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Short communication

Assay of zofenopril and its active metabolite zofenoprilat by liquid chromatography coupled with tandem mass spectrometry

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Abstract

Zofenopril is a pro-drug designed to undergo metabolic hydrolysis yielding the active free sulfhydryl compound zofenoprilat, which is an angiotensin converting enzyme (ACE) inhibitor, endowed also with a marked cardioprotective activity. A simple, highly sensitive specific LC–MS–MS method was developed for the determination of zofenopril and zofenoprilat in human plasma. In order to prevent oxidative degradation of zofenoprilat and its internal standard, their free sulfhydryl groups were protected by treatment with *N*-ethylmaleimide (NEM), which produced the succinimide derivatives. The compounds and their corresponding fluorine derivatives, used as internal standards, were extracted from plasma with toluene. The reconstituted dried extracts were chromatographed and then monitored by a triple-stage-quadrupole instrument operating in the negative ion spray ionization mode. The method was validated over the concentration range of 1–300 ng/ml for zofenopril and 2–600 ng/ml for zofenoprilat. Inter- and intra-assay precision and accuracy of both zofenopril and zofenoprilat were better than 10%. The limit of quantitation was 1 ng/ml with zofenopril and 2 ng/ml with zofenoprilat. Extraction recovery proved to be on average 84.8% with zofenopril and 70.1% with zofenoprilat. Similar recoveries were shown by the above two internal standards. The method was applied to measure plasma concentrations of zofenopril and zofenoprilat in 18 healthy volunteers treated orally with zofenopril calcium salt at the dose of 60 mg.

Keywords: Zofenopril; Zofenoprilat

1. Introduction

In 1960 some scientists observed that a peptide family contained in the venom of pit vipers was able to inhibit both the synthesis of angiotensin II and the catabolism of bradykinin [1]. Non-peptide substances were then synthesised to mime the above activity, generating the class of angiotensin converting enzyme (ACE) inhibitors [2].

Zofenopril is a new ACE inhibitor, recently registered. Zofenopril, like most compounds of this class, in the body is hydrolysed to the active metabolite zofenoprilat, which contains a free thiolic group [3,4].

Several methods have been employed to assay both parent compounds and active metabolites of ACE inhibitors, including gas chromatography, HPLC, both requiring derivatization, and RIA [3]. Jemal et al. [5] have assayed zofenopril and zofenop-

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rilat with a relatively complex gas chromatographic method with single quadrupole mass-selective detection.

The aim of this paper is to describe a new, reliable and very sensitive LC–MS–MS method for the assay of zofenopril and zofenoprilat, which was validated in human plasma and does not require any chemical derivatization, other than the required chemical stabilization of the thiolic group of zofenoprilat and its internal standard.

2. Materials and methods

2.1. Chemicals

Zofenopril (I) calcium salt, zofenoprilat (II) dicyclohexylamine salt and the corresponding fluorine derivative analogue internal standards (III, IV) (Fig. 1) were kindly supplied by Menarini Industrie Farmaceutiche Riunite, Florence, Italy.

Solvents and reagents of analytical or HPLC grade, were supplied by BDH (Poole, UK), Carlo Erba (Milan, Italy), E. Merck (Darmstadt, Germany) and Fluka Chemie (Buchs, Switzerland).

HPLC grade water was produced with a Milli-Q apparatus (Bedford, MA, USA). Gases for triple–quadrupole were produced by nitrogen and air generators (N_2 purity 99.999, air dew point −60°C) manufactured by Claind (Lenno, Italy).

N-Ethylsuccinimide derivatives of salified zofenoprilat (V) and its internal standard (VI) were obtained by treating 7 mg of II and IV with 1.75 ml of *N*-ethylmaleimide, NEM, (25 mg/ml in 0.06 *M* phosphate buffer pH 7) in a 10 ml volumetric flask. After 1 h in the dark at room temperature acetone was added into the above flask to reach the 10 ml volume, corresponding to a concentration of 450 μg/ml of II and 460 μg/ml of IV, as free acids.

2.2. Standard solutions

Standard stock solutions of I (500 μg/ml as free acid) and III (100 μg/ml) were prepared in methanol. The preparation of acetonic standard stock solutions of V and VI is described above.

Working standard solutions containing both I and V were obtained by subsequent appropriate dilutions in methanol.

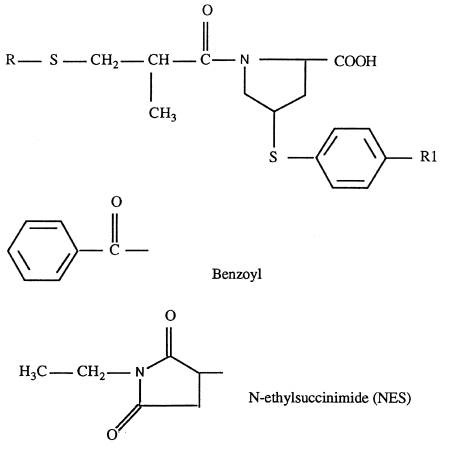
Working solutions containing both the internal standards were obtained by diluting aliquot parts of stock solutions with methanol (10 μg/ml for III, 15 μg/ml for VI).

20 μl of working standard solutions were added to 1 ml blank human plasma to prepare the calibration sample.

Quality control (QC) samples were prepared by adding to 1 ml of blank human plasma, 20 μl of standard solutions originated from separately weighed and diluted standards I and V.

2.3. Extraction procedure

The extraction procedure was that adopted by Jemal et al. [5] with some modifications as follows. One ml of plasma, 20 μl of the working solution of the internal standards, 1 ml of 6 *N* H_3PO_4–2% tetrabutylammonium hydrogen sulphate and 7 ml of toluene were placed into a 12 ml screw-cap PTFE lined tube. The tube was shaken for 15 min on a rotating mixer at 32 rpm and then centrifuged at 1500 *g* for 5 min.



Compound	R	R1	M.W. (DA)	Q1 Parent ion (m/z)	Q3 Product ion (m/z)
I Zofenopril	benzoyl	H	429	428	137
II Zofenoprilat	H	H	325	-	-
III Zofenopril I.S.	benzoyl	F	447	446	137
IV Zofenoprilat I.S.	H	F	343	-	-
V II-NES	NES	H	450	449	290
VI IV-NES	NES	F	468	467	308

Fig. 1. Chemical structures and ions monitored of the analytes.

The organic phase was separated and evaporated to dryness under a gentle stream of nitrogen at 60°C. The residue was dissolved with 200 μl of methanol:water (50:50 v/v) and transferred into an autosampler vial.

2.4. Chromatographic conditions

HPLC was performed using a ''series 200 Micro Pump'' solvent delivery system and a ''series 200 Autosampler'' from Perkin-Elmer (Foster City, CA, USA). The analytical column was a Luna C18 (75× 4.6 mm I.D., 3 μm, Phenomenex, Torrance, CA, USA) preceded by a 5 μm C18 guard column (10× 3.2 mm I.D., Hichrom Ltd., Theale, UK).

The compounds were eluted using acetonitrile and 26 m*M* ammonium acetate (adjusted to pH 4.5 with glacial acetic acid) as mobile phases according to the following gradient program: the acetonitrile concentration was maintained at 20% for 0.5 min, then increased to 80% in 2.5 min and remained constant for 3 min, followed by reequilibration to initial condition for 3 min. The flow-rate was 0.4 ml/min and each run time was 9 min.

2.5. Mass spectrometry conditions

The HPLC was coupled to a Perkin-Elmer SCIEX API 365 triple–quadrupole mass spectrometer (Foster City, CA, USA) equipped with a Turbo Ion Spray source. Probe temperature was set at 450°C with nitrogen as curtain gas (position 12) and nebulizer gas (position 10). Air was used as an auxiliary gas. Samples were analyzed in the negative ion mode with operative voltages for curtain plate orifice and the ring of −35 and −200 V respectively.

Multiple reaction monitoring (MRM) detection was employed using nitrogen as the collision gas (position 2) with a dwell time of 300 ms for each transition. The mass spectrometer operated with unit resolution for both Q1 and Q3 (e.g., 0.7 Da at 50% height).

Data were acquired and processed using the PE Sciex software package MacQuan (version 1.6).

Peak area ratios obtained from MRM of precursor to product ion transitions for I, V and the corresponding internal standards, were utilized for the construction of calibration curves using weighted ($1/C^2$) linear least square regression of the plasma concentrations and the measured area ratios.

2.6. Method validation

Quantitation was achieved from seven point calibration curves covering the range 1–300 ng/ml for I and 2–600 ng/ml for II, as detailed in Table 1.

Quality control (QC) samples for determining intra- and inter-assay precision and accuracy were prepared at three concentration levels 2.5, 25 and 250 ng/ml for I and 5, 50 and 500 ng/ml for II.

The stability of I and II in QC samples was investigated after storage for 24 h at room tempera-

Table 1
Standard curve statistics of zofenopril and zofenoprilat Mean values of five findings

Zofenopril	Nominal concentrations (ng/ml)							Calibration curve parameters		
	300	200	60	20	6	2	1	Slope $\times 10^{-3}$	Intercept $\times 10^{-3}$	r
	Back-calculated concentrations (ng/ml)									
Mean	283.39	192.37	60.64	20.99	6.37	1.93	1.01	6.7841	0.3895	0.9984
SD	2.463	3.267	1.269	0.155	0.083	0.089	0.025	0.033	–	–
Precision (C.V.%)	0.9	1.7	2.1	0.7	1.3	4.6	2.5	0.49	–	–
Accuracy (%)	−5.5	−3.8	+1.1	+4.9	+6.2	−3.5	+1.0	–	–	–
Zofenoprilat	Nominal concentrations (ng/ml)							Calibration curve parameters		
	600	400	120	40	12	4	2	Slope $\times 10^{-3}$	Intercept $\times 10^{-3}$	r
	Back-calculated concentrations (ng/ml)									
Mean	576.87	381.92	119.66	41.88	12.74	3.92	2.00	4.4037	0.6903	0.9985
SD	8.279	8.238	2.211	0.885	0.246	0.197	0.052	0.114	–	–
Precision (C.V.%)	1.4	2.2	1.8	2.1	1.9	5.0	2.6	2.59	–	–
Accuracy (%)	−3.9	−4.5	−0.3	+4.7	+6.2	−2.0	0.0	–	–	–

ture, after three freeze–thaw cycles and in reconstituted samples left for at least 24 h at room temperature on an autosampler.

The recovery of I, II and internal standards was calculated by comparing the analytical results for extracted QC samples with samples at the same analyte concentrations obtained by spiking extracted blank plasma samples with analyte working standard solutions.

3. Results

3.1. LC–MS–MS

The negative ion mass spectra (Q1) of the analytes indicated the predominant presence of the deprotonated molecules $[M-H]^-$ of these compounds (Fig. 1).

For I and its internal standard (III) the MS–MS product mass spectra (Q3) of deprotonated molecules showed the presence of intense product ion at m/z 137 for both the compounds that corresponds to the *S*-benzoyl group fragment.

The Q3 mass spectra of V and its internal standard (VI) showed predominant product ions at 290 m/z and 308 m/z respectively, most likely resulted by the loss of S-NES group from the corresponding deprotonated parent molecules.

By monitoring the parent→product ion pairs at m/z 428→137 for I, 446→137 for III, 449→290 for V and 467→308 for VI in the multi reaction monitoring mode, highly sensitive assay for I and II was developed.

Under the reported chromatographic conditions I and III are eluted at about 6 and 5.3 min and are not separated from their corresponding internal standards.

Fig. 2A and B depicts typical LC–MS–MS chromatograms of extracted human plasma standard samples both blank and spiked with the analytes at the LOQ.

3.2. Calibration curve

Table 1 shows the back-calculated concentrations of I (range 1–300 ng/ml) and II (range 2–600 ng/ml) and the parameters obtained from the corresponding calibration curves. The mean accuracy (%) of back-calculated concentrations ranged from −5.5% to +6.2% and the precision was ≤4.6% for I. With II, accuracy ranged from −4.5% to +6.2% and precision was ≤5.0%. The mean coefficient of correlation (r) was 0.9984 with I and 0.9985 with II.

3.3. Intra- and inter-assay precision and accuracy

Intra-assay precision was ≤5.1 and ≤2.4% with I and II, respectively. Accuracy ranged from −4.0% to +8.0% and from −3.9% to +8.4% with the two analytes in the same above order.

Inter-assay data were as follows: for I the precision was ≤3.7% and accuracy ranged from −4.6% to +10.0%.

For II the precision was ≤3.9% and the accuracy ranged from −3.3% to +6.6%.

3.4. Limit of quantitation

The limit of quantitation (LOQ) proved to be as low as 1 ng/ml for I and 2 ng/ml for II. At these concentrations the precision and the accuracy were 7.3% and −2.0% for I and 6.9% and +0.2% for II, respectively.

The limit of detection was 50 pg/ml for I and 100 pg/ml for II, evaluated considering a signal-to-noise ratio of 3.

3.5. Specificity

No significant interfering peaks were found at the retention times of I, II and their internal standards in any extracts of control plasma and in human study pre-dose samples.

3.6. Stability

No significant change was detected in I and II concentrations in extracted plasma samples, maintained in final solution and at room temperature for at least 24 h in autosampler vials prior to injection. Similarly no significant change was detected in I and II concentrations in plasma stored at room temperature for 24 h and after three freeze–thaw cycles.

(A)

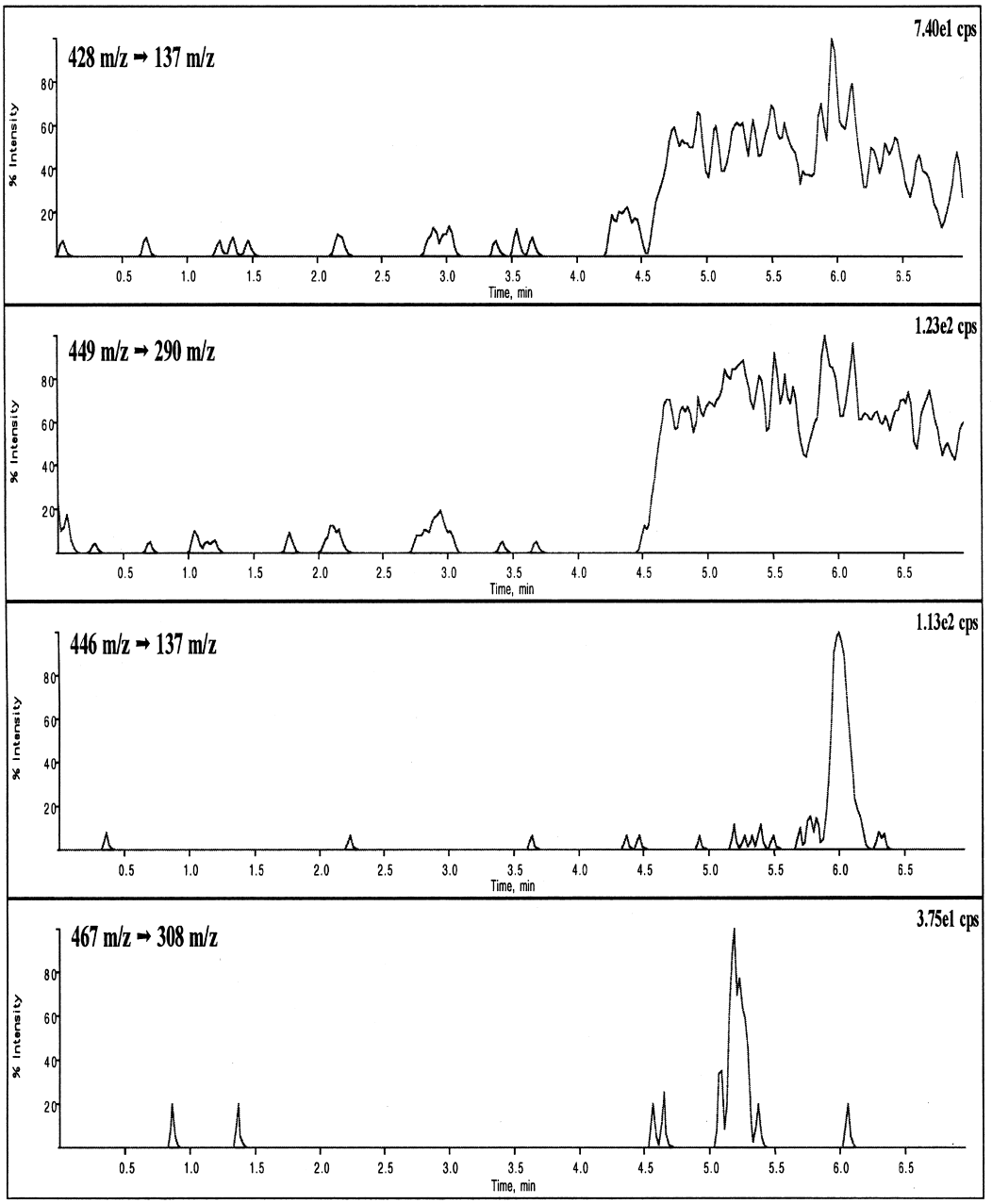


Fig. 2. (A) Chromatograms of blank human plasma sample. Traces from top to bottom: zofenopril, zofenoprilat–NES, zofenopril I.S., zofenoprilat–NES I.S. (B) Chromatograms of human plasma sample at the LOQ. Traces from top to bottom: zofenopril (1 ng/ml), zofenoprilat–NES (2 ng/ml), zofenopril I.S. (200 ng/ml), zofenoprilat–NES I.S. (300 ng/ml).

(B)

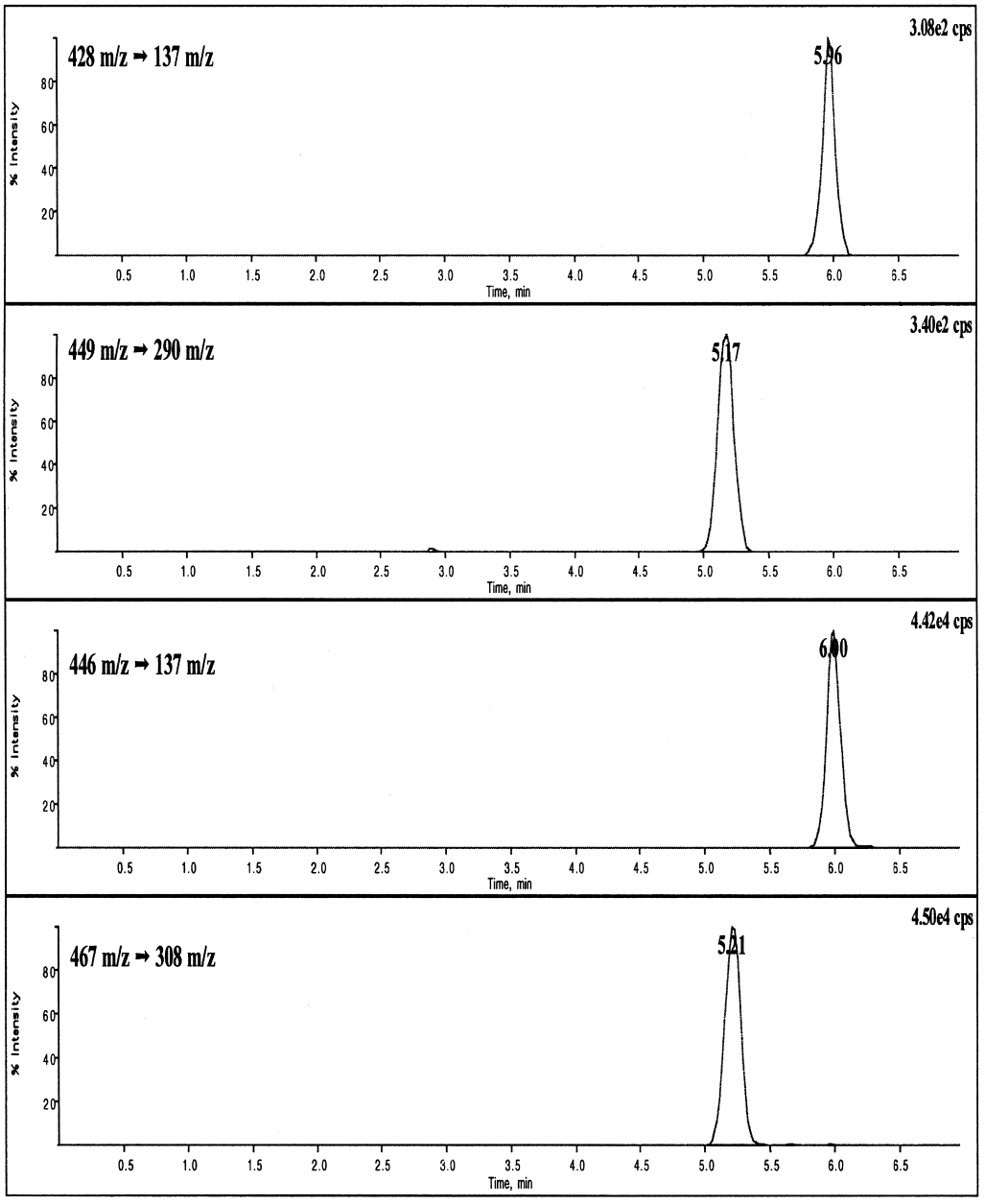


Fig. 2. (*continued*)

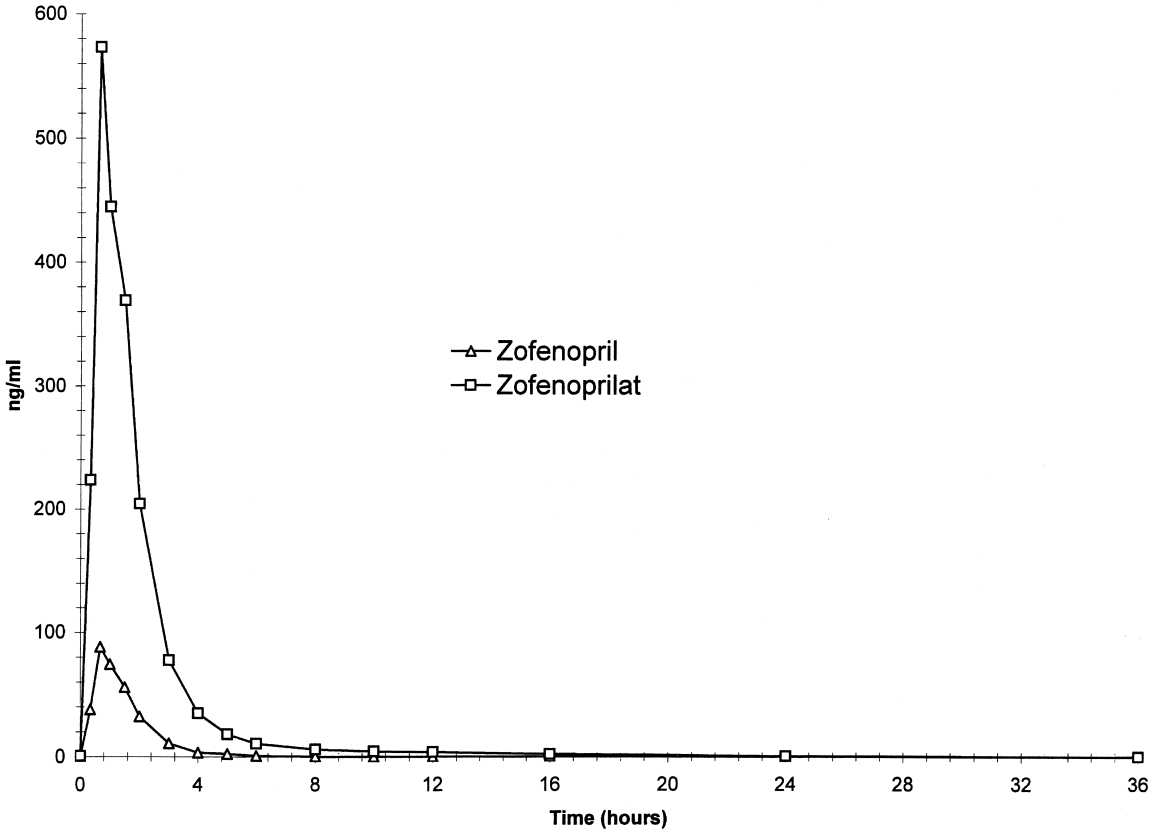


Fig. 3. Plasma concentration-time curves of zofenopril and zofenoprilat after treatment with 60 mg of zofenopril calcium salt. Mean values of 18 findings.

3.7. Recovery

The recovery in the investigated concentration range was on average 84.8% for I with a C.V.% ranging from 0 to 1.2 and 70.1% for II with a C.V.% ranging from 0.8 to 4.4.

The extraction recovery of III was 84.9% with a C.V.% of 1.3 at the concentration of 200 ng/ml. The extraction recovery of VI was 71.5% with a C.V.% of 1.0 at the concentration of 300 ng/ml.

3.8. Pharmacokinetic investigation in healthy volunteers

The investigation was carried out on 18 Caucasian healthy volunteers of both sexes aging from 19 to 52 years. Subjective and objective examinations including vital signs, blood analysis and complete urine analysis were performed to determine the volunteers' general health conditions. The same examinations were repeated at the end of the trial. The study protocol was approved by the Ethics Committee of Canton Ticino (Switzerland) and volunteers provided written informed consent prior to trial initiation.

Volunteers were administered a 60 mg single oral dose of zofenopril calcium salt in tablets, following an overnight fast. The tablet was swallowed with 250 ml of non gaseous mineral water. Volunteers were not allowed to drink any other liquid starting from 1 h before to 2h after dosing. Fasting continued for 4 h after administration.

Venous blood was sampled at 0, 30, 45 min, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24 and 36 h.

Each sample volume was 10 ml and was collected, by indwelling catheter, into heparinised test tubes containing 20 mg of NEM. After centrifugation

resulting plasma samples were stored in freezer at −20°C pending assay.

Fig. 3 shows the mean plasma concentration–time behaviour of both zofenopril and zofenoprilat. As expected, concentrations of zofenoprilat prevailed over those of the parent compound [6].

4. Discussion

Both HPLC and GC assays of ACE inhibitors present a peculiar difficulty, as they do not possess adequate chromophore groups for detection and thus they need to be derivatized [3]. This has justified the proliferation of RIA assays of these compounds. The RIA and mainly HPLC and GC assays of ACE inhibitors are complicated and time-consuming.

Our method requires a liquid–liquid extraction of the analytes, followed by the concentration of the extract and its injection. The method of Jemal et al. [5] requires a liquid–liquid extraction, followed by an SPE clean-up on a C18 reverse phase column and a further derivatisation procedure before injection into GC–MS.

The method described in this paper is routinely used in our laboratory to assay thousands of samples related to pivotal pharmacokinetic trials of zofenopril.

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References

[1] S.H. Ferreira, Br. J. Pharmacol. Chemother. 24 (1965) 164.
[2] D.W. Cushman, F.L. Wang, W.C. Fung et al., Br. J. Clin. Pharmacol. 28 (1989) 115S.
[3] J.G. Kelly, K. O'Malley, Clin. Pharmacokinet. 19 (1990) 177.
[4] X. Liu, R.M. Engelman, J.A. Rousou et al., Cardiov. Drugs Ther. 6 (1992) 437.
[5] M. Jemal, E. Ivashkiv, D. Teitz, A.I. Cohen, J. Chromatogr. 428 (1988) 81.
[6] A. Marzo, L. Dal Bo, P. Mazzucchelli et al., Arzneim. Forsch. 49 (1999) 992.