes were then capped and stored in a freezer.

Isolation and purification

Stabilized and frozen blood samples were thawed at room temperature or kept in a refrigerator overnight. After centrifugation, the acetone solution from each tube was decanted from the solids into a 20 ml scintillation vial, and the acetone was evaporated at 65 °C in a sample concentrator with a scintillation vial adapter (Brinkmann Instruments). To each tube containing the solids, 10 ml of aqueous acetone (15% water in acetone) was added, the precipitated blood cells dislodged with a spatula, the mixture shaken for 5 min and the phases separated by centrifugation. The second acetone extract was combined with the first extract in the scintillation vial, and evaporation continued until no acetone odor could be detected, leaving behind approximately 2.5 ml of an aqueous solution in the scintillation vial. After cooling to room temperature, 10 ml of 0.1N hydrochloric acid was added to the scintillation vial. The extract could be further purified or frozen but stored for no longer than 72 h. Further purification was achieved by a method previously described[7] for the isolation and purification of **2**. In summary, the extract in 0.1N hydrochloric acid was passed through an XAD-2 column and the compounds **2**, **3**, **5** and **6** eluted with purified ethyl acetate, which was back extracted into 5% sodium bicarbonate and then extracted again into ethyl acetate. The ethyl acetate was removed by evaporation to obtain a dry clean residue.

Methylation and reconstitution

The dried extracts were methylated with methanolic hydrochloric acid as previously described.[5] After removing the reagents by evaporation, the dried methylated residue was reconstituted with 200 µl of toluene.

Table 1. Extraction of 2 and 3 from 3 ml of blood with acetone

Extraction number	Solvent	Extracted (%) 2	3
1st	15 ml acetone	87.6	78.5
2nd	10 ml aqueous acetone	11.2	18.8
3rd	10 ml aqueous acetone	2.2	2.6

Procedure

The calibration curve, each for captopril and S-benzoyl captopril, was established at the beginning of the study. Daily, a control sample, which represents the calibration point of 1000 ng of **1**, 333 ng of **3**, 500 ng of **4** and 167 ng of **6** per ml of blood, 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 as described previously.[6]

The standard curve for captopril was constructed by plotting area ratios of the analyte **7** to the internal standard **9** versus the amount ratios of **1** and **4**, the amount being the total nanograms of **1** and **4** in each calibration standard. The standard curve for S-benzoyl captopril was similarly constructed by using the areas of **8** and **10** and the amounts of **3** and **6**. A typical calibration curve for captopril gave a slope of 1.595, coefficient of correlation of 0.9998 and y-intercept of −0.0007288. The corresponding values for the S-benzoyl captopril curve were 1.551, 0.99980 and −0.02001, respectively.

RESULTS AND DISCUSSION

In blood, hydrolysis of **3** to **1** was observed after 10 days of storage at −20 °C. Hence, the blood samples were stabilized with acetone within 72 h of collection. For an efficient simultaneous isolation of **2** and **3** from 3 ml of blood, two extractions with acetone were needed (Table 1). After evaporation of acetone, adding 0.1N hydrochloric acid and extracting with 10 ml of ethyl acetate or methylene chloride did not produce adequately clean extracts. Hence, the hydrochloric acid sample solution was further purified by passing through an extraction column. Bond Elut® columns (Analytichem International) C18, C8, C2, cyclohexyl, cyanopropyl, carboxylic acid, quaternary amine, aminopropyl, silica and benzenesulfonic acid were investigated. All the columns absorbed both **2** and **3** quantitatively, but only the last three produced clean methanolic eluate but with low recoveries only. XAD-2 columns gave pure extracts with good recoveries.

The fragmentation pattern of **7** and **8** was determined using the peakfinder program (Fig. 3) and the diagnostic fragment ions have been identified (Fig. 2), with the base peak at m/z 128 for **7** and m/z 105 for **8**. To avoid potential interferences from co-eluting background components from blood, the somewhat lower intensity m/z 230 fragment ion common to both analytes was selected for quantitative measurements in the SIM mode.

Typical SIM chromatograms are shown in Fig. 4. Whereas **8** or its internal standard **10** gave a single peak, **7** or its internal standard **9** each gave two peaks, the sum of which was used in area computation. The reaction of the optically pure captopril diastereomer with NEM, in non-stereospecific manner, leads to the formation of a diastereomer pair. The resolving power of the capillary system employed was adequate to resolve the two diastereomers. On the other hand, the reaction of desmethyl analogue of captopril (**11**) with NEM does not

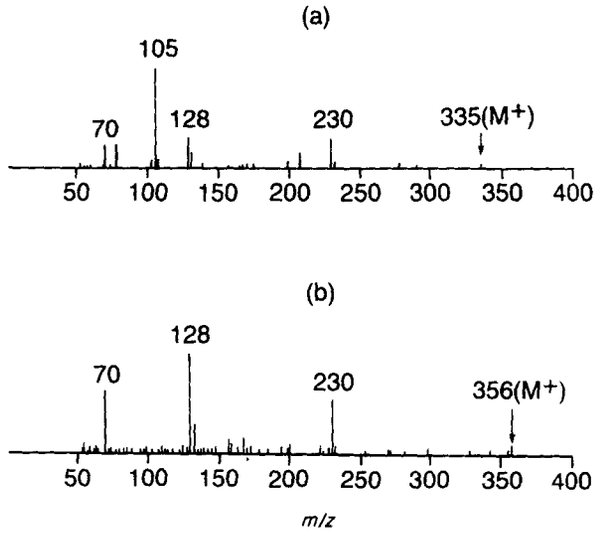

Figure 3. Electron impact spectra of the methyl ester derivatives of (a) S-benzoyl captopril and (b) captopril *N*-ethylsuccinimide.

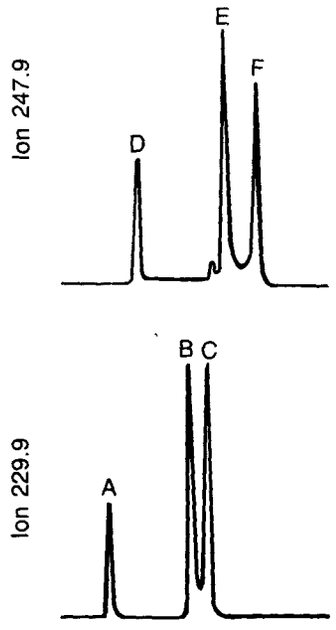

Figure 4. SIM chromatograms of sample extract, m/z 229.9 and 247.9 plotted separately. Compounds **1**, **3**, **4** and **6** are 1000, 333, 500 and 167 ng ml^{-1} of blood, respectively.

Peak	Compound	Retention time
A	**8**	5.17
B	**7**	5.68
C	**7**	5.80
D	**10**	5.34
E	**9**	5.92
F	**9**	6.11

lead to a separable pair because of the absence of a neighboring chiral center to the succinimide.

Compound 7 was baseline separated from **8**, allowing simultaneous analysis of captopril and S-benzoyl captopril using a common EI fragment ion. The high efficiency of the moderately polar capillary column provided the resolving power, with a peak width at one-half amplitude of approximately 2 s. The lower polarity capillary column SE-54 provided only marginal GC resolution.

Capillary GC with the splitless injection can be performed by either a 'solvent effect' or by 'cold trapping' of the solute.[8-13]. To achieve a solvent effect with toluene, a low initial column temperature, 80 °C, would have to be used which would produce the undesirable 'band broadening in space' which would have to be eliminated by the use of the 'retention gap'.[14,15] With the initial column temperature of 190 °C, no solvent effect was achieved but the components **7**, **8**, **9** and **10** were cold trapped at the head of the column. After an isothermal period of 1.0 min, the column temperature was increased at a rapid rate of 25 °C min^{-1} to initiate the elution of the components, resulting in narrow width chromatographic peaks of approximately 2 s. Monitoring only two fragment ions, m/z 230 and 248, a maximum sampling dwell time of 50 ms could be used permitting the sampling of an eluting GC peak 20 times, in order to obtain a good integration accuracy.

Some bias resulted when a low-concentration solution was injected immediately after a very concentrated sample. Under the cold trapping splitless injection conditions, with the capillary column at a temperature above the boiling point of the solvent and with the slow carrier flow in the inlet, the vaporized sample expanded into the colder parts of the injection zone, where some of the solutes (**7**, **8**, **9** and **10**) became trapped. On a subsequent injection, the vaporized solvent redissolved some of the trapped components, which were carried into the column eluting at the same retention times of the **7**, **8**, **9** and **10**, present in the injected sample. Since the amount trapped may depend on the injection temperature, the rate of injection, the inlet purge off time and the carrier flow, the potential bias should be evaluated under the prevailing conditions.

Under the conditions specified, the limit of quantification was estimated to be approximately 3 ng ml^{-1} of blood for both captopril and S-benzoyl captopril, which is significantly better than the limits established for packed column GC/MS,[6] 13 ng ml^{-1} of blood. Sensitivity could be further enhanced by reconstituting with 20 μl of toluene instead of 200 μl.

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