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Note

Determination of S-benzoyl captopril in human urine by capillary gas chromatography with electron-capture detection*

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

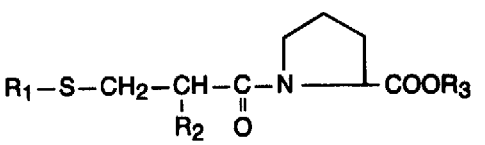

Fig. 1. Structures of compounds.

Compound	R_1	R_2	R_3
S-Benzoyl captopril (I)	C_6H_5—C=O	CH_3	H
Internal standard (II)	C_6H_5—C=O	H	H
Captopril (III)	H	CH_3	H
IV	C_6H_5—C=O	CH_3	CH_3
V	C_6H_5—C=O	H	CH_3

Published methods for the determination of captopril in body fluids utilize packed-column gas chromatography (GC) [5], packed-column gas chromatography—mass spectrometry [6, 7] and high-performance liquid chromato-

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graphy [8]. The method developed for the measurement of S-benzoyl captopril utilizes a simple and unique sample clean-up based on liquid—liquid extraction followed by solid-phase extraction. The methyl ester derivative (compound IV, Fig. 1) is chromatographed by high-resolution capillary GC using splitless cold trapping injection.

Compound I as the methyl ester (IV) has sufficient inherent electron-capture response to produce a quantitative measurement from less than 25 pg injected. The limit of quantitation is 10 ng/ml of urine.

EXPERIMENTAL

Gas chromatography

A Hewlett-Packard 5840 gas chromatograph equipped with a constant-current electron-capture detector and an autosampler was used. The fused-silica capillary column (10 m × 0.22 mm I.D., 0.20 μm film thickness) was coated with bonded cyanopropylphenylmethylpolysiloxane phase, CP sil 19 CB (Chrompack). The carrier gas was helium with an inlet pressure of 137 kPa (20 p.s.i.g.) and the make-up gas for the electron-capture detector was 5% methane in argon, at a flow-rate of 30 ml/min. The oven temperature was operated isothermally at 220°C for 4.0 min after injection and then heated at a rate of 4°C/min to 250°C, and then held at the final temperature for 2 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 injecton. The injector and detector temperatures were maintained at 280°C and 300°C, respectively.

Extraction

Cyanopropyl Bond-Elut® columns (Analytichem International) were used for solid-phase extraction. The columns were fitted into a 10-place Vac-Elut® (Analytichem International) vacuum manifold. Plasipak syringe barrels (Fisher) were connected to the Bond-Elut columns to act as reservoirs for larger volumes of samples and extraction solvents.

Reagents and chemicals

The internal standard, desmethylated analogue of S-benzoyl captopril (compound II, Fig. 1) was synthesized at the Department of Organic Chemistry. Compounds I and II were characterized pharmaceutical-grade materials (E.R. Squibb & Sons).

The methylating reagent was prepared from an instant methanolic hydrochloric acid kit (Applied Science Lab.). Methanolic hydrochloric acid was prepared by reacting 5 ml of anhydrous methanol with 0.25 ml of acetyl chloride. The solution may be kept refrigerated for no longer than one week.

Solution 1, used for the preparation of control urine, was prepared by dissolving 4.0 g of diethylenetriamine pentaacetic acid in 500 ml of 0.08 *M* sodium hydroxide, and solution 2 was prepared by dissolving 53 g of citric acid monohydrate and 32 g of oxalic acid dihydrate in 500 ml of water. Control urine was obtained by mixing 1.0 ml of solution 1 and 15 ml of solution 2 with 85 ml of a composite urine collected from several drug-free healthy individuals.

The control urine was kept in a freezer. The protocol for urine collection required the addition of oxalic acid, citric acid and diethylenetriamine pentaacetic acid to prevent the oxidation of liberated captopril, which was not to be measured in this procedure. For measurement of I, urine samples acidified with orthophosphoric acid to pH 2 behaved similarly following the same extraction procedure, described below.

Toluene and acetone, glass-distilled, were obtained from Burdick & Jackson Labs.

S-Benzoyl captopril (I) and internal standard (II) stock solutions were prepared separately by accurately weighing approximately 12 mg of each and dissolving in 25 ml of acetone. Diluted stock solutions, prepared by a 1:50 dilution with acetone, were stable for at least three weeks when stored in a refrigerator. Calibration standards were obtained by transferring appropriate volumes of the diluted stock standard and a fixed amount of the diluted stock internal standard to culture tubes, evaporating the acetone, and adding 5.0 ml of control urine. A typical calibration set consisted of a 0 and seven other values ranging from 20 to 300 ng of the standard per ml of urine, each containing 200 ng of the internal reference standard per ml of urine.

Sample preparation

Purification of urine samples and calibration standards was accomplished by a liquid—liquid extraction followed by a solid-phase extraction.

Liquid—liquid extraction. Urine samples were either thawed at room temperature or kept in a refrigerator overnight. A 5.0-ml portion was transferred to a culture tube containing 1000 ng of the internal standard and 3 g of sodium chloride. To each tube, 8.0 ml of toluene were added, the mixture was shaken for 5 min and the phases were separated by centrifugation. A 5.0-ml portion of the toluene phase was further purified by the solid-phase extraction described below.

Solid-phase extraction. The required number of cyanopropyl Bond-Elut columns, with attached syringe barrels, were conditioned with 5.0 ml of methanol followed by 5.0 ml of toluene. Immediately, the 5.0 ml of toluene extract was passed through the column, retaining compounds I and II. Each column was then washed with 10 ml of toluene followed by elution with 1.0 ml of acetone directly into an autosampler vial. Acetone was removed at about 30°C under a slow stream of nitrogen.

Methylation and reconstitution. To each vial a 0.1-ml portion of methanolic hydrochloric acid was added, the vial was sealed with PTFE-lined caps, vortexed and then heated at 60°C for 10 min. The cooled vial was then uncapped and the reagent removed by evaporation at 50°C under a stream of nitrogen. The dried sample was reconstituted with 1.0 ml of toluene, the vial was capped, the contents mixed and then loaded onto the autosampler. At this point the reconstituted samples could be stored in a freezer for later measurement.

Procedure

Daily calibration standards were run at the beginning and end of each day. Typical injection volumes were 1—2 μl.

Standard curves were constructed by plotting peak area ratios of the analyte (IV) to the internal standard (V) versus the amount ratios of I to II, the amount being the total nanograms of I or II in each calibration standard. A typical calibration curve gave a slope of 1.266, coefficient of correlation of 0.9993 and y-intercept of −0.005962.

RESULTS AND DISCUSSION

Extraction

Various extraction schemes were investigated to optimize the isolation of compounds I and II (Tables I and II). In Scheme 1, where acidified urine was passed through the extraction columns prior to liquid–liquid extraction, recovery generally increased with decreasing polarity of the column, but the reverse was true for cleanliness. This simple procedure could be used for samples with relatively large amounts of compound I, where the cleanliness of the extract is less critical. An attempt to improve cleanliness by adding acetone to the urine before passing through the columns or rinsing with methanolic water prior to elution was successful but resulted in lower recovery. In Scheme 2, the ethyl acetate extract was reconstituted with the less polar toluene because the analyte was not adequately retained on the columns, due to higher solubility in ethyl acetate. The recovery from Scheme 2 was, however, poor due to incomplete dissolution of the sticky residue after the evaporation of the ethyl acetate. In Scheme 3, where the ethyl acetate extract is reconstituted

TABLE I

EXTRACTION PROCEDURES FROM URINE

Bond-Elut columns are silica-based, to which the organic layers are bonded. C_{18} = Octadecyl; C_8 = octyl; C_2 = ethyl; CH = cylcohexyl; PH = phenyl, CN = cyanopropyl; CBA = carboxylic acid; SI = silica; NH_2 = aminopropyl; 2OH = diol; SAX = quaternary amine; SCX = sulfonic acid.

Scheme 1.	Condition the columns C_{18}, C_2, CH, PH, CN, CBA, SI, NH_2, 2OH, SAX and SCX with 5.0 ml of methanol followed by 5.0 ml of 0.1 *M* hydrochloric acid. Pass 0.5 ml of acidified urine through each column. Rinse with 10 ml of 0.1 *M* hydrochloric acid and elute with 1.0 ml of acetone or methanol.
Scheme 2.	Extract 2.0 ml of acidified urine, after adding 3 g of sodium chloride, with 6.0 ml of ethyl acetate. Separate the ethyl acetate layer, evaporate, reconstitute with 2.0 ml of toluene. Condition the columns C_2, CH, PH, CN, CBA and SI with 5.0 ml of methanol followed by 5.0 ml of toluene. Pass 2.0 ml of the reconstituted toluene solution through each column. Rinse with 10 ml of toluene and elute with 1.0 ml of acetone or methanol.
Scheme 3.	Extract exactly as in Scheme 2, but reconstitute with 2.0 ml of 0.1 *M* hydrochloric acid. Condition the columns with 5.0 ml of methanol followed by 5.0 ml of 0.1 *M* hydrochloric acid. Pass 2.0 ml of the reconstituted 0.1 *M* hydrochloric acid solution through each column. Rinse with 10 ml of 0.1 *M* hydrochloric acid and elute with 1.0 ml of acetone or methanol.
Scheme 4.	Extract 5.0 ml of acidified urine, after adding 3 g of sodium chloride, with 8.0 ml of toluene. Condition the columns C_2, CH, PH, CN, CBA, SI and NH_2 with 5.0 ml of methanol followed by 5.0 ml of toluene. Pass 5.0 ml of the toluene extract through each column. Rinse with 10 ml of toluene. Elute with 1.0 ml of acetone or methanol.

TABLE II

EXTRACTION RESULTS

Column A designates degree of cleanliness as seen chromatographically and column B designates recovery of compound I Cleanliness: +++ = very good, ++ = good, + = fair. Recovery: +++ = nearly 100%, ++ = above 50%, + = below 50%, — = nearly 0%. ND = Not determined.

Column	Scheme 1		Scheme 2		Scheme 3		Scheme 4	
	A	B	A	B	A	B	A	B
C_{18}	+	+++	ND	ND	ND	ND	ND	ND
C_2	+	+++	++	+	++	++	+++	+
CH	+	+++	++	+	++	++	+++	+
PH	+	+++	++	+	++	+++	+++	+
CN	+	++	++	+	++	++	+++	+++
CBA	+	+++	++	+	++	++	+++	++
SI	++	+	ND	ND	ND	ND	+++	++
NH_2	ND	ND	ND	ND	ND	ND	+++	—
2OH	++	+	ND	ND	ND	ND	ND	ND
SAX	+++	+	ND	ND	ND	ND	ND	ND
SCX	+++	+	ND	ND	ND	ND	ND	ND

with 0.1 *M* hydrochloric acid, both the recovery and cleanliness were satisfactory. This procedure was not adopted because it involves a number of steps. In Scheme 4, where the toluene extract was passed through the columns, very good cleanliness was obtained. The recovery improved with extraction columns of medium polarity. There was no significant difference in cleanliness or recovery whether acetone or methanol was used for elution. Acetone was chosen for ease of evaporation. Modification of Scheme 4 using 2—5% amyl alcohol in toluene for liquid—liquid extraction resulted in greatly reduced cleanliness and recovery for all the extraction columns. Scheme 4 using the CN column was finally adopted.

Gas chromatography

There was excellent proportionality between concentration and response for compounds IV and V in the range of 25—1000 pg injected. Above the 1000-pg level, the response/concentration decreased gradually with increase in concentration.

A typical sample chromatogram is shown in Fig. 2. Chromatograms of control urine and pre-dose sample urine showed absence of any interferences with compounds IV and V.

The concentration of I in the non-zero samples ranged from 8 to 730 ng/ml of urine. For samples above 300 ng/ml, the 1.0-ml toluene solution for injection was diluted so that the concentration fell in the linear region of the detector response.

Capillary GC with the splitless injection can be performed by either a solvent effect or by cold trapping of the solute [9—14]. 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 [15, 16].

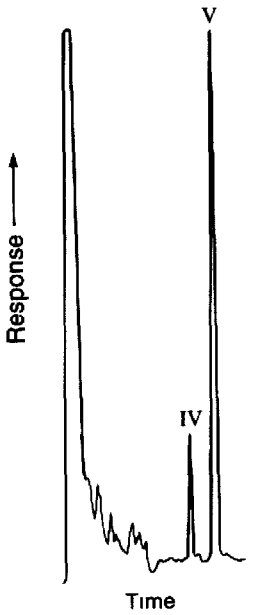

Fig. 2. Chromatogram of extract from 5.0 ml urine, 32 ng/ml I in urine. Peaks: IV = S-benzoyl captopril methyl ester (7.0 min); V = internal standard methyl ester (8.3 min).

To achieve complete cold trapping of IV and V, the initial column temperature should be less than 190°C. While complete trapping was not achieved with the temperature program starting at 220°C, extract impurities eluted well away from IV and V. The peak width of V was approximately 8 sec, only somewhat larger than would have been achieved with complete cold trapping.

Electron-capture response

The methyl esters of S-benzoyl esters IV and V show surprisingly strong electron-capture response. Most reports indicating the electron-capture nature of thio compounds are usually associated with aryl thio but not alkyl thio compounds [17, 18]. As a corollary, our experience is that the thiobenzoyl ester but not the thioacetyl ester has electron-capture activity. Since methylation does not contribute at all to the detectability, the method is very specific and sensitive, allowing extremely good signal-to-noise detection. For example, typically 1/1000 of the urine extract injected yields quantitative responses from 25 pg injected, allowing quantitation of 10 ng/ml of urine. The limit of detection could be lowered by reconstituting injection solutions with 100 μl of toluene.

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