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Quantitative determination of benazepril and benazeprilat in human plasma by gas chromatography–mass spectrometry using automated 96-well disk plate solid-phase extraction for sample preparation

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Abstract

An analytical method for the determination of benazepril and its active metabolite, benazeprilat, in human plasma by capillary gas chromatography–mass-selective detection, with their respective labelled internal standard, was developed and validated according to international regulatory requirements. After addition of the internal standards, the compounds were extracted from plasma by solid-phase extraction using automated 96-well plate technology. After elution, the compounds were converted into their methyl ester derivatives by means of a safe and stable diazomethane derivative. The methyl ester derivatives were determined by gas chromatography using a mass-selective detector at m/z 365 for benazepril and benazeprilat and m/z 370 for the internal standards. Intra- and inter-day accuracy and precision were found to be suitable over the range of concentrations between 2.50 and 1000 ng/mL.

Keywords: Benazepril; Benazeprilat

1. Introduction

Benazepril hydrochloride, 3-{[1-ethoxycarbonyl-3-phenyl-(1*S*)-propyl]amino}-2,3,4,5-tetrahydro-2-oxo-1-(3*S*)-benzazepine-1-acetic acid hydrochloride, is a prodrug-type angiotensin-converting enzyme (ACE) inhibitor which, on absorption, is hydrolyzed to a pharmacologically active metabolite, the dicarboxylic acid (benazeprilat).

The previously published gas chromatography–mass spectrometry (GC–MS) method [1,2] was based on manual solid-phase extraction (SPE) using glass columns prepared in-house, which was time-consuming, required 1.0 mL of plasma and derivatization with diazomethane, which was prepared daily in ether.

Our goal was to modify the previous method to reduce the time of preparation using a 96-well plate directly adaptable on an automatic Packard MultiProbe, to halve the volume of plasma without modifying the lower limit of quantitation (LLOQ), to considerably reduce the volume of solvent for conditioning, washing and eluting, and to use a new methylation reagent, trimethylsilyldiazomethane, a less hazardous, but equally effective, substitute for

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diazomethane, which has been shown to react quickly and cleanly at room temperature [3,4].

The described method was validated according to the Guidance for Bioanalytical Method Validation [5,6], including determination of selectivity, accuracy, precision, recovery, calibration curve and stability of analyte in spiked samples.

2. Experimental

2.1. Chemicals and reagents

The chemical structures of benazepril, benazeprilat and the corresponding deuterium-labelled internal standards are shown in Fig. 1, and were supplied by Novartis (Basle, Switzerland).

All the chemicals were of analytical grade. Hexane and toluene (Pestipur SDS) were obtained from Solvants Documentation Synthèse (Pépin, France). Methyl *tert.*-butyl ether (ref. 20247) and 2 mol/L trimethylsilyldiazomethane solution in hexane (ref. 92738) were from Fluka (Saint-Quentin Fallavier, France). Hydrochloric acid (0.1 mol/L; Titrisol 9973), 0.5 mol/L sulphuric acid (Titrisol 9981), and sodium carbonate (Merck 6392) were from Merck (Merck, Darmstadt, Germany). 3M Empore C_{18}-SD 96-well disk plates containing 12 mg C_{18} were obtained from Varian (Les Ulis, France). Drug-free human plasma was obtained from ''Les Etablissements Français du Sang'' (Rungis, France), where blood was collected from volunteers in tubes containing heparin. After centrifugation, the plasma was transferred and stored at −18 °C.

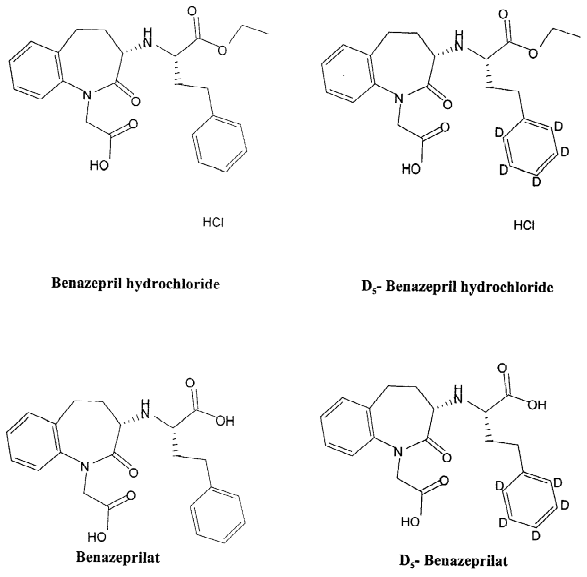

Fig. 1. Chemical structures of benazepril, benazeprilat and their respective labelled internal standards.

2.2. Standard solutions

The stock solution of benazepril hydrochloride was prepared by dissolving 1.085 mg in 10 mL of water. The stock solution of benazeprilat was prepared by dissolving 1 mg in 10 mL of 0.75% ammoniac. Appropriate serial dilutions of the stock solutions with water were then made in order to prepare the spiking solutions to be used for calibration samples, at concentrations ranging from 2.50 to 1000 ng/mL of benazepril base and benazeprilat.

The internal standard (I.S.) stock solutions were prepared by dissolving 1.085 mg of D_5-benazepril hydrochloride in 10 mL of water and 1 mg of D_5-benazeprilat in 0.75% ammoniac, respectively. Further dilution of the stock solutions with water resulted in the internal standard spiking solution (150 ng/100 μL). All solutions were stored at about +5 °C for 1 month.

2.3. Equipment

A Hewlett-Packard 5890 Series II GC apparatus, equipped with a capillary inlet system and an HP 7673 automatic sampler, was used (Hewlett-Packard, Palo Alto, CA, USA). The column was a 15 m×0.25 mm I.D. fused-silica capillary column coated with cross-linked methylsilicone with a film thickness of 0.25 μm (Resteck Rtx-1; Restek, Evry, France). The carrier gas was helium with an inlet pressure of 55 kPa (8 p.s.i.g.) with a split flow of 50 mL/min and a septum purge of 3.0 mL/min. Sample introduction was performed in the splitless mode at an injection temperature of 280 °C with a 30 s splitless period. The column was initially at 190 °C for 0.5 min and the temperature was then raised at a rate of 30 °C/min to 290 °C.

A Hewlett-Packard 5970B mass-selective detector (MSD) was interfaced to the GC apparatus, with the

capillary column inserted directly into the ion source. The MSD was calibrated with the Autotune program at the beginning of each day using perfluorotributylamine (PFTBA). The GC–MSD interface was maintained at 280 °C. The detector was turned from 3.5 to 5 min after injection. The selected ions monitored for the methyl ester derivatives were *m/z* 365 for benazepril and benazeprilat and *m/z* 370 for D_5-benazepril and D_5-benazeprilat.

A ChemStation was used to control the GC and injector instruments using the software Hewlett-Packard version G1701AA for data acquisition and the software version G1710BA for data processing. A Packard MultiProbe II liquid handling robotic system was used (Packard Instruments, Meriden, CT, USA) using the WinPrep software.

2.4. Calibration standards and quality control samples

For calibration standards, aliquots of working solutions were added to 0.5 mL of drug-free human plasma to produce reference samples in the range of concentrations 2.50–1000 ng/mL for benazepril and benazeprilat. For quality control samples, aliquots of working solutions were added to 0.5 mL of drug-free human plasma to produce reference samples in the range of concentrations 2.50–900 ng/mL for benazepril and benazeprilat. For calibration standards and quality control samples, a constant amount of internal standard (150 ng/100 μL) was added to each sample.

2.5. Extraction from plasma

To 0.5 mL plasma in a polypropylene tube were successively added an aliquot of the appropriate standard solution (only for calibration and validation),100 μL of I.S. solution (150 ng) and 100 μL of 0.1 mol/L hydrochloric acid. All tubes were then placed on the platform of the Packard MultiProbe II and a 96-well disk plate was placed on top of a vacuum manifold.

The 96-well disk plate was conditioned automatically with 100 μL of methanol, and 300 and 80 μL of 0.1 mol/L hydrochloric acid. Then, 500 μL of the prepared samples were loaded onto the 96-well disk plate. The loaded samples were washed with 100 μL of water. The analytes and internal standards were eluted twice with 500 μL of methanol. The eluates were transferred into extraction tubes and 300 μL of trimethylsilyldiazomethane solution was added. The reaction was run at room temperature in a dry bath for 30 min and the reaction mixture was evaporated to dryness under nitrogen at 40 °C.

To the dry derivatized plasma sample were added 0.5 mL of 0.5 mol/L sulphuric acid and 1 mL of hexane. The mixture was shaken mechanically for 5 min at 240 r.p.m. and centrifuged at 1600 *g* for 2 min. The organic phase was discarded. The aqueous phase was alkalinized with 1 mL of 2 mol/L sodium carbonate and shaken with 2 mL methyl *tert.*-butyl ether for 5 min at 240 r.p.m. After centrifugation at 1600 *g* for 5 min, the organic phase was evaporated to dryness under nitrogen at 40 °C. The residue was dissolved in 100 μL of toluene and a 2 μL aliquot was injected onto the chromatograph.

3. Results and discussion

3.1. Derivatization

The derivatization reaction using trimethylsilyldiazomethane with methanol provides a less-hazardous method for preparing methyl esters under mild conditions. Trimethylsilyldiazomethane is commercially available, obviating the need to synthesize diazomethane daily. It is a stable and safe substitute for either hazardous diazomethane or corrosive reagents containing boron trifluoride. Trimethylsilyldiazomethane reacts quickly at room temperature with carboxylic acid in the presence of methanol to give methyl esters in yields suitable for analytical GC.

3.2. Mass spectra

The electron impact mass spectra of the methyl ester derivatives of benazepril, benazeprilat and their respective labelled internal standards after derivatization with trimethylsilyldiazomethane are identical to those previously obtained after derivatization with diazomethane [1,2]. Molecular ions were observed for the four compounds, but the base peaks were at *m/z* 365 for the derivatives of benazepril and

benazeprilat, and at m/z 370 for the derivatives of their respective internal standards. Fragment ions at m/z 365 and 370 were selected for quantitative measurements in the SIM mode. These fragments are obtained by cleavage of the carboxyethyl [M−73] or carboxymethyl group [M−59] in the side chain.

3.3. Specificity

The specificity of the analytical method was investigated by preparing and analyzing blank samples prepared from six different batches of human plasma. The specificity was assessed by comparing the apparent signal for benazepril and benazeprilat and for D_5-benazepril and D_5-benazeprilat in blank samples to the mean signal obtained for samples spiked with a concentration of benazepril and benazeprilat at the LLOQ, and D_5-benazepril and D_5-benazeprilat at the working concentration. The following criteria for specificity were used to assess the method suitability: interference in the blank at the retention of the analyte should not exceed 20% of the response at the LLOQ and interference at the retention of the I.S. should not exceed 5% of the response at the working concentration.

Representative selected ion current profiles for extracts of drug-free human plasma and for the same plasma spiked with benazepril, benazeprilat and I.S. are shown in Figs. 2 and 3. As can be seen, the compounds of interest were separated from co-extracted endogenous plasma components. Similar profiles were observed for six different batches of human plasma and the above mentioned criteria were respected.

3.4. Extraction recovery

The extraction yields from plasma were estimated at three different concentrations: 7.50, 250 and 900 ng/mL for benazepril and benazeprilat. The mean extraction efficiencies were 30.5 and 62.4% for benazepril and benazeprilat, respectively. I.S. extraction efficiencies estimated at the working concentration (150 ng) were 39.4 and 56.3% for D_5-benazepril and D_5-benazeprilat, respectively.

Under the extraction conditions specified in Section 2.5, clean eluates were obtained, but with low extraction yields for benazepril. In spite of these low

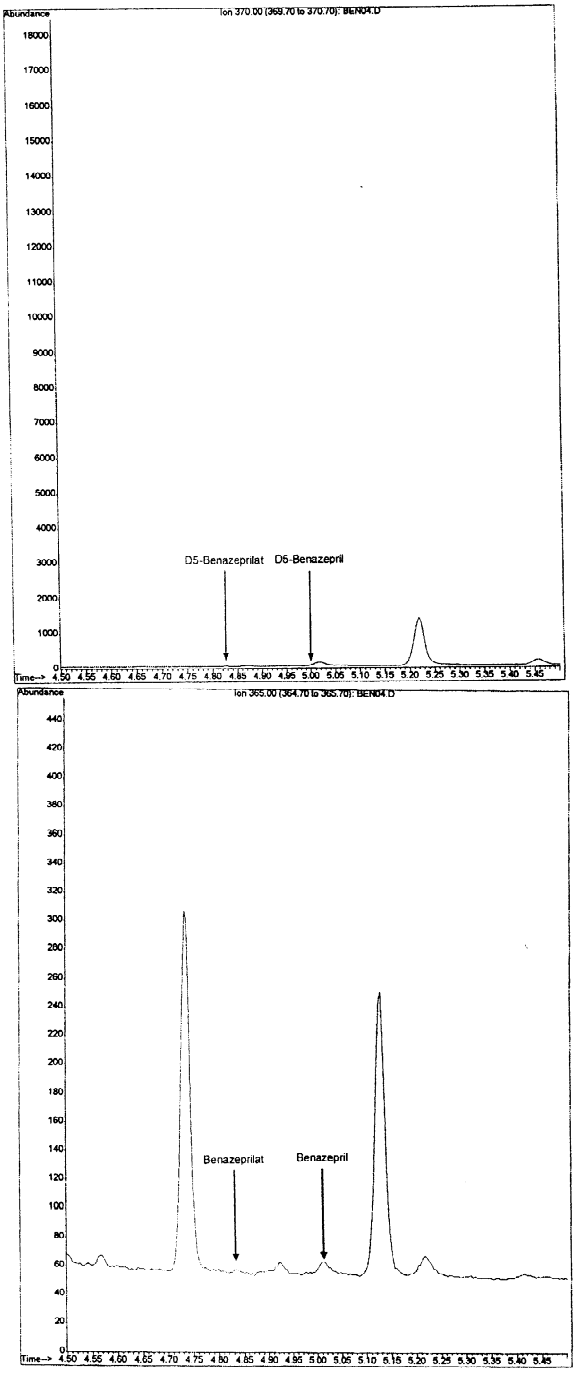

Fig. 2. Examples of selected ion current profiles for an extract of 1 mL drug-free human plasma.

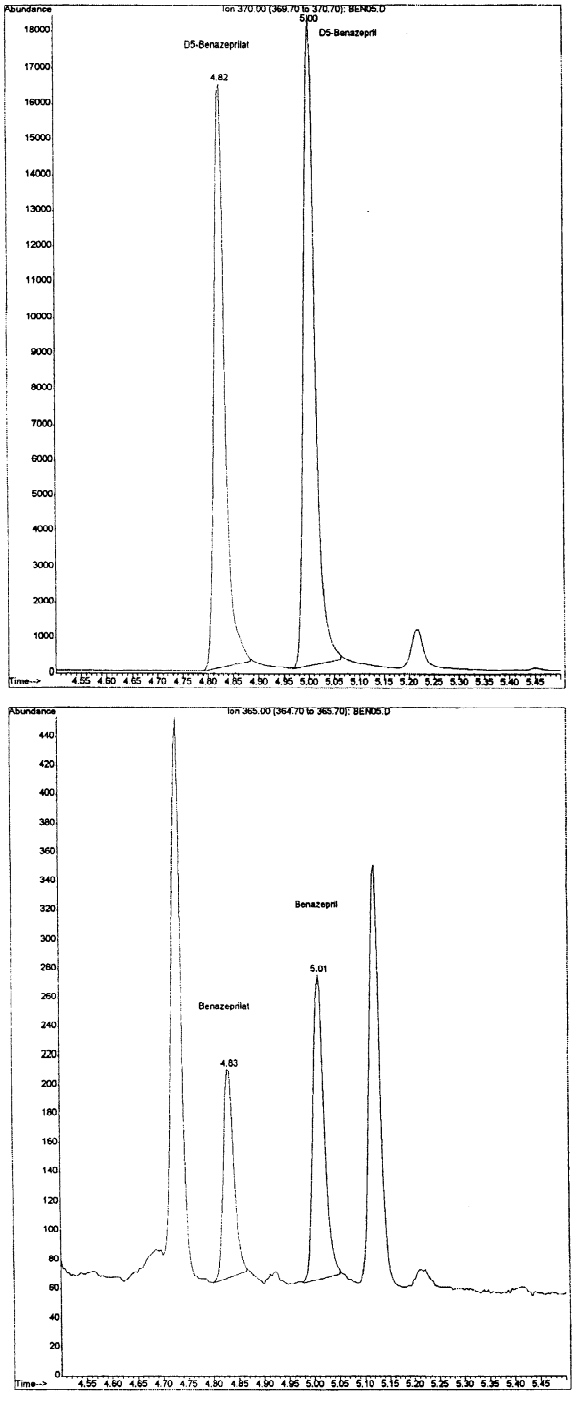

Fig. 3. Examples of selected ion current profiles: an extract of 0.5 mL plasma spiked with 1.25 ng (LLOQ) of benazepril and benazeprilat and 150 ng of labelled internal standards.

yields, they were reproducible and independent of the concentration tested. The use of a labelled I.S. with the same extraction yield avoids nonreproducible extraction yields between benazepril and I.S.

3.5. Calibration curves

Daily calibration standards were prepared at six different concentrations, in duplicate, in the range 2.50–1000 ng/mL for benazepril and benazeprilat. Calibration curves ($y = ax + b$) were represented by plots of the peak area ratios (y) of the methyl ester derivative of benazepril or benazeprilat to the methyl ester derivative of the I.S. versus the concentration (x) of the calibration standards, and were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model (Fig. 4). Concentrations in quality control samples were calculated from the

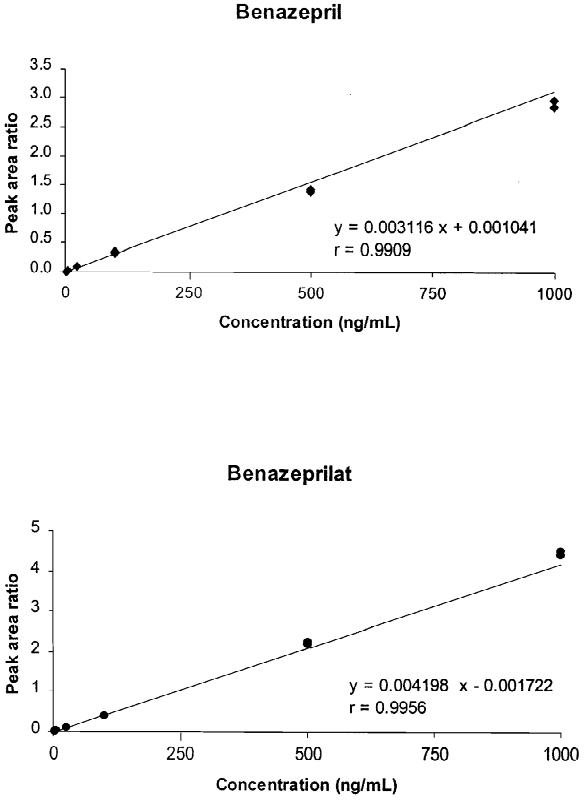

Fig. 4. Representative calibration curves for the determination of benazepril and its active metabolite, benazeprilat, in human plasma.

Table 1
Back-calculated concentrations of calibration curves

Compound	Nominal conc. (ng/mL)	Mean accuracy[a] ($n=6$) (%)	Precision RSD[b] (%)
Benazepril	2.50	97.7	7.4
	5.00	103	4.4
	25.0	108	4.0
	100	106	4.4
	500	93.8	4.8
	1000	93.6	3.4
Benazeprilat	2.50	103	3.6
	5.00	94.7	2.6
	25.0	95.7	3.2
	100	95.9	2.5
	500	105	2.1
	1000	104	3.9

[a] Accuracy: determined concentration expressed as a percentage of the nominal concentration.
[b] RSD, relative standard deviation (%).

resulting peak area ratios and the regression equation of the calibration curve.

Inter-day repeatability was determined on three different days. Good agreement between the nominal and the back-calculated concentration for calibration samples was observed. Inter-day variability is presented in Table 1. The precision ranged from 2.1 to 7.4% and mean accuracies were within 8% of the nominal value for the two compounds.

3.6. Accuracy and precision

The accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest (2.50 ng/mL), near the lowest (7.50 ng/mL), near the middle (200 ng/mL) and the highest (900 ng/mL) concentrations of the calibration range. Accuracy was determined by calculating the mean recovery for the determined concentrations as a percentage of the nominal concentrations in standard samples. Precision was assessed from the relative standard deviation (RSD) as a percentage of the mean recovery. The following validation criteria for accuracy and precision were used to assess method suitability: mean recoveries should be within 85–115%, except at the LLOQ, where it should be within 80–120%; RSD should not exceed 15%, except at the LLOQ, where it should not exceed 20% [2,3].

Series of five quality control samples were prepared at four different concentrations in the range 2.50–900 ng/mL for benazepril and benazeprilat, by spiking drug-free plasma with the corresponding working solutions. As shown in Table 2, the results met the acceptance criteria.

3.7. Lower limit of quantitation (LLOQ)

The analyte response at the LLOQ should be at least five times the response of the blank. The LLOQ is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability. As indicated in the previous section, the mean recovery should be within 80–120% of the expected value with a RSD not exceeding 20%. The lowest concentration of 2.50

Table 2
Intra- and inter-day accuracy and precision of the method

Measurement	Given (ng/mL)	Benazepril		Benazeprilat	
		Mean accuracy[a] ($n=5$) (%)	Precision RSD[b] (%)	Mean accuracy ($n=5$) (%)	Precision RSD (%)
Intra-day	2.50	106	2.1	106	2.1
	7.50	111	2.7	111	2.7
	250	95.7	3.6	95.7	3.6
	900	95.3	1.9	95.3	1.9
Inter-day	2.50	103	3.5	103	3.5
	7.50	107	5.1	107	5.1
	250	95.5	2.5	95.5	2.5
	900	93.6	2.0	93.6	2.0

[a] Accuracy: determined concentration expressed as a percentage of the nominal concentration.
[b] RSD, relative standard deviation.

ng/mL, the accuracy and precision of which (Table 1) were within the proposed criteria, is quoted as the LLOQ.

3.8. Stability

Stock solutions were found to be stable for at least 40 days at +5 °C. The stability of benazepril and benazeprilat was investigated by analyzing quality control human samples and reconstituted extracts, which were stored under varying conditions, in triplicate at low (7.50 ng/mL) and high concentration (900 ng/mL), together with freshly prepared C standards and QC samples. At or below −18 °C, in frozen human plasma (spiked samples), benazepril and benazeprilat were found to be stable for at least 3 months. Extracts were found to be stable on the autosampler at room temperature for at least 24 h. Benazepril and benazeprilat were found to be stable in human plasma after three freeze–thaw cycles (two different thawing methods: one night at about +5 °C or 20 min at about +37 °C).

3.9. Automation and application of the method

Automation of the 96-well disk plate system can be as simple as positioning the plate and vacuum manifold on the deck of the Packard MultiProbe II. The typical time for sample extraction of a 96-well plate is about 45 min, without taking into account derivatization and the re-extraction time.

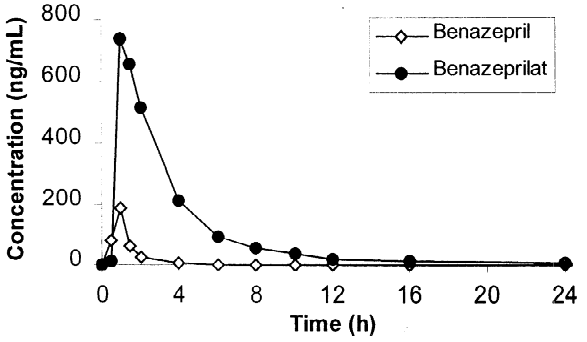

Fig. 5. Plasma concentrations of benazepril and benazeprilat after a single oral dose of 20 mg benazepril as a capsule to one subject.

Compared with the previous method [2], the volume of plasma required for the assay is halved without modifying the LLOQ of the two compounds. The volumes of solvent required for conditioning, washing and eluting were considerably smaller.

The 96-well disk plate adaptable to the Packard MultiProbe II allows the elimination of manual pipetting steps. Automation reduces human error and improves precision and accuracy.

The method was applied to a clinical trial where benazepril and benazeprilat were determined in 1080 samples. Fig. 5 shows the plasma concentration profile obtained for one healthy subject after administration of a 20 mg benazepril capsule.

4. Conclusions

A GC–MS technique has been developed and validated for quantifying benazepril and its active metabolite, benazeprilat, in human plasma in the range 2.50–1000 ng/mL according to internationally accepted criteria. The procedure has a good throughput, with a combination of automated sample extraction with a 96-well disk plate using a Packard MultiProbe II and derivatization with a new methylation reagent, trimethylsilyldiazomethane, avoiding the daily preparation of diazomethane.

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