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Note

Determination of benazepril, a new angiotensin-converting enzyme inhibitor, and its active metabolite, benazeprilat, in plasma and urine by capillary gas chromatography–mass-selective detection

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Benazepril·HCl, 3-{[1-ethoxycarbonyl-3-phenyl-(1*S*)-propyl]amino}-2,3,4,5-tetrahydro-2-oxo-1-(3*S*)-benzazepine-1-acetic acid hydrochloride (I·HCl; CGS 14 824 A) is a potent angiotensin-converting enzyme (ACE) inhibitor. The compound, a dicarboxylic acid monoethylester, is a prodrug which, on absorption, is hydrolysed to pharmacologically active metabolite, the dicarboxylic acid (II; CGS 14 831).

To follow the pharmacokinetics of the new drug, different analytical methods have been developed: gas chromatography–mass spectrometry (GC–MS) was used for the specific and simultaneous determination of unchanged benazepril and active benazeprilat in plasma and urine [1]. Based on the inhibition of ACE by compound II, two slightly different bioassays, i.e. an enzyme inhibition high-performance liquid chromatographic method and a radioenzymatic method, were used for the specific determination of the metabolite II in plasma and urine [2].

We have now adapted the GC–MS method of Kaiser et al. [1] to a Hewlett-Packard gas chromatograph with mass-selective detection (MSD). The previ-

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ously described extraction and derivatization procedures were maintained. However, the packed column used for the chromatographic separation of the derivatives of compounds I and II was replaced by a fused-silica capillary column. Thereby a complete separation of the derivatives was achieved, in contrast to the incomplete chromatographic resolution obtained with the packed column. Consequently, we improved the sensitivity of the method, especially for unchanged compound I, by a factor of 3–8. This paper describes the modified method and its within-day and day-to-day validation.

EXPERIMENTAL

Reagents and chemicals

Benazepril·HCl (I·HCl), its metabolite benazeprilat (II), and the corresponding deuterium-labelled internal standards (III·HCl and IV) were supplied by Ciba-Geigy (Basle, Switzerland).

A solution of diazomethane in diethyl ether was prepared from N-nitroso-N-methyl-*p*-toluenesulphonamide (Cat. No. 73970; Fluka, Buchs, Switzerland) according to the method of Fales et al. [3]. Amberlite XAD-2, particle size 0.2–0.25 mm, was obtained from Serva (Heidelberg, F.R.G.), and pre-packed glass columns from Merck (Extrelut 1-15371; Merck, Darmstadt, F.R.G.).

Dichloromethane, diethyl ether, hexane and toluene (Pestipur, SDS, Peypin, France) were of analytical grade. Tetrahydrofuran (Merck 8110), 25% (w/v) ammonia solution (Merck 5432), hydrochloric acid 0.1 *M* (Titrisol 9973), sulphuric acid 0.5 *M* (Titrisol 9981), sodium carbonate (Merck 6392) and pH 3 buffer (Titrisol 9883) were from Merck. Brine was a saturated solution of sodium chloride (Merck 6404) in water.

Equipment

A Hewlett-Packard 5890A gas chromatograph equipped with a capillary inlet system was used. The column was a 12.5 m×0.2 mm I.D. fused-silica capillary column coated with cross-linked methylsilicone (Hewlett-Packard 19091A, option 101). The carrier gas was helium with an inlet pressure of 55 kPa (8 p.s.i.g.). Splitless injection was used with a 0.30-min splitless period. The injection temperature was 250°C. The column was initially set at 210°C for 0.50 min, and the temperature was then raised at 50°C/min to 290°C. This temperature was maintained during the chromatography. At the end of the analysis, the temperature decreased automatically to 210°C.

A Hewlett-Packard 5970B mass selective detector was interfaced with the 5890A gas chromatograph, and the capillary column was inserted directly into the ion source. The interface was maintained at 280°C. The detector was calibrated with the Autotune® program from Hewlett-Packard at the beginning of each day, using perfluorotributylamine (PFTBA). The detector was turned on from 3.5 to 5.0 min after injection, and the electron multiplier voltage applied was 400 V above the Autotune value. The selected ions monitored were *m/z* 365 for the methyl ester derivative of compound I and the dimethyl ester derivative of compound II,

and *m/z* 370 for the methyl ester derivative of compound III and the dimethyl ester derivative of compound IV.

Extraction and derivatization

For plasma samples, glass columns with a sintered glass filter were filled with 400 mg of Amberlite XAD-2. A rack was used to simplify the pre-treatment of Amberlite XAD-2 columns and the clean-up of samples. The rack accommodates up to eight columns simultaneously. A vacuum source was used to draw solvents through each column. The Amberlite XAD-2 was washed with 3 ml of methanol, then degassed with 5 ml of methanol and washed three times with 5 ml of water. The chromatographic system was acidified with 3 ml of 0.1 *M* hydrochloric acid. The supernatant of the centrifuged plasma sample was applied to the Amberlite XAD-2 column. The column was washed three times with 5 ml of water. The absorbed compounds were eluted with 4 ml of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40°C, and the residue dried in a vacuum desiccator over phosphorus pentoxide for at least 30 min.

For urine samples, pre-packed glass columns (Extrelut 1) were washed twice with 4 ml of dichloromethane–ethanol (95:5, v/v) and dried at 50°C for at least 12 h. Then 1 ml of each spiked urine sample was transferred to the top of the column and allowed to soak for 10 min. The column was eluted with 4 ml of dichloromethane–ethanol (95:5, v/v); 5 min later the column was eluted with a second portion of 4 ml of eluent, and the combined eluates were evaporated to dryness under a stream of nitrogen at 40°C. The residue was dried as described for plasma samples.

The plasma or urine extract was reconstituted in 0.1 ml of tetrahydrofuran, and 0.3 ml of a solution of diazomethane in diethyl ether was added. The mixture was shaken mechanically (Infors shaker) for 30 min at 300 rpm and evaporated to dryness.

To the dry derivatized plasma sample were added 0.5 ml of 0.5 *M* sulphuric acid and 1 ml of hexane. The mixture was shaken mechanically for 5 min at 300 rpm and centrifuged at 1600 *g* for 2 min. The organic phase was discarded. The aqueous phase was alkalinized with 1 ml of 2 *M* sodium carbonate, and shaken with 1 ml of diethyl ether–dichloromethane (2:1, v/v) for 5 min at 300 rpm. After centrifugation at 1600 *g* for 2 min, the organic phase was transferred to a conical vial and evaporated to dryness under a stream of nitrogen at 40°C. The residue was reconstituted in 10 μl of toluene, and 2 μl were injected into the gas chromatograph. The residue of the derivatized urine sample was dissolved in 1 ml of diethyl ether and shaken for 5 min at 300 rpm. The solution was transferred into a conical vial and evaporated to dryness under a stream of nitrogen at 40°C. The residue was reconstituted in 5 μl of toluene and 2 μl were injected into the gas chromatograph.

Gas chromatography–mass spectrometry

A fused-silica capillary column (12.5 m×0.2 mm I.D.) was chosen for GC of ester derivatives of compounds I and II. Compared with the method published by Kaiser et al. [1], the capillary column produced a complete resolution of the two

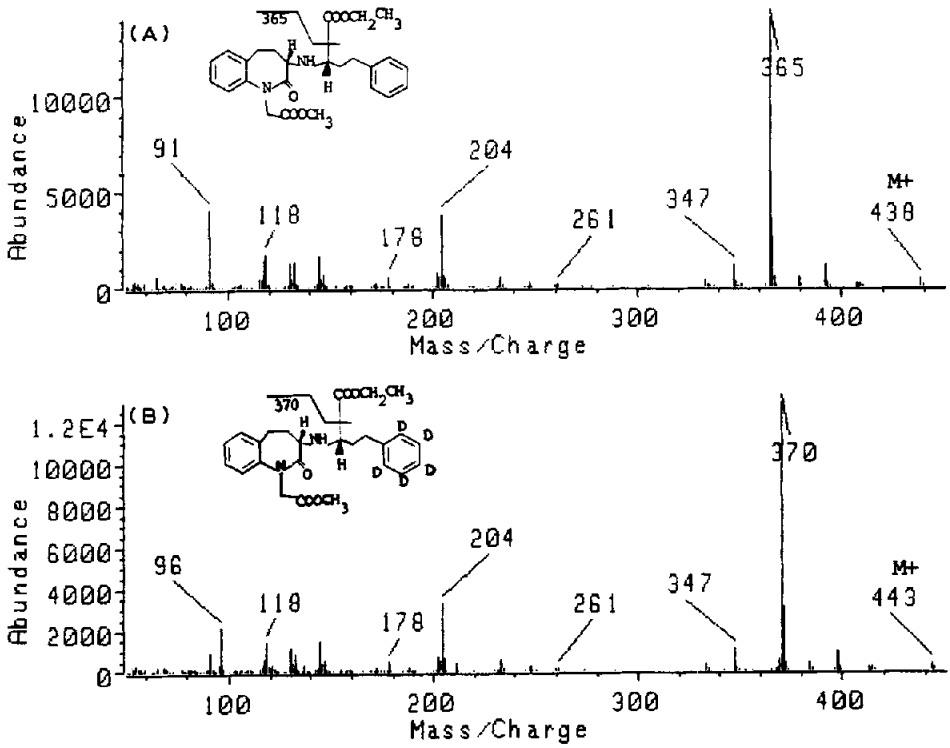

Fig. 1. Electron-impact spectra of (A) the methyl ester derivatives of benazepril and (B) its labelled analogue.

compounds. The retention times of the derivatives were ca. 4.5 and 4.7 min with the capillary column, compared with 2.25 and 2.5 min with the packed column [1].

Electron-impact spectra of the methyl ester derivatives of compound I and its labelled analogue III are shown in Fig. 1. Spectra of the dimethyl ester derivatives of compound II and its labelled analogue IV are shown in Fig. 2. Molecular ions were observed for all four compounds: *m/z* 438 for the methyl ester derivative of compound I and *m/z* 443 for the derivative of compound III; *m/z* 424 for the dimethyl ester derivative of compound II and *m/z* 429 for the derivative of compound IV. The base peaks were *m/z* 365 for the derivatives of compounds I and II and *m/z* 370 for the derivatives of compounds III and IV. These fragments were obtained after cleavage of the carboxyethyl (M−73) and carboxymethyl group (M−59) in the side-chain. Fragment ions *m/z* 365 and *m/z* 370 were selected for quantitative measurements in the selected-ion monitoring mode.

RESULTS

Plasma and urine interferences

The extract of blank human plasma showed a clean baseline at *m/z* 365 and 370; typical selected-ion current profiles obtained from human plasma are shown in Fig. 3.

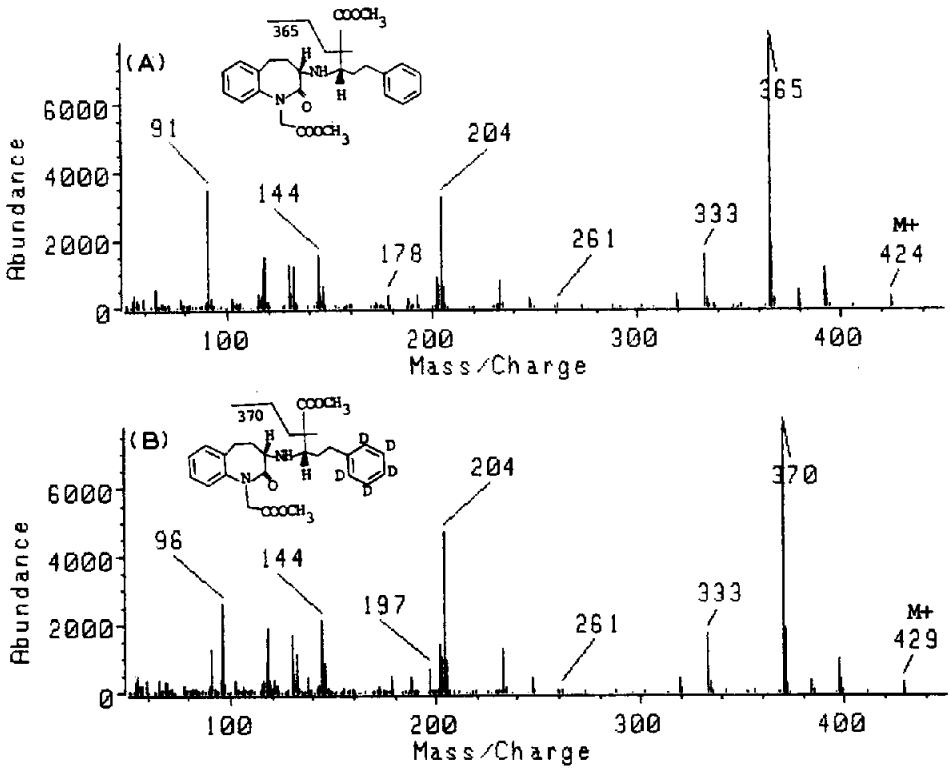

Fig. 2. Electron-impact spectra of (A) the dimethyl ester derivatives of benazeprilat and (B) its labelled analogue.

The extract of blank human urine showed a clean baseline at *m/z* 365 and *m/z* 370; typical selected-ion current profiles obtained from human urine samples are shown in Fig. 4.

Calibration curves

Calibration curves were obtained by plotting the peak-area ratio of compounds I or II (*m/z* 365) and the respective internal standard III or IV (*m/z* 370) versus the concentration of compounds I or II (conc.). Their equations were calculated by using weighted linear least-squares regression with a weighting factor of $1/(\text{conc.})^2$.

In plasma samples, the linear calibration range was 5.42–217.00 nmol/l for compound I, which corresponds to the regression equation $y = 0.011\,84x - 0.001\,12$, and 6.55–259.56 nmol/l for compound II, which corresponds to the regression equation $y = 0.008\,32x + 0.000\,19$.

In urine samples, the linear calibration range was 10.85–542.50 nmol/l for compound I, which corresponds to the regression equation $y = 0.005\,91x + 0.031\,98$, and 52.92–1310.40 nmol/l for compound II, which corresponds to the regression equation $y = 0.004\,42x - 0.003\,56$.

Every day, the validity of the calibration curves was checked by analysis, in duplicate, of samples spiked with a low and with a high amount of the compounds.

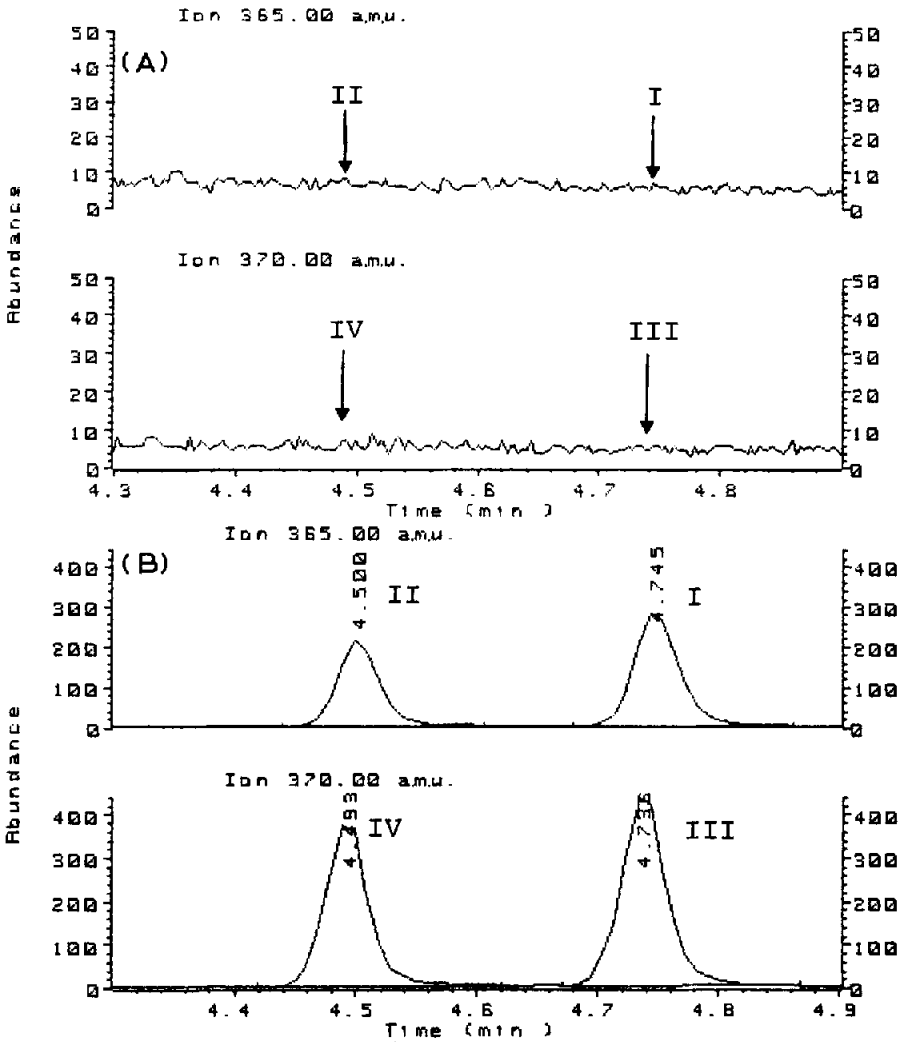

Fig. 3. Selected-ion current profiles of (A) an extract of 1 ml of blank human plasma and (B) the same plasma spiked with 66 pmol of benazeprilat (II), 155 pmol of [$^2H_5$]benazeprilat (IV), 54 pmol of benazepril (I) and 122 pmol of [$^2H_5$]benazepril (III).

If these spiked samples gave results deviating too much (accuracy >115% or <85%), a new calibration curve was obtained. For routine analysis, the calibration curves were valid for 1 week.

Limit of quantitation

The limit of quantitation in human plasma was estimated at 5.4 nmol/l for compound I and 6.5 nmol/l for compound II. In human urine, it was 11 nmol/l for compound I and 53 nmol/l for compound II. Lower concentrations could still be detected with a coefficient of variation above 10%.

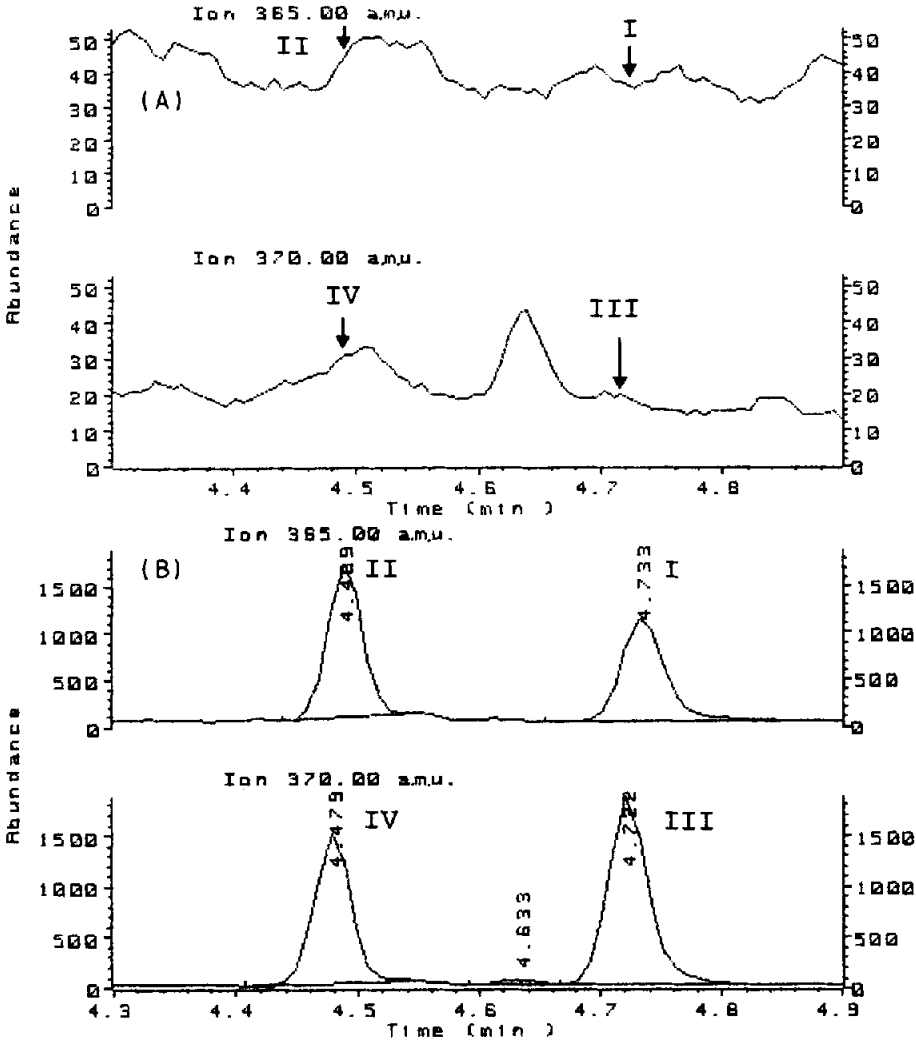

Fig. 4. Selected-ion current profiles of (A) an extract of 1 ml of blank human urine and (B) the same urine spiked with 260 pmol of benazeprilat (II), 310 pmol of [$^2H_5$]benazeprilat (IV), 108 pmol of benazepril (I) and 243 pmol of [$^2H_5$]benazepril (III).

CONCLUSION

The proposed technique permits the quantitative determination of benazepril and its metabolite in human plasma at concentrations down to 5.4 nmol/l for benazepril and 6.5 nmol/l for benazeprilat, in human urine at concentrations down to 11 nmol/l for benazepril and 53 nmol/l for benazeprilat. It is specific, reproducible and sensitive for determination of benazepril and its metabolite in pharmacokinetic, bioavailability and clinical pharmacology studies.

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