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Liquid chromatographic–electrospray tandem mass spectrometric method for the simultaneous quantitation of the prodrug fosinopril and the active drug fosinoprilat in human serum

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Abstract

A sensitive, specific, accurate and reproducible LC–MS–MS method was developed and validated for the simultaneous quantitation of the prodrug fosinopril and its active drug fosinoprilat in human serum. The method employed acidification of the serum samples to minimize the hydrolysis of fosinopril to fosinoprilat prior to purification by solid-phase extraction to isolate the two analytes and the two internal standards from human serum. The extracted samples were analyzed by turbo ionspray LC–MS–MS in the positive ion mode. Chromatography was performed on a polymer-based C_{18} column (Asahipak™ ODP PVA-C_{18}, 2×50 mm) using gradient elution with methanol and 10 m*M* ammonium acetate, pH 5.5. The calibration curve, 1.17 to 300 ng/ml, was fitted to a weighted ($1/x$) linear regression model. Serum quality control (QC) samples used to gauge the accuracy and precision of the method were prepared at concentrations of 5.00, 100, 250 and 500 ng/ml of each analyte. The inter-assay accuracies were within 6% (DEV) for both analytes. The intra- and inter-assay precisions were within 7% and 11% (RSD), respectively, for both analytes. The hydrolysis of fosinopril to fosinoprilat during sample processing was ≤6%. This degree of conversion would cause little error in the analysis of post-dose serum samples since such samples are known to contain low levels of the prodrug compared to the drug.

Keywords: Fosinopril; Fosinoprilat

1. Introduction

Fosinopril (Fig. 1), an anti-hypertensive agent belonging to the class of angiotensin-converting enzyme (ACE) inhibitors, has a dual and compensatory route of excretion [1–5]. Fosinopril is a phosphinic acid ester prodrug which undergoes in vivo hydrolysis to the active drug, fosinoprilat (Fig. 1). We report in this paper an LC–MS–MS method developed and validated for the simultaneous quantitation of fosinopril and fosinoprilat in human serum. The previously published methods, which were based on radioenzymatic assay [6], GC with nitrogen–phosphorus detection [7] and radioimmunoassay

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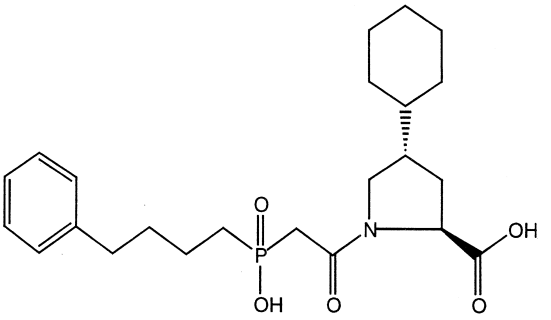
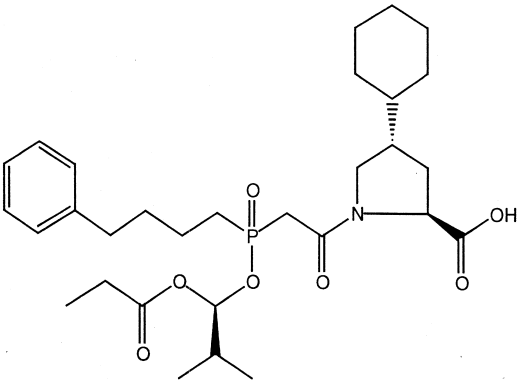


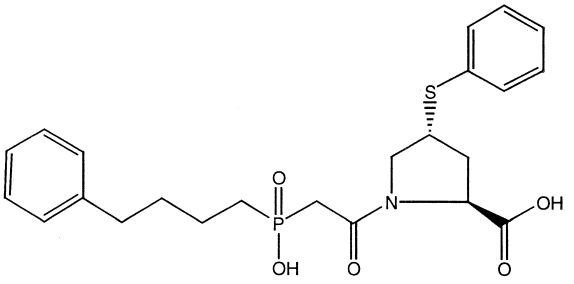
$C_{23}H_{34}NO_5P$
Exact Mass: 435.22
Mol. Wt.: 435.49
C, 63.43; H, 7.87; N, 3.22; O, 18.37; P, 7.11

Fosinoprilat



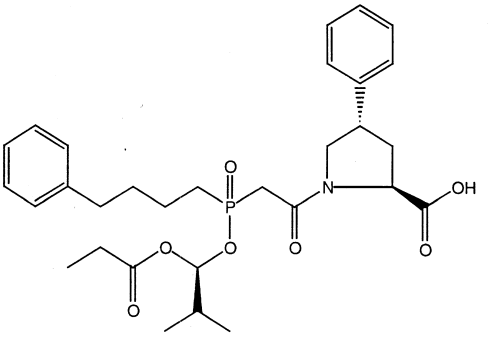
$C_{30}H_{46}NO_7P$
Exact Mass: 563.30
Mol. Wt.: 563.66
C, 63.92; H, 8.23; N, 2.48; O, 19.87; P, 5.50

Fosinopril



$C_{23}H_{28}NO_5PS$
Exact Mass: 461.14
Mol. Wt.: 461.51
C, 59.86; H, 6.12; N, 3.03; O, 17.33; P, 6.71; S, 6.95

SQ-SQ-27133 (int std for fosinoprilat)



$C_{30}H_{40}NO_7P$
Exact Mass: 557.25
Mol. Wt.: 557.61
C, 64.62; H, 7.23; N, 2.51; O, 20.08; P, 5.55

SQ-33055 (int std for fosinopril)

Fig. 1. Chemical structures of fosinoprilat, fosinopril and the two internal standards.

[8], were capable of only measuring fosinoprilat. To measure fosinopril by these methods, the prodrug had to be hydrolyzed to fosinoprilat under alkaline conditions.

2. Experimental

2.1. Chemicals and reagents

Fosinopril, fosinoprilat, SQ-33055 (internal standard for fosinopril), and SQ-27133 (internal standard for fosinoprilat) were characterized products obtained from Bristol-Myers Squibb Pharmaceutical Research Institute. Control human serum was purchased from Biological Specialty (Colmar, PA, USA). Acetonitrile and methanol were from Baxter/Scientific Products (McGaw Park, IL, USA). Ammonium acetate was from J.T. Baker (Danvers, MA, USA) and high purity (NANOpure) water was generated from a Barnstead Model D733 1 Ultrapure Water System (Dubuque, IA, USA). Phosphoric acid was from EM Industries (Gibbstown, NJ, USA) and Prosil-28 was from PCR (Gainsville, FL, USA). Ethanol was obtained from Quantum Chemical (Tuscola, IL, USA).

A solution of 10 m*M* ammonium acetate, pH 4.6, was prepared by dissolving 770 mg of ammonium acetate in 1000 ml of water and adjusting the pH to 4.6 using glacial acetic acid. HPLC eluent A (75:25 water:methanol containing 10 m*M* ammonium acetate), pH 5.5 was prepared by dissolving 770 mg of ammonium acetate in 750 ml of water and 250 ml of methanol. The pH was adjusted to 5.5 with glacial acetic acid. HPLC eluent B (10 m*M* ammonium acetate in methanol) was prepared by dissolving 770 mg of ammonium acetate in 1000 ml of methanol. The extract reconstitution solution consisted of 7:3 eluents A and B. A 1.0 N stock solution of phosphoric acid was prepared by diluting with water, 23 ml of phosphoric acid to 1 l. The 0.1 N and 0.2 N solutions were prepared by diluting 100 and 200 ml, respectively, of the 1.0 N stock to 1 l using water. Prosil-28 working solution was prepared by diluting 10 ml of Prosil-28 concentrate to 1 l with water. Glass scintillation vials (20 ml) were silanized by immersion in the Prosil-28 working solution for 1 min. The vials were then removed and rinsed with water several times and allowed to air-dry. Note: Initially the reference standard stock solutions (Section 2.3) were prepared in silanized glass vials; however, it was later found that polypropylene containers were also acceptable.

2.2. Materials and equipment

Bond-Elut® cyclohexyl SPE columns (200 mg, 3 ml) were purchased from Varian Associates (Harbor City, CA, USA). A RT-6000D refrigerated centrifuge from Du Pont (Wilmington, DE, USA) and TurboVap LV evaporator from Zymark (Hopkinton, MA, USA) were used. The autosampler vials consisted of 1-ml glass 8×40 mm vials, polypropylene 200-μl inserts, springs for inserts and polyethylene stoppers, purchased from Sun Brokers (Wilmington, NC, USA). Screw-cap polypropylene tubes were purchased from Sarstedt (Newton, NC, USA). Twenty-ml glass scintillation vials were from Kimble Glass (Vineland, NJ, USA). The HPLC column used was a polyvinyl alcohol copolymer bonded C_{18} column (Asahipak™ ODP PVA-C_{18}, 5 μm, 2×50 mm) from Keystone Scientific (Bellefonte, PA, USA). Precolumn solvent filters, PEEK frits for precolumn filters, and PEEK tubing were from Upchurch Scientific (Oak Harbor, WA, USA). Liquid nitrogen and argon (U.H.P) were obtained from Empire Airgas (Elmira, NY, USA). The autosampler used was Waters 717+ autosampler (Millipore, Milford, MA, USA). Shimadzu LC-10AD pumps (Columbia, MD, USA) were used in tandem for gradient elution. The mass spectrometer was a PE-SCIEX API III^+ triple quadrupole mass spectrometer from PE-SCIEX (Concord, Ontario). The data system used was API Standard Software, v2.5, run on a Macintosh Quadra 800.

2.3. Preparation of standard and quality control samples

Stock solution #1, fosinopril (1 mg/ml), was prepared by dissolving an accurately weighed amount (3–5 mg) of fosinopril in a silanized 20-ml glass scintillation vial in a sufficient amount of ethanol to yield a 1 mg/ml solution. Stock solution #2 (fosinoprilat, 1 mg/ml), stock solution #3 (SQ-27133, 1 mg/ml), and stock solution #4 (SQ-33055, 1 mg/ml) were prepared similarly by dissolving accurately weighed amounts (3–5 mg) of fosinoprilat, SQ-27133 and SQ-33055, respectively, in etha-

nol. A combined working solution (fosinopril and fosinoprilat, 50 μg/ml each) was prepared in a silanized 20-ml glass scintillation vial by combining 0.5 ml of stock solution #1, 0.5 ml of stock solution #2 and 9.0 ml of ethanol. Fosinopril working solution (50 μg/ml) was prepared in a silanized 20-ml glass scintillation vial by combining 0.5 ml of stock solution #1 and 9.5 ml of ethanol. Fosinoprilat working solution (50 μg/ml) was prepared in a silanized 20-ml glass scintillation vial by combining 0.5 ml of stock solution #2 and 9.5 ml of ethanol. Quality control (QC) samples were prepared using working solutions prepared from stocks other than those used for the preparation of the standard curve working solutions. The internal standard working solution (SQ-27133 and SQ-33055, 1.5 μg/ml each) was prepared in a 100-ml volumetric flask by diluting 0.150 ml of stock solution #3 and 0.15 ml of stock solution #4 to 100 ml with ethanol.

The calibration curve consisted of nine serum standards. Standard 9 (300 ng/ml of fosinopril and fosinoprilat) was prepared in a polypropylene tube by adding 60 μl of the combined standard working solution to 10 ml of serum. Standards 1 to 8 were prepared by diluting, in a serial fashion, 2.5 ml of the next higher standard with 2.5 ml of human serum. This resulted in serum standards with concentrations of 1.17, 2.34, 4.69, 9.38, 18.75, 37.5, 75.0, and 150.0 ng/ml. QC-4 (500 ng/ml, dilution QC), QC-3 (250 ng/ml) and QC-2 (100 ng/ml) were prepared in volumetric flasks by diluting the specified amounts of the combined QC working solution to 50 ml with human serum. QC-1 (5 ng/ml) was prepared in a volumetric flask by diluting 1.0 ml of QC-3 to 50.0 ml with human serum. Fosinopril-only and fosinoprilat-only QC samples were also prepared to monitor in-process stability. QC-8 (250 ng/ml of fosinoprilat) was prepared in a volumetric flask by diluting 125 μl of fosinoprilat QC working solution (50 μg/ml) to 25.0 ml with human serum. QC-7 (250 ng/ml of fosinopril) was prepared in a volumetric flask by diluting 125 μl of fosinopril QC working solution (50 μg/ml) to 25.0 ml with human serum. QC-6 (5 ng/ml of fosinoprilat) was prepared in a volumetric flask by diluting 500 μl of QC-8 to 25.0 ml with human serum. QC-5 (5 ng/ml of fosinopril) was prepared in a volumetric flask by diluting 500 μl of QC-7 to 25.0 ml with human serum.

2.4. Sample extraction

Two ml of 0.2 N phosphoric acid was added to a 13×100 mm polypropylene tube and placed in an ice-water bath. Immediately prior to extraction, serum sample (1 ml) was added to the tube followed by the internal standard working solution and the tubes were vortexed. The cyclohexyl SPE cartridges were conditioned with 3 ml of methanol followed by 3 ml of 0.1 N phosphoric acid. The diluted samples were added to the cartridge and aspirated through the cartridge at a low flow-rate (approx. 1–2 ml/min). The cartridge bed was washed with 3 ml of 0.1 N phosphoric acid and 3 ml of 10 m*M* ammonium acetate, pH 4.6 and dried under high vacuum. The extracted components were eluted into 13×100 mm polypropylene tubes using 1.5 ml of 10 m*M* ammonium acetate in methanol at a low flow-rate (approx. 1–2 ml/min). The eluent was evaporated to dryness under nitrogen at 40°C. The dry extract was reconstituted in 75 μl of reconstitution solution, centrifuged and transferred to the autosampler vials. Note: instead of pipetting all samples to be extracted into the 0.2 N phosphoric acid, followed by addition of internal standard and extraction, only the samples within an extraction set were pipetted into the 0.2 N phosphoric acid, followed by internal standard and extraction. The remaining samples were stored in the refrigerator after completely thawing, and removed only prior to extraction. This procedure has been found to stabilize fosinoprilat and had no effect upon fosinopril.

2.5. Chromatographic and mass spectrometric conditions

A 2×50 mm Asahipak™ ODP PVA-C_{18} HPLC column was used in conjunction with a spacer (in place of guard cartridge) and in-line PEEK frit. The column was maintained at ambient temperature, the flow-rate was 200 μl/min and 5 μl of the sample was injected. A gradient elution was used by on-line mixing of HPLC eluents A and B (section 2.1) in the following manner: at time 0 min 30% B, at time 1.0 min 95% B, at time 3.0 min 95% B, and at time 5.0 min 30% B. Due to the large dead volume of the autosampler, the run-time was set to 10 min to allow for sufficient column re-equilibration.

The mass spectrometer was operated in the turbo ionspray positive-ion mode. The sprayer voltage, the orifice voltage and declustering potential were +4200 V, 35 V and 4 V, respectively. The interface heater was at 65°C and the turbo ionspray was operated at ambient temperature, since a loss of sensitivity was observed at higher temperatures. The nebulizer gas (nitrogen), curtain gas (nitrogen) and turbo ionspray gas (nitrogen) were set at 65 p.s.i., 1.2 l/min and 4.0 l/min, respectively. The argon collision gas thickness was 2.50×10^{14} atom per cm^2 and the collision energy was 23 eV. The dwell time was 175 ms and the count controller (CC) was set to 10. Four selected-reaction-monitoring (SRM) transitions were used, using the ammonium adduct, $(M+NH_4)^+$, as the precursor ion for the two analytes and two internal standards: m/z 581.3→m/z 436.2 for fosinopril; m/z 575.3→m/z 430.2 for SQ-33055 (internal standard of fosinopril); m/z 453.2→m/z 390.2 for fosinoprilat and m/z 479.2→m/z 416.2 for SQ-27133 (internal standard of fosinoprilat). Peak widths of the precursor and product ions were approximately 0.7 a.m.u. at half-height in the MS–MS mode. Peak area ratios were used for calculation

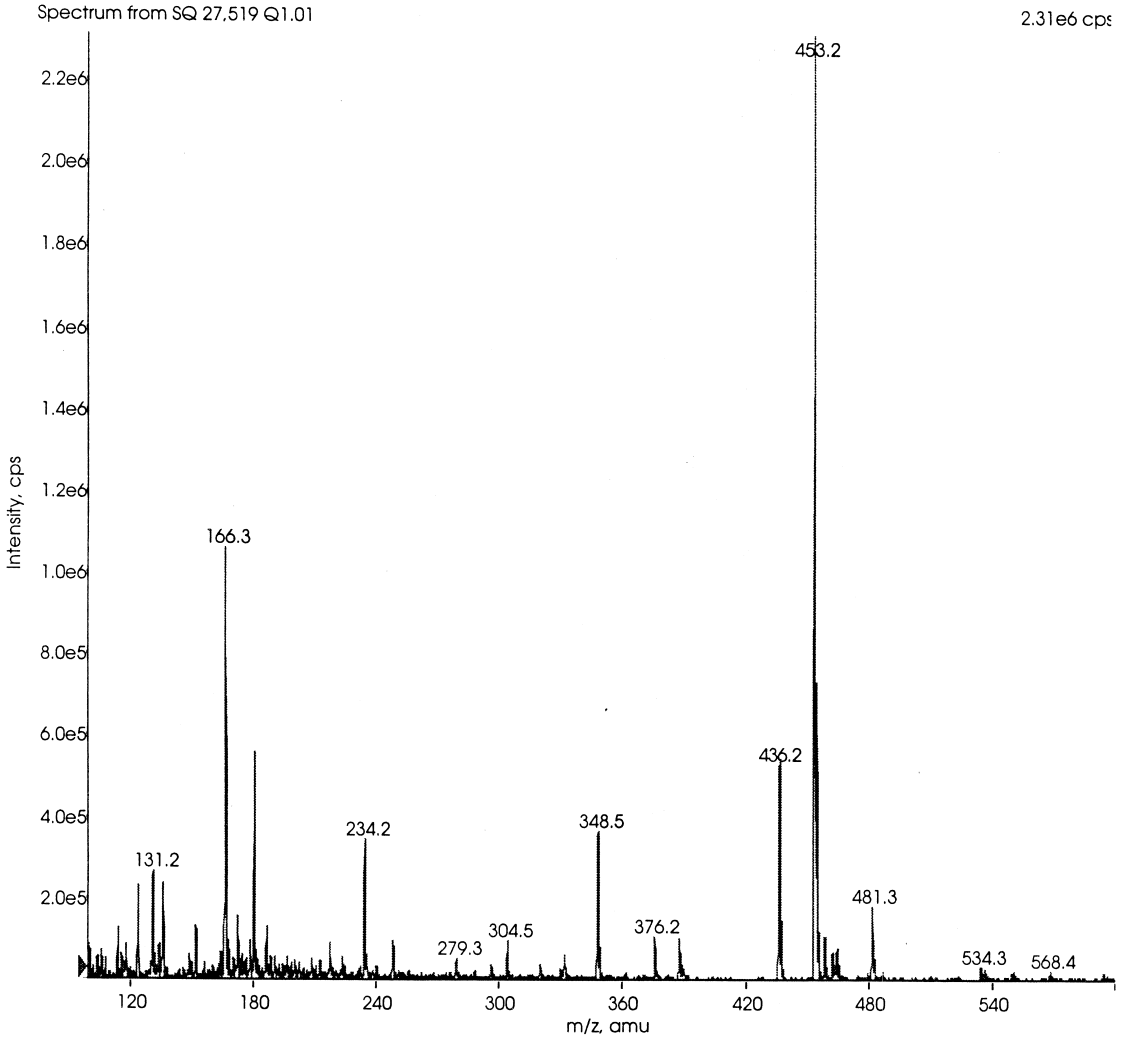


Fig. 2. Full-scan mass spectrum of fosinoprilat ($[M+NH_4]^+$=453.2) obtained using positive-ionization LC–MS.

and the calibration curve was fitted to a weighted $(1/x)$ linear regression model.

2.6. Method validation

The accuracy at the lower limit of quantitation (LLQ, 1.17 ng/ml) was assessed by spiking eight individual samples at the LLQ and analyzing them as unknowns against a standard curve. For the determination of intra- and inter-assay precision and inter-assay accuracy, QC samples at four concentrations were assayed in replicates of six on four different days. The specificity of the assay was determined by including in each analytical run, two blank serum samples and two serum samples to which only the internal standard was added. The bench-top stability at 4°C of fosinopril and fosinoprilat in serum QC samples was examined by pipetting two sets of the QCs into 0.2 N phosphoric acid in an ice bath, extracting one set immediately, and storing the remaining set in the ice bath for 7 h, after which time these samples were extracted. As a result of this experiment, an additional bench-top stability experiment was performed in which one set of QCs was pipetted into 0.2 N phosphoric acid in an ice bath and immediately extracted and the other set was

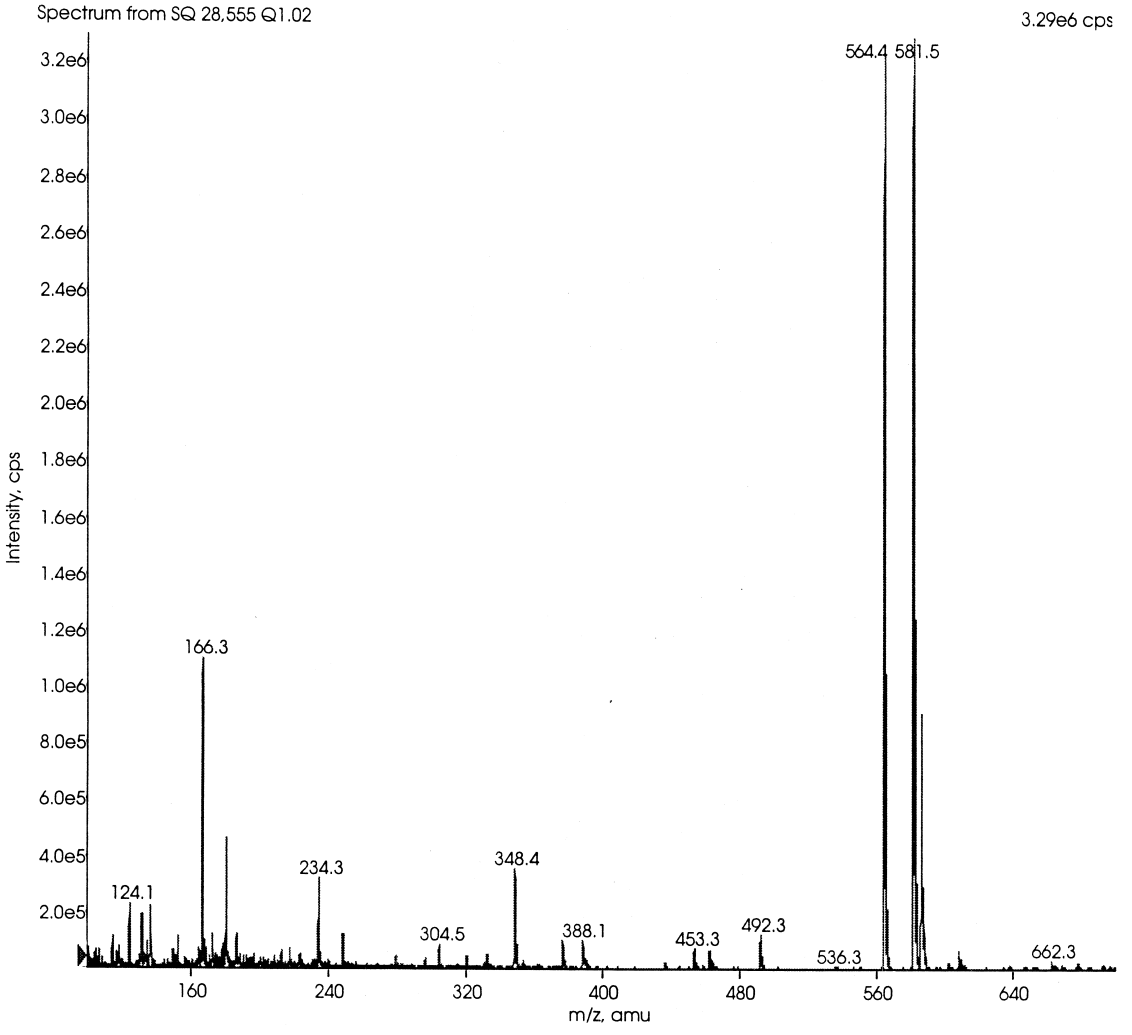


Fig. 3. Full-scan mass spectrum of fosinopril ($[M+NH_4]^+=581.5$) obtained using positive-ionization LC–MS.

placed into a refrigerator for 6 h, after which time it was pipetted into 0.2 N phosphoric acid in an ice bath and immediately extracted. All QCs were assayed in replicates of three. The freezer storage stability of fosinopril and fosinoprilat in serum at −20°C was examined by assaying a set of QCs in replicates of three on days 0, 50 and 181. The stability of the analytes in serum QC samples which had been subjected to repetitive freeze–thaw episodes was investigated. Samples which had been thawed and refrozen 0, 1 or 2 times were assayed in replicates of three. The stability of fosinopril and fosinoprilat in reconstituted serum extract was investigated by re-injecting a set of previously-assayed standards and QCs which had been stored at ambient temperature for 22 h.

3. Results and discussion

An LC–MS–MS method was developed and validated to enable quantitation of fosinopril and fosinoprilat in human serum and was shown to be sensitive, specific, accurate, precise and reproducible. The full-scan ionspray mass spectra are presented in Figs. 2–5. The spectra show significant responses

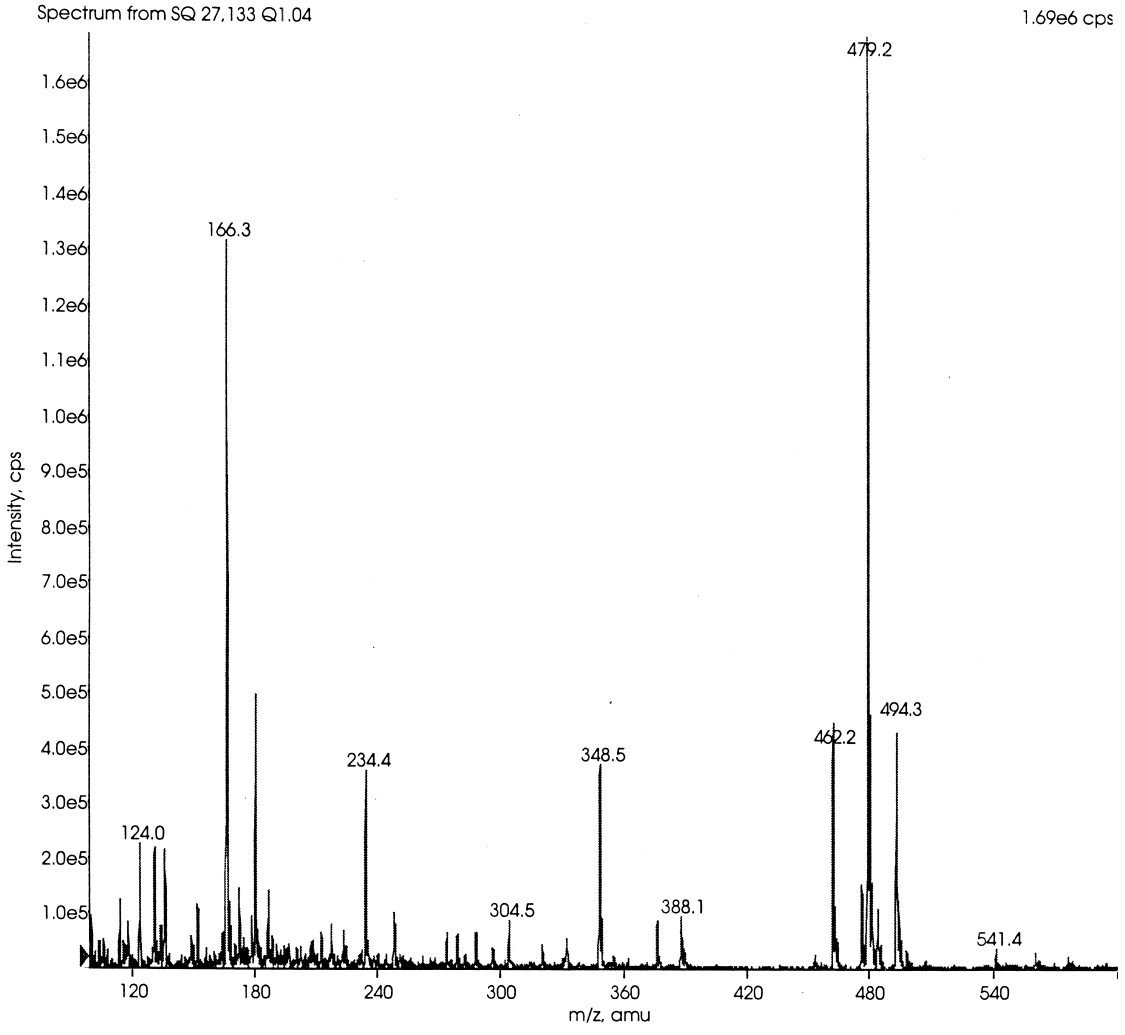


Fig. 4. Full-scan mass spectrum of SQ 27,133 ($[M+NH_4]^+$=479.2) obtained using positive-ionization LC–MS.

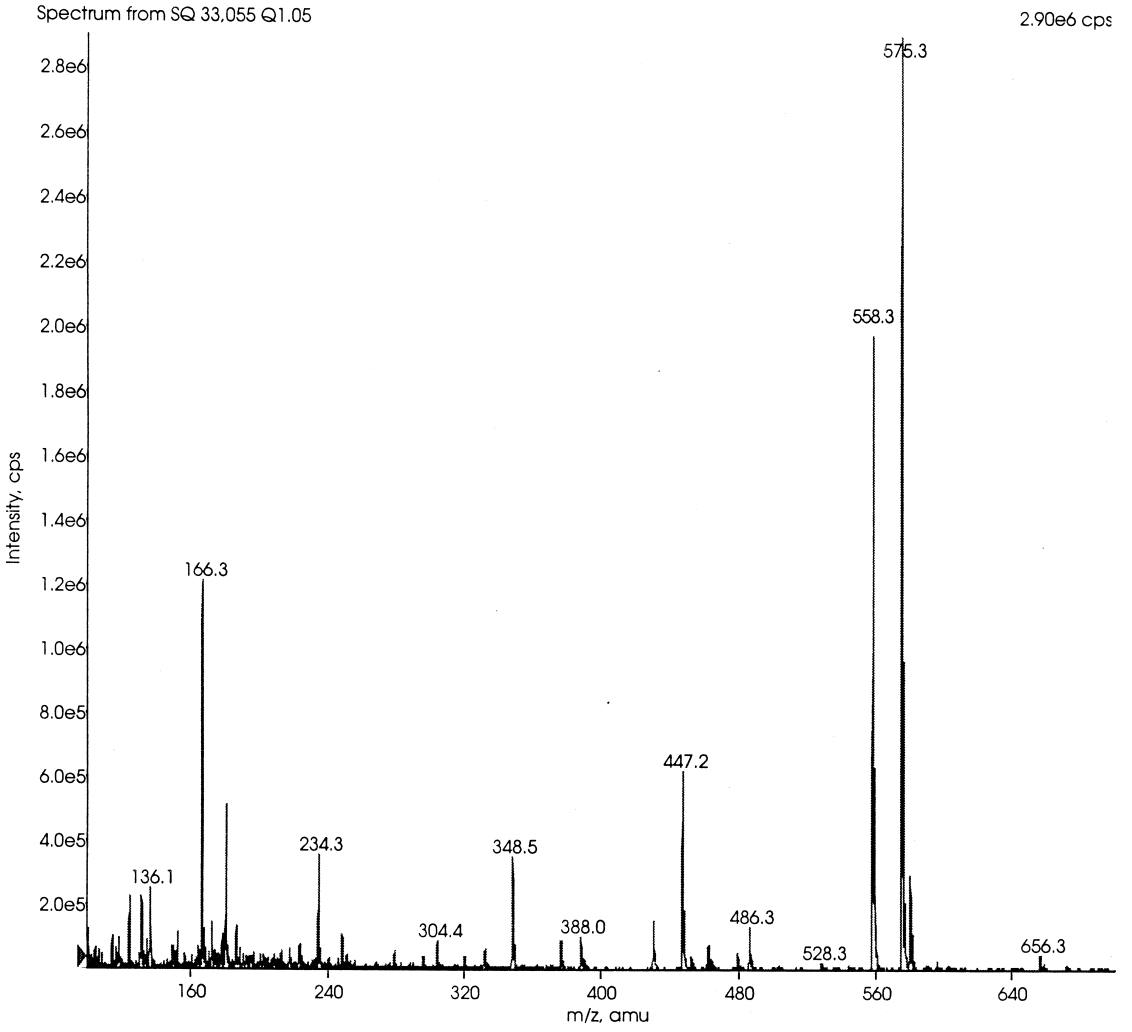


Fig. 5. Full-scan mass spectrum of SQ 33055 ($[M+NH_4]^+=575.3$) obtained using positive-ionization LC–MS.

from the protonated and ammoniated adduct species of the analytes. Selected reaction monitoring (SRM) of the collision-induced dissociation (CD) fragmentation of the ammoniated analytes to product ions was used for quantitation. The product-ion spectra of all analytes are shown in Figs. 6 and 7. The proposed fragmentation mechanisms of fosinopril and fosinoprilat to the SRM product ions monitored are presented in Fig. 8. Identical mechanisms (not shown) can be proposed to explain the SRM product ions monitored for the corresponding internal standards.

Silica-based HPLC columns were initially assessed during method development. However, significant tailing was observed for fosinoprilat, presumably due to interactions between the phosphinic acid and the silanol groups. This tailing was greatly improved with the selected polymeric column. Typical chromatograms of the blank sample, lowest calibration standard and highest calibration standard are presented in Figs. 9–11. The assay was highly specific and only trace chromatographic interferences were observed in blank samples. Minor chromatographic carryover was observed; however, after injection of the highest standard, these peaks were always much smaller than the lowest standard.

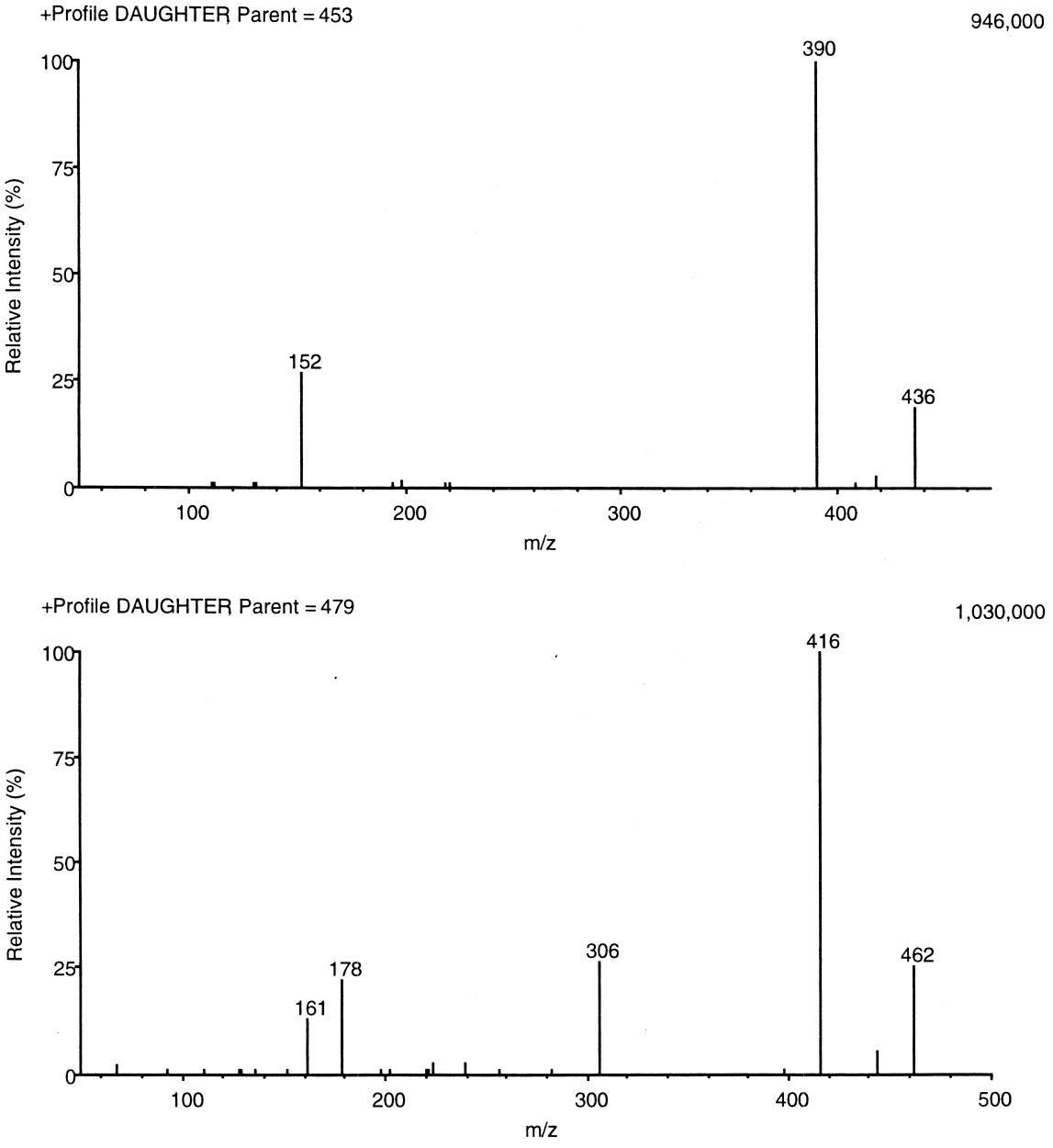


Fig. 6. Full-scan product-ion spectra of the $[M+NH_4]^+$ ions of fosinoprilat (top) and its internal standard, SQ 27,133 (bottom).

The LLQ experiment demonstrated that acceptable accuracy and precision were observed at the lowest standard concentration of 1.17 ng/ml for both analytes in human serum. As listed in Table 1, the deviations (%Dev.) for the individual LLQ samples were within 16% for both analytes. The mean %Dev. was within 4.6% and the C.V. was within 8.5%. The summary results of the analysis of QC samples from the accuracy and precision experiments are presented in Tables 2 and 3. The inter-assay accuracy, as

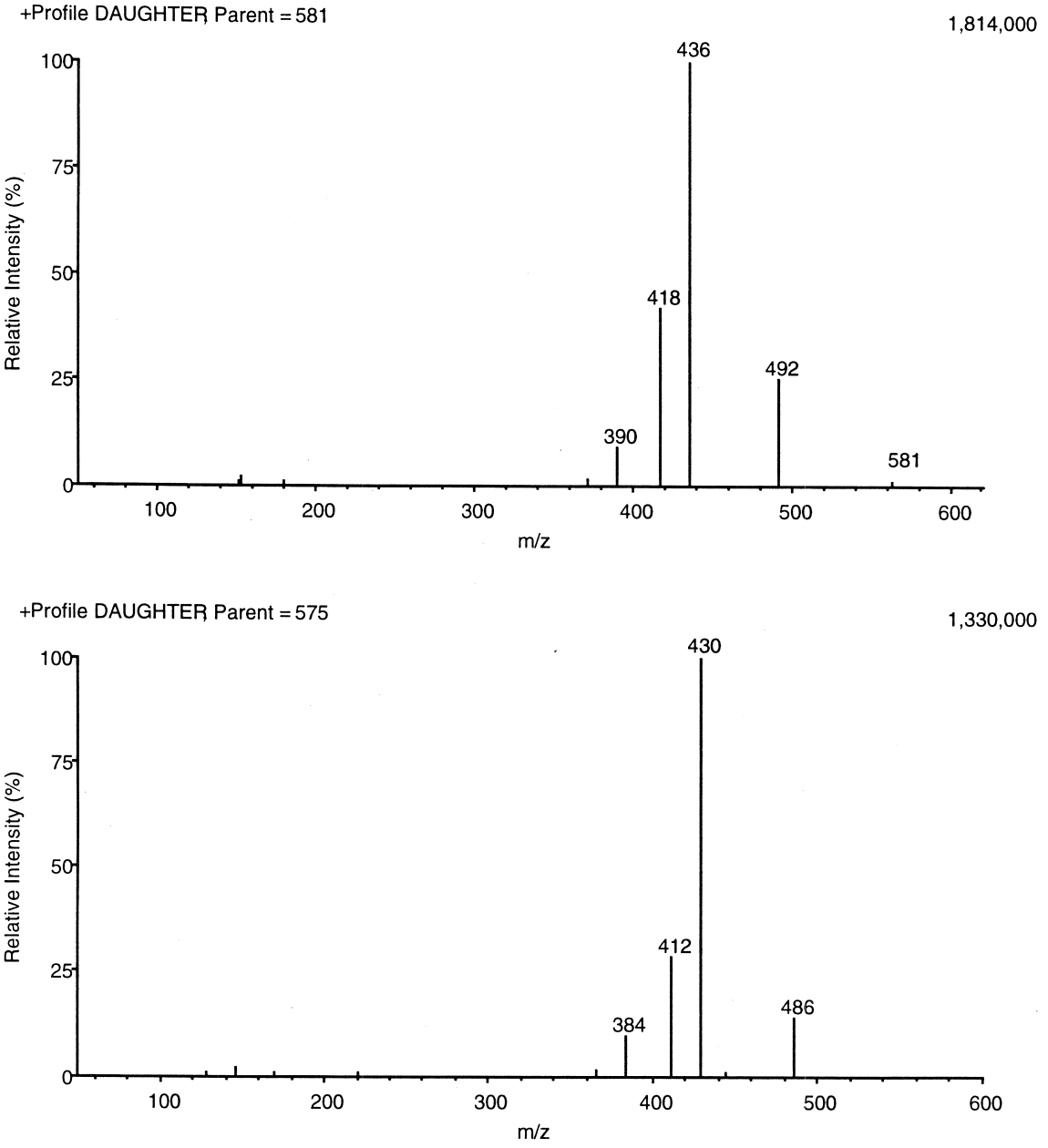


Fig. 7. Full-scan product-ion spectra of the $[M+ NH_4]^+$ ions of fosinopril (top) and its internal standard, SQ 33055 (bottom).

measured by mean deviation, was within 6% for both analytes. The inter-assay precision was within 11% (RSD) and the intra-assay precision was within 7% (RSD) for both analytes.

It was necessary to acidify the serum standards and QC samples to reduce the rate of hydrolysis of fosinopril to fosinoprilat. In addition, keeping the serum samples at 4°C (instead of room temperature)

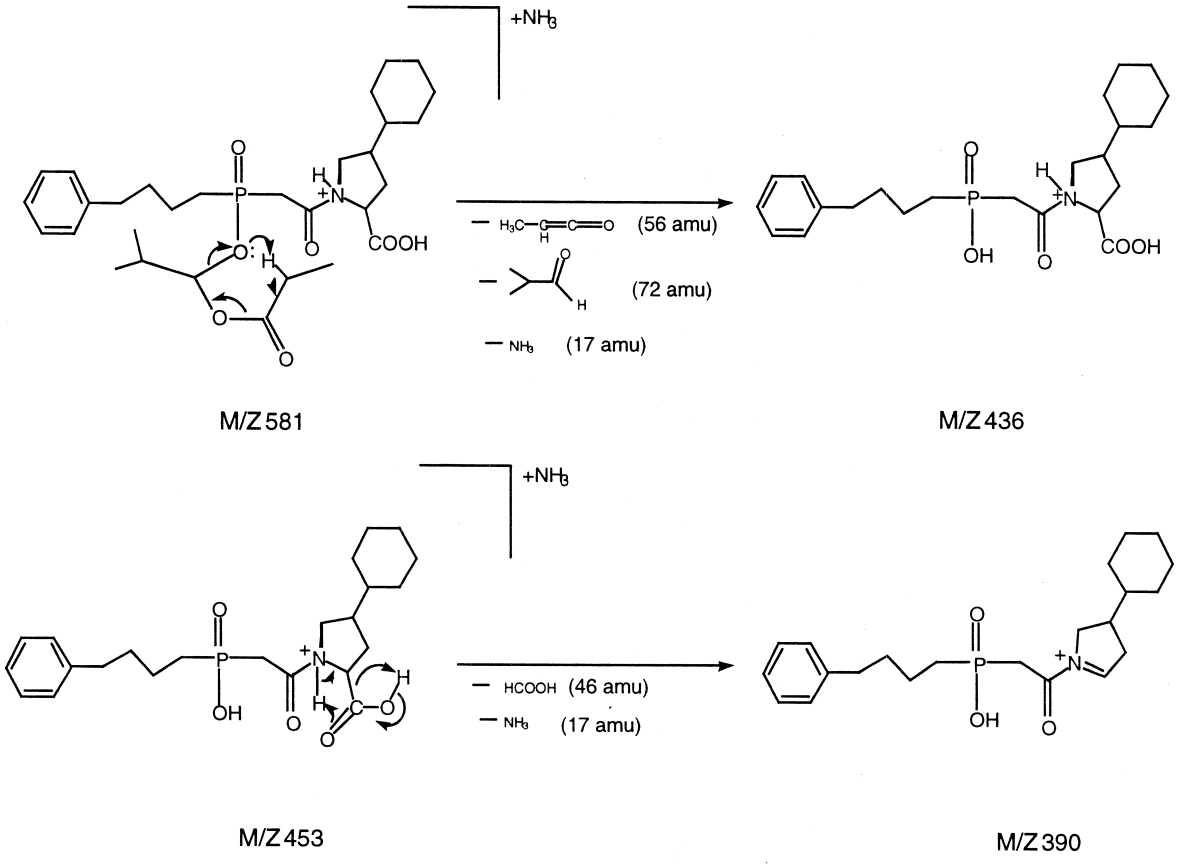


Fig. 8. Proposed CID fragmentation mechanisms for the $[M+ NH_4]^+$ ions of fosinopril (m/z 581) and fosinoprilat (m/z 453).

Table 1
Lower limit of quantitation for fosinopril and fosinoprilat in human serum

Sample	Fosinopril		Fosinoprilat	
	Measured conc. (ng/ml)	Dev. (%)	Measured conc. (ng/ml)	Dev. (%)
	1.23	5.1	1.10	−6.0
	1.25	6.8	1.19	1.7
	1.30	11	1.23	5.1
	1.33	14	1.12	−4.3
	1.24	6.0	1.14	−2.6
	1.26	7.7	1.05	−10
	1.10	−6.0	1.36	16
	1.08	−7.7	1.09	−6.8
Mean	1.22	4.6	1.16	−0.85
%C.V.	7.3		8.5	

minimized the rate of hydrolysis. Hence, the bench-top stability in human serum at 4°C was first investigated after adding the phosphoric acid solution to the serum samples. The results shown in Tables 4 and 5 indicate that fosinopril is stable (deviations within ±13%) under these conditions but fosinoprilat is not (deviations within ±43%). As a result of these findings, the stability of the serum samples at 4°C prior to adding the phosphoric acid solution was studied. As summarized in Tables 6 and 7, fosinoprilat was stable (deviations within ±18%) without compromising the fosinopril stability (deviations within ±16%). Consequently, this modification was used for all subsequent work. It is evident from Tables 5 and 7 that in the 250 ng/ml fosinopril-only

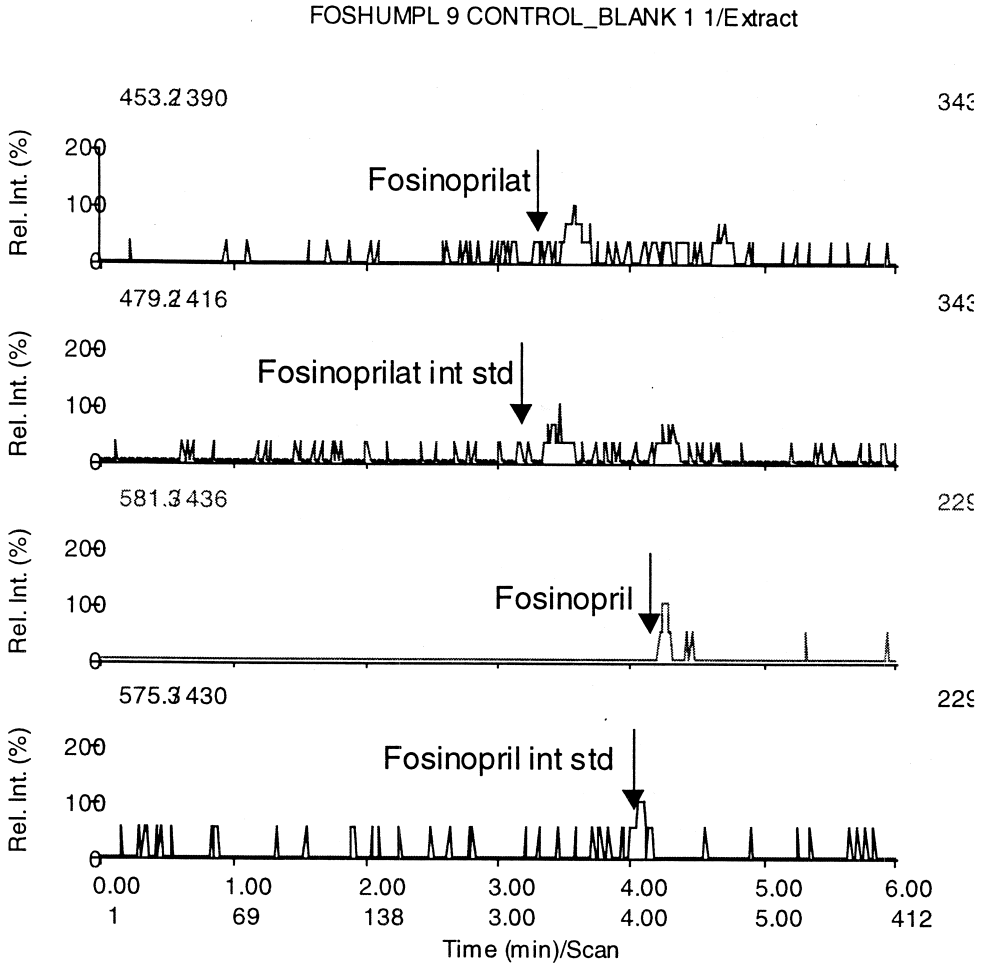


Fig. 9. Selected-reaction-monitoring chromatograms of the blank serum sample, with the four SRM channels shown for (from top to bottom): fosinoprilat, fosinoprilat internal standard, fosinopril, and fosinopril internal standard. int std=internal standard.

Table 2
Intra-assay and inter-assay accuracy and precision for fosinopril in human serum

Nominal conc. (ng/ml)	Mean measured conc. (ng/ml)	Dev. (%)	C.V (%)	
			Intra-assay precision	Inter-assay precision
5	5.27	5.4	4.9	4.2
100	105.0	5.0	5.1	11.0
250	247.0	−1.3	3.1	5.3
500	483.0	−3.5	4.9	4.1

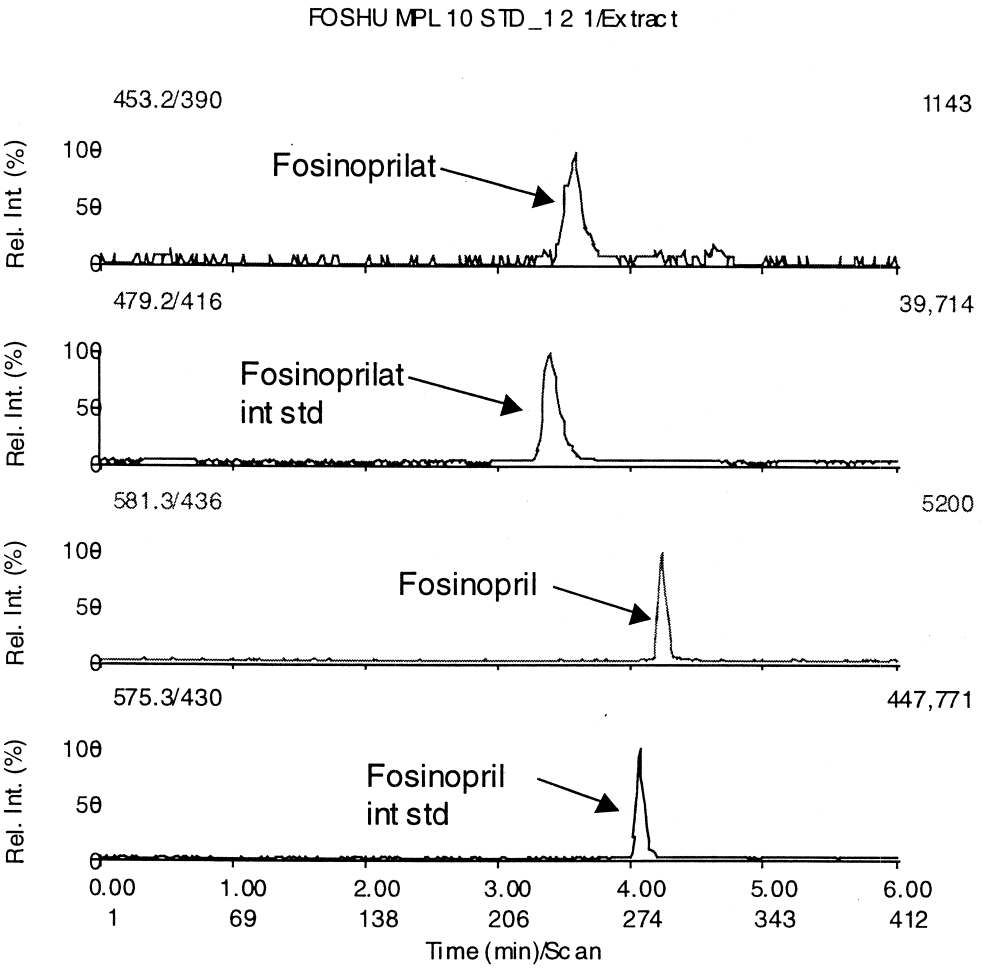


Fig. 10. Selected-reaction-monitoring chromatograms of the low serum standard (1. 17 ng/ml), with the four SRM channels shown for (from top to bottom): fosinoprilat, fosinoprilat internal standard, fosinopril, and fosinopril internal standard. int std=internal standard.

Table 3
Intra-assay and inter-assay accuracy and precision for fosinoprilat in human serum

Nominal conc. (ng/ml)	Mean measured conc. (ng/ml)	Dev. (%)	C.V. (%)	
			Intra-assay precision	Inter-assay precision
5	4.9	−2.0	6.5	0.0
100	98.4	−1.6	4.8	10.0
250	235.0	−5.8	3.2	8.4
500	475.0	−5.0	3.4	8.7

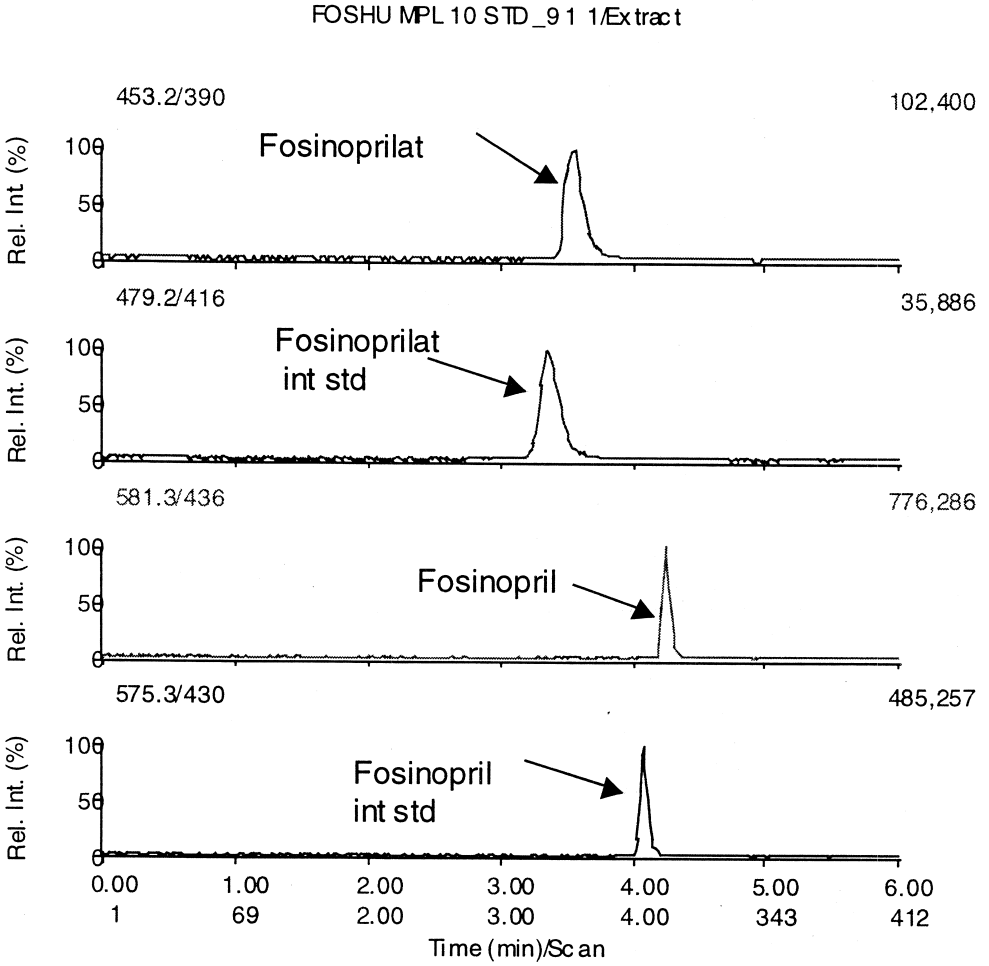


Fig. 11. Selected-reaction-monitoring chromatograms of the high serum standard (300 ng/ml), with the four SRM channels shown for (from top to bottom): fosinoprilat, fosinoprilat internal standard, fosinopril, and fosinopril internal standard. int std=internal standard.

QCs, a small percentage of fosinopril is being converted to fosinoprilat (approx. 4–6%). Thus, some conversion occurs in spite of acidification. This relatively small conversion is not a problem in the analysis of post-dose serum samples obtained from humans dosed with fosinopril, since the concentrations of fosinopril in study samples are low compared to the concentrations of fosinoprilat [1].

The results from the study of long-term freezer storage stability at −20°C of serum QC samples showed that both fosinopril and fosinoprilat were stable for at least 181 days. The data for fosinopril and fosinoprilat obtained from the serum freeze–thaw experiment showed that both fosinopril and fosinoprilat were stable for at least two freeze–thaw cycles. The results of the reconstituted sample stability showed that both fosinopril and fosinoprilat were stable in reconstituted samples.

4. Conclusions

The LC–MS–MS method developed for the simultaneous quantitation of fosinopril and fosinoprilat in human serum was shown to be accurate, precise, sensitive, specific and reproducible. Under the acidic conditions used for serum sample processing, the hydrolysis of fosinopril to fosinoprilat was

Table 4
Bench-top stability of fosinopril in human serum after addition of phosphoric acid

Fosinopril nominal conc. (ng/ml)	Replicate No.	0-h		7-h	
		Fosinopril measured conc. (ng/ml)	Dev. (%)	Fosinopril measured conc. (ng/ml)	Dev. (%)
5.0 (both analytes)	1	5.16	3.1	5.04	0.7
5.0 (both analytes)	2	4.58	−8.3	4.90	−2.1
5.0 (both analytes)	3	5.00	−0.1	4.37	13.0
5.0 (fosinopril only)	1	4.86	−2.8	5.00	0.0
5.0 (fosinopril only)	2	4.31	−14.0	5.11	2.2
5.0 (fosinopril only)	3	4.52	−9.6	4.97	−0.6
0.0 (fosinoprilat only)	1	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	2	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	3	<LLQ	N/A	<LLQ	N/A
250.0 (both analytes)	1	226.0	−9.5	235.0	−6.0
250.0 (both analytes)	2	227.0	−9.3	229.0	−8.5
250.0 (both analytes)	3	229.0	−8.4	227.0	9.0
250.0 (fosinopril only)	1	229.0	−8.5	220.0	−12.0
250.0 (fosinopril only)	2	219.0	−12.0	223.0	11.0
250.0 (fosinopril only)	3	218.0	−13.0	220.0	−12.0
0.0 (fosinoprilat only)	1	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	2	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	3	<LLQ	N/A	<LLQ	N/A

Table 5
Bench−top stability of fosinoprilat in human serum after addition of phosphoric acid

Fosinoprilat nominal conc. (ng/ml)	Replicate No.	0-h		7-h	
		Fosinoprilat measured conc. (ng/ml)	Dev. (%)	Fosinoprilat measured conc. (ng/ml)	Dev. (%)
5.0 (both analytes)	1	4.65	−7.0	4.01	−20
5.0 (both analytes)	2	5.37	7.3	3.42	−32
5.0 (both analytes)	3	4.71	−5.8	3.88	−22
0.0 (fosinopril only)	1	<LLQ	N/A	<LLQ	N/A
0.0 (fosinopril only)	2	<LLQ	N/A	<LLQ	N/A
0.0 (fosinopril only)	3	<LLQ	N/A	<LLQ	N/A
5.0 (fosinoprilat only)	1	4.37	−12.0	2.86	43
5.0 (fosinoprilat only)	2	4.36	−13.0	3.03	−39
5.0 (fosinoprilat only)	3	4.70	−6.0	3.14	−37
250.0 (both analytes)	1	243.00	−2.8	183.00	−27
250.0 (both analytes)	2	225.00	−9.8	211.00	16
250.0 (both analytes)	3	215.00	−14.0	223.00	−11
0.0 (fosinopril only)	1	12.50	N/A	11.80	N/A
0.0 (fosinopril only)	2	11.70	N/A	10.60	N/A
0.0 (fosinopril only)	3	12.40	N/A	13.10	N/A
250.0 (fosinoprilat only)	1	181.00	−27.6	155.36	−38
250.0 (fosinoprilat only)	2	190.00	−23.8	158.82	−36
250.0 (fosinoprilat only)	3	194.00	−22.4	142.64	43

Table 6
Bench-top stability of fosinopril in human serum prior to addition of phosphoric acid

Fosinopril nominal conc. (ng/ml)	Replicate No.	0-h		6-h	
		Fosinopril measured conc. (ng/ml)	Dev. (%)	Fosinopril measured conc. (ng/ml)	Dev. (%)
5.0 (both analytes)	1	5.29	5.7	5.81	16.0
5.0 (both analytes)	2	4.89	−2.3	4.82	−3.6
5.0 (both analytes)	3	5.05	1.1	5.37	7.4
5.0 (fosinopril only)	1	5.77	15.0	5.28	5.6
5.0 (fosinopril only)	2	5.10	2.0	4.61	−7.8
5.0 (fosinopril only)	3	5.30	5.9	4.69	−6.2
0.0 (fosinoprilat only)	1	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	2	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	3	<LLQ	N/A	<LLQ	N/A
250.0 (both analytes)	1	249.00	−0.3	250.00	−0.1
250.0 (both analytes)	2	243.00	−2.8	242.00	−3.3
250.0 (both analytes)	3	232.00	−7.2	229.00	−8.6
250.0 (fosinopril only)	1	258.00	3.2	247.00	−1.1
250.0 (fosinopril only)	2	248.00	−0.9	245.00	−2.1
250.0 (fosinopril only)	3	244.00	−2.6	245.00	−1.9
0.0 (fosinoprilat only)	1	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	2	<LLQ	N/A	<LLQ	N/A
0.0 (fosinoprilat only)	3	<LLQ	N/A	<LLQ	N/A

Table 7
Bench-top stability of fosinoprilat in human serum prior to addition of phosphoric acid

Fosinoprilat nominal conc. (ng/ml)	Replicate No.	0-h		6-h	
		Fosinoprilat measured conc. (ng/ml)	Dev. (%)	Fosinoprilat measured conc. (ng/ml)	Dev. (%)
5.0 (both analytes)	1	5.57	11.0	5.77	15.0
5.0 (both analytes)	2	5.27	5.3	5.50	10.0
5.0 (both analytes)	3	5.90	18.0	5.72	14.0
0.0 (fosinopril only)	1	<LLQ	N/A	<LLQ	N/A
0.0 (fosinopril only)	2	<LLQ	N/A	<LLQ	N/A
0.0 (fosinopril only)	3	<LLQ	N/A	<LLQ	N/A
5.0 (fosinoprilat only)	1	5.60	12.0	5.48	9.4
5.0 (fosinoprilat only)	2	5.19	3.7	5.37	7.3
5.0 (fosinoprilat only)	3	5.20	3.9	5.65	13.0
250.0 (both analytes)	1	263.00	5.3	286.0	14.0
250.0 (both analytes)	2	253.00	1.2	250.0	0.1
250.0 (both analytes)	3	247.00	−1.2	263.0	5.2
0.0 (fosinopril only)	1	9.93	N/A	12.8	N/A
0.0 (fosinopril only)	2	9.45	N/A	10.9	N/A
0.0 (fosinopril only)	3	10.00	N/A	10.1	N/A
250.0 (fosinoprilat only)	1	249.00	−0.3	271.0	8.3
250.0 (fosinoprilat only)	2	243.00	−2.9	287.0	15.0
250.0 (fosinoprilat only)	3	240.00	−4.0	246.0	−1.8

relatively insignificant in that this degree of conversion would cause no significant error in the analysis of post-dose human serum samples for fosinoprilat since these samples are known to contain very little of the prodrug compared to the drug. There was some observed instability of fosinoprilat in the acidified serum samples; however, this was minimized by shortening the time between sampling and extraction.

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