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Development of liquid chromatographic method for fosinoprilat determination in human plasma using microemulsion as eluent

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Abstract

Fosinopril sodium presents a prodrug for the active angiotensin converting enzyme (ACE) inhibitor, fosinoprilat. The dual elimination of fosinoprilat by the liver and the kidney distinguishes fosinopril from other angiotensin converting enzyme inhibitors. Such ways of elimination are important for antihypertensive therapy of patients on haemodialysis. The paper presents development and evaluation of a new and sensitive liquid chromatographic (LC) method for the analysis of fosinoprilat in plasma obtained from patients on haemodialysis. A microemulsion system mixture as mobile phase has been used for the separation and analysis of fosinoprilat in plasma samples. The plasma samples were injected directly onto the HPLC system (Waters Breeze) after appropriate sample dilution with mobile phase. Separations were performed on the Bakerbond ENV 4.6 mm × 150 mm, 5 μm particle size column with UV detection at 220 nm. The flow rate was 1.00 mL min^{-1}. The mobile phase consisted of 1.0% (w/v) of diisopropyl ether, 2.0% (w/v) of sodium dodecyl sulphate (SDS), 6.0% (w/v) of *n*-propanol and 91% (w/v) of aqueous 25 mM di-sodium hydrogen phosphate, pH adjusted to 2.8 with 85% orthophosphoric acid. The developed method was then subjected to method validation according to the criteria stated in the FDA bioanalytical method validation guidance. The results for specificity, linearity, low limit of quantification (LLOQ), precision, accuracy and stability were within the accepted criteria. The unique approach applied in this paper makes possible the determination of fosinoprilat even in the presence of metabolites of other drugs, so the method can be used for obtaining the reliable results in a fast and simple way.

Keywords: Fosinoprilat; Liquid chromatography; Human plasma

1. Introduction

Fosinopril sodium is the ester prodrug of the active angiotensin converting enzyme (ACE) inhibitor, fosinoprilat (SQ 27519). It contains a phosphinate group capable of specific binding to the active site of angiotensin converting enzyme [1]. Fosinopril sodium is designated chemically as sodium [1[S^*(R^*)],2α,4β]-4-cyclohexyl-1-[[[2-methyl-1-(1-oxo-propoxy)propoxy](4-phenylbutyl)phosphinyl]acetyl]-L-proline [2] and its structure as well as structure of fosinoprilat and cilazapril (internal standard) are shown in Fig. 1.

Following oral administration, fosinopril is poorly absorbed [3]. The absolute absorption of fosinopril averaged 36% of an oral dose. After absorption the prodrug is almost completely de-esterified in the liver and the gastrointestinal mucosa to form the active diacid fosinoprilat [4,5]. Fosinoprilat is highly protein-bound (≥95%), has a relatively small volume of distribution, and has negligible binding to cellular components in blood. After single and multiple oral dose plasma levels are directly proportional to the dose of fosinopril. Time to peak concentrations are independent of dose and are achieved in approximately 3 h [1]. By our method, fosinopril was virtually undetectable in the plasma 2 h post-dose as a result of rapid metabolism of fosinopril to fosinoprilat [6].

In present literature there is only one paper concerning analysis of fosinoprilat in human serum using liquid chro-

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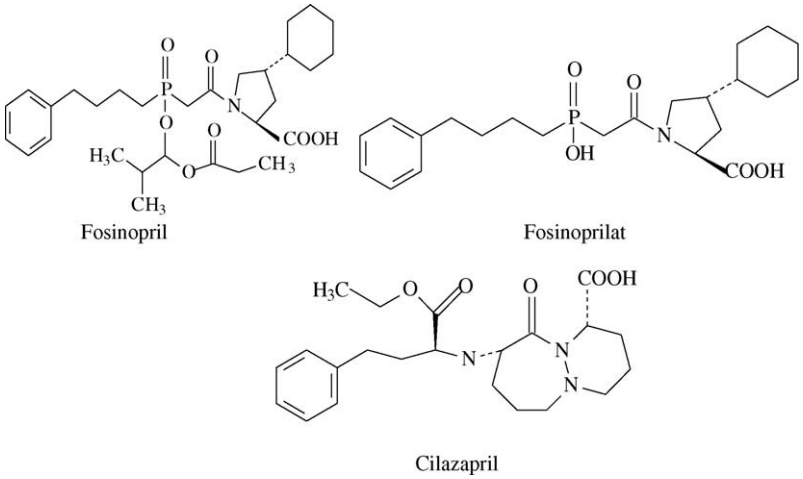

Fig. 1. Structural formulae of the investigated substances.

matographic electrospray tandem mass spectrometric method [7]. The pharmacokinetics and pharmacodynamics of fosinoprilat was investigated in haemodialysis and peritoneal dialysis patients by a radioimmmunoassay (RIA) [6,8,9].

In bioanalysis it has always been important to minimize the sample preparation in order to eliminate all the possible errors and losses. A direct injection of biofluids is preferred whenever possible. However, the presence of organic solvents in the mobile phase in the concentration above 5% tend to precipitate proteins and makes direct HPLC analysis imposible. El-Sherbiny et al. proposed liquid chromatographic (LC) method, with microemulsion as eluent, for the analysis of bumetanide and acebutolol in plasma and urine [10]. Our developed method enables direct injection of plasma after dilution with mobile phase because proteins were solubilised in microemulsion.

Microemulsions are clear, thermodynamically stable, isotropic mixtures containing oil, water, surfactant and, most often a medium chain alcohol acting as a co-surfactant. They can be used as a pseudo-single-phase solvent or two-phase solvent consisting of microemulsion phase and an aqueous or organic phase. In the single phase oil-in-water (o/w) or water-in-oil (w/o) microemulsions, which are denoted as L_2 and L_1, respectively [11], the partitioning and the interfacial adsorption of the solute in the microheterogeneous systems are responsible for the chemical reactivity and separation mechanisms [11]. In the previous reports [12–14] an excellent potential of application of microemulsion as mobile phase in LC analysis was proved.

Having in mind the enhanced solubilisation capacity of microemulsions, as well as its unique properties as separation media, we investigated the possibility of determination of fosinoprilat in plasma without previous solid phase extraction (SPE). The aim of this work was to develop, validate and apply an efficient and novel LC method for the determination of fosinoprilat in human plasma employing oil-in-water microemulsion as eluent.

2. Experimental

2.1. Reagents and standards

All reagents used were of an analytical grade. Sodium dodecyl sulphate, was obtained from *Sigma* (St. Louis, MO, USA). Diisopropyl ether and *n*-propanol—HPLC grade were manufactured from *Riedel-deHäen* (Sleeze, Germany). Water—HPLC grade, di-sodium hydrogen phosphate *J.T. Baker* (Deventer, The Netherlands) and orthophosphoric acid *Carlo Erba* (Milan, Italy) were used to prepare the mobile phase. The reference standards of fosinopril and fosinoprilat were obtained from Bristol-Myers Squibb (Roma, Italy). Cilazapril (working standard) was obtained from *Hemofarm Concern*, Vrsac, Serbia and Montenegro and it was used as internal standard.

2.2. Preparation of stock solutions

Primary stock solution of fosinoprilat for preparation of standards and quality controls (QC) was prepared by dissolving the reference standard substance in microemulsion, used as mobile phase, to obtain the concentration of 90 $\mu g\,mL^{-1}$. The internal standard stock solution was prepared by dissolving cilazapril in microemulsion mobile phase to produce a concentration of 0.60 $mg\,mL^{-1}$.

Working solutions of fosinoprilat were prepared in microemulsion by appropriate dilution at: 1.2, 2.25, 4.5, 9.0, 18.0, 36.0, 45.0, 63.0 and 81.0 $\mu g\,mL^{-1}$. All the solutions contained internal standard in the concentration of 60 $\mu g\,mL^{-1}$. All solutions are stable during the investigation.

2.3. Calibration curves

Calibration curve was prepared by spiking 500 μL of human blank plasma each with 250 μL of one of the above-

mentioned working solutions to produce the calibration curve points equivalent to 0.40, 0.75, 1.5, 3.0, 6.0, 12.0, 15.0, 21.0 and 27.0 $\mu g\,mL^{-1}$ of fosinoprilat. Each sample so contained 20 $\mu g\,mL^{-1}$ of the internal standard. Zero plasma samples were prepared by diluting internal standard in 500 μL of human plasma to obtain the final concentration 20 $\mu g\,mL^{-1}$. A plasma blank sample was prepared by mixing 500 μL of human blank plasma with 250 μL of microemulsion.

2.4. Quality control samples

Quality control samples were prepared at three different levels. QC samples were prepared daily by spiking 500 μL of human blank plasma each with 250 μL of corresponding working solution to produce a final concentration equivalent to 0.75 (low), 6.0 (medium) and 21 $\mu g\,mL^{-1}$ (high) of fosinoprilat and 20 $\mu g\,mL^{-1}$ of internal standard.

2.5. Chromatographic conditions

The chromatographic system Waters Breeze (*Waters Corporation*, Milford, Ireland) consisted of Waters 1525 Binary HPLC Pump, Waters 2487 UV–vis detector and Breeze Software, Windows XP, for data collection. Separations were performed on the Bakerbond ENV 4.6 mm × 150 mm, 5 μm particle size column with UV detection at 220 nm. The column temperature was 25 °C. The flow rate was 1 mL min^{-1}. The samples were introduced through a Rheodyne injector valve with a 20 μL sample loop. For the calculation of statistic parameters the Microsoft Excel 2000 was used.

The mobile phase was prepared by mixing 10 g of diisopropyl ether, 20 g of SDS, 60 g of *n*-propanol and 910 mL of aqueous 25 mM di-sodium hydrogen phosphate in 1000 mL volumetric flask. The mixture was then sonicated for 30 min to aid dissolution. The pH of the resulting optically transparent microemulsion was adjusted to 2.8 with 85% orthophosphoric acid. Microemulsion was stable for several months. The obtained mobile phase was filtered through a 0.45 μm membrane filter *Alltech* (Loceren, Belgium).

2.6. Method validation

In order to carry out sample analysis in a Good Laboratory Practice (GLP)-compliant manner, the developed method was validated according to currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [15]. The following parameters were considered:

(1) The method's *specificity* was tested by screening blank human plasma. The plasma blank sample was tested for interference using the proposed chromatographic conditions and compared with those obtained with a solution of the analyte in microemulsion at a concentration close to the lower limit of quantitation (LLOQ).
(2) For *sensitivity* determination, the lowest standard concentration in the calibration curve was considered as the LLOQ, and was to meet the following criteria: LLOQ response should be five times the response of the blank and the LLOQ response should be identifiable, discrete and reproducible with precision of 20% and accuracy of 80–120%. The five samples in concentration 0.40 $\mu g\,mL^{-1}$ was investigated as the LLOQ. Reproducibility and precision were determined.
(3) For the determination of *linearity*, standard calibration curve of at least seven points (non-zero standards) should be used. In addition, a blank and zero plasma samples were also analyzed to confirm absence of interferences. These two samples were not used to construct the calibration function. Four out of seven none zero standards including LLOQ and upper limit of quantitation (ULOQ) were to meet the following acceptance criteria: no more than 20% deviation at LLOQ and no more than 15% deviation for standards above the LLOQ. The acceptance criteria for the correlation coefficient should be 0.998 or more, otherwise the calibration curve should be rejected. Linearity was tested for the range of concentrations 0.40–27.0 $\mu g\,min^{-1}$.
(4) The *intra-day precision and accuracy* of the assay was measured by analyzing five spiked samples of fosinoprilat at each QC level (0.75, 6.0 and 21 $\mu g\,min^{-1}$). The *inter-day precision* and accuracy was determined over three days by analyzing five spiked samples of fosinoprilat at each QC level. Normal acceptance criteria for accuracy are that the percent deviation of the mean concentration falls within ±15% of the nominal value except at the LLOQ, where it should not deviate by ±20%. The coefficient of variation (%CV) of each QC samples must be within 15%. The coefficient of variation (%CV) ranges define the intra- and inter-day precision with normal acceptance criteria being that the CV should not exceed 15%.
(5) *Short-term stability*: QC plasma aliquots were thawed and kept at room temperature for 5 h based on the expected duration that samples will be maintained at room temperature in the intended study during sample processing. Samples were analyzed as mentioned above.
(6) *Freeze and thaw stability*: QC plasma samples containing fosinoprilat were tested after three freeze (−20 °C) and thaw (room temperature) cycles.

3. Results and discussion

Bioanalysis, perhaps more than many other types of analysis, involves unknown factors as analyte concentrations are low, matrices are complex and may contain unknown and variable components. The reliability of modern methods is therefore remarkable given that analytes are often at concentrations of one a billion or less in the parts per billion range. After extraction the analyte of interest may be present in extremely low concentration especially if the recovery is low.

In bioanalysis it has always been a desirable to minimize the sample preparation. The ability of micellar and microemulsion systems to solubilise proteins proved to be advantageous and enabled the direct injection of biofluids into an HPLC system and LC analysis applying micellar solutions or microemulsions as eluent. This is of course useful for solutes being present in concentrations sufficiently high so that no further concentration before detection is needed. According to previous reports after administration of 20 mg of fosinopril sodium the renally impaired patients reached peak plasma concentrations of 3.87 $\mu g\,mL^{-1}$ within 3 h and the healthy volunteers 3.24 $\mu g\,mL^{-1}$ in 3 h [16]. Preliminary investigations showed that even lower plasma concentrations of fosinoprilat can be easily detected at 220 nm, after proper dilution of plasma samples with microemulsion used as eluent in LC analysis.

Using microemulsion in bioanalysis is advantageous because of solubilisation capacity of microemulsions and possibility of fine tuning of selectivity. When microemulsions are used as eluent in HPLC separation, solutes influenced by the partitioning between the charged oil droplet and the aqueous buffer phase. The retention of the more lipophilic analytes will be influenced by the partition into the microemulsion droplets, while the retention of the more hydrophilic substances will be primarily governed by the stationary phase of the column packing material. As previously stated, fosinopril was not expected to be present in plasma samples collected beyond 2 h post dose.

The major problem in developing such kind of method was to retain fosinoprilat in order to avoid any matrix interference. In conventional RP-HPLC separations the retention of analytes is related to their hydrophobicity. When an analyte is ionised its retention is strongly influenced by pH of the mobile phase. Retention of fosinoprilat was strongly affected by changing of microemulsion mobile phase pH. Its retention time was almost doubled with lowering the pH of the mobile phase from 4.5 to 2.5. This is the same effect that would be observed in conventional RP-HPLC separation. Finally, the pH of the mobile phase was adjusted to 2.8 because of estimating in order to attain good selectivity in the reasonable run time. Selectivity of the separation was not so significantly affected by the type and content of the inner phase of microemulsion eluent and for that reason diisopropyl ether was chosen as an internal phase and the content was 1.0% (w/v). The retention of fosinoprilat decreased by increase of SDS concentration from 1.0% (w/v) to 3.0% (w/v) leading to co-elution with unknown and variable components of plasma. However, the type and concentration of co-surfactant had the biggest influence on the separation. The replacement of *n*-butanol with *n*-propanol, tetrahydrofuran and acetonitrile caused drastic increase in retention time thus affecting selectivity. Short-chain alcohol, such as *n*-propanol, are completely miscible with water and influence mobile phase hydrophobicity, modeling in that way the hydrophobic interaction between solute and stationary phase, e.g. affected the retention of fosinoprilat and the internal standard. The best

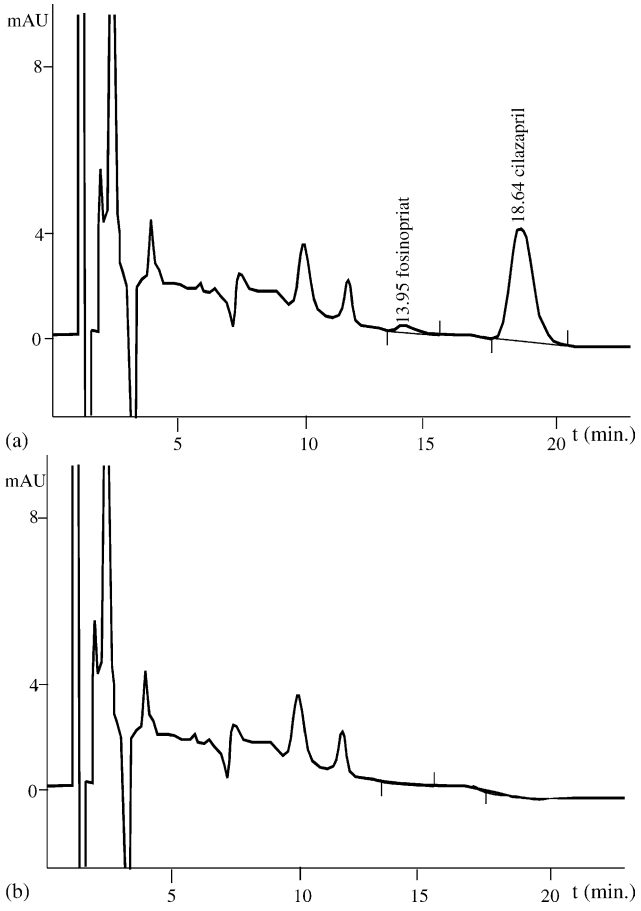

Fig. 2. Representative chromatogram of: (a) medium QC sample and (b) blank plasma (mobile phase consisted of 1.0% (w/v) of diisopropyl ether, 2.0% (w/v) of sodium dodecyl sulphate (SDS), 6.0% (w/v) of *n*-propanol and 91% (w/v) of aqueous 25 mM di-sodium hydrogen phosphate, pH adjusted to 2.8 with 85% orthophosphoric acid; flow rate 1 mL min^{-1}; $\lambda = 220$ nm and temperature 20 °C).

separation and selectivity were obtained with microemulsion containing 6% (w/v) of *n*-propanol as co-surfactant and 2% (w/v) of SDS as surfactant. A representative chromatogram of QC sample containing 0.75 $\mu g\,mL^{-1}$ of fosinoprilat and 20 $\mu g\,mL^{-1}$ of cilazapril is presented in Fig. 2.

The developed bioanalytical method was than subjected to method validation according to FDA and ICH guidelines [17]. In order to prepare validation step pre-validation was executed. The pre-validation step allowed us to: define limit of detection (LOD) for fosinoprilat (0.2 $\mu g\,mL^{-1}$), identify linear response function of the calibration curve, estimate the LLOQ, define the range and the number of calibration levels and to evaluate the selectivity. Before starting experiments the preparation of the stock solution and its dilutions, the QC standards were also defined [18].

Proposed method proved to be specific. The specificity of the method was evaluated by inspection of the chromatogram of a blank sample. No significant interfering peaks were observed at the retention times of fosinoprilat and the internal standard. All the unknown constituents of plasma eluted in

Table 1
Calibration curve parameters

Parameters	Results
Concentration range ($\mu g\,mL^{-1}$)	0.4–27.0
$y = ax + b$	$y = 0.0419x - 0.0029$
r	0.9996
S_a	0.000462
S_b	0.004783
t_b	0.5986

r: correlation coefficient, a: slope, b: intercept, S_a: standard deviation of slope, S_b: standard deviations of the intercept, t_b: value obtained from Student's t-test ($t_{tab.} = 1.859$; $p = 0.05$).

12 min at different retention times of the analytes. Fosinopril, as a prodrug of fosinoprilat, was eluted at 21.0 min.

In order to test method's sensitivity, the LLOQ was measured. Acceptable accuracy (from 93.6% to 111.5% of the nominal concentration) and good precision (9.72% CV) were obtained.

The calibration curve was obtained by unweighted least-squares linear regression of the peak area ratio of fosinoprilat to cilazapril, versus the concentration of fosinoprilat over the range 0.40–27.0 $\mu g\,mL^{-1}$. Linearity was confirmed by calculating the important calibration curve parameters of three different runs which are presented in Table 1: slope (a), intercept (b), correlation coefficient or Pearson's coefficient (r) [19], standard deviation of slope (S_a) and standard deviation of intercept (S_b) which are presented in Table 1.

Data for intra-day precision and accuracy of the assay, measured by analyzing five spiked samples of fosinoprilat at each QC level, are presented in Table 2. QC samples at all levels were analyzed in five replicates in one run. The data in Table 2 show that the method is precise (CV within 4.7%) and accurate (from 96.02% to 103.51% of the nominal concentration).

Results obtained for inter-day precision and accuracy, determined over three days by analyzing five spiked samples of fosinoprilat at each QC level, are given in Table 3.

The results for both, intra- and inter-day precision and accuracy, are within the acceptance criteria.

Table 2
Intra-day accuracy and precision for fosinoprilat determination in spiked plasma samples

	Fosinoprilat concentration in plasma		
	0.75 $\mu g\,mL^{-1}$ (low)	6.0 $\mu g\,mL^{-1}$ (medium)	21.0 $\mu g\,mL^{-1}$ (high)
	0.740	5.737	21.431
	0.721	6.526	21.380
	0.709	6.126	22.218
	0.710	6.066	22.459
	0.721	6.011	21.201
Mean value	0.720	6.093	21.738
SD ($n = 5$)	0.012	0.284	0.561
Precision as RSD (%)	1.7	4.7	2.6
Accuracy as %R	96.02	101.55	103.51

Table 3
Inter-day accuracy and precision for fosinoprilat determination in spiked plasma samples

	Fosinoprilat concentration in plasma		
	0.75 $\mu g\,mL^{-1}$ (low)	6.0 $\mu g\,mL^{-1}$ (medium)	21.0 $\mu g\,mL^{-1}$ (high)
Day 1	0.740	5.737	21.431
	0.721	6.526	21.380
	0.709	6.126	22.218
	0.710	6.066	22.459
	0.721	6.011	21.201
Day 2	0.696	5.904	20.388
	0.709	5.868	21.589
	0.701	5.894	22.034
	0.689	6.006	20.218
	0.666	6.010	21.276
Day 3	0.675	6.012	21.940
	0.828	6.068	22.751
	0.834	6.042	20.814
	0.737	6.242	23.016
	0.828	6.038	22.159
Mean value	0.731	6.037	21.658
SD ($n = 15$)	0.055	0.179	0.817
Precision as RSD (%)	7.5	3.0	3.8
Accuracy as %R	97.45	100.61	103.13

Short-term stability investigation using the QC samples at all levels indicated reliable stability of fosinoprilat at room temperature of at least 5 h (Table 4).

Using QC samples at all levels, the data in Table 5 show that fosinoprilat is stable following three freeze/thaw cycles.

The stability study of fosinoprilat in human plasma showed reliable stability behavior as the mean of the results of the tested samples were within the acceptance criteria. These findings indicated that storage of fosinoprilat's plasma samples at −20 °C is adequate, and no stability-related problems would be expected during the samples routine analysis.

The method was applied to analyze plasma samples obtained after the administration of a single dose of 10 mg Monopril® tablets (inactive ingredients include: lactose, microcrystalline cellulose, crospovidone, povidone and sodium stearyl fumarate) to patients with renal disfunction. The analyses were accomplished in accordance with the FDA

Table 4
Data showing short term stability (room temperature for 5 h) of fosinoprilat at all QC levels

	QC recovery (%)		
	Low	Medium	High
Run #1	93.85	100.24	105.08
Run #2	102.44	96.04	101.07
Run #3	90.66	97.86	101.21
Run #4	102.58	95.73	100.04
Run #5	91.43	100.88	102.04
Mean value	96.19	98.15	101.89
SD	5.88	2.36	1.92
RSD (%)	6.1	2.4	1.9

Table 5
Data showing freeze and thaw stability (three cycles) of fosinoprilat in human plasma at different all QC levels

	QC recovery (%)		
	Low	Medium	High
Cycle 1	102.50	98.86	102.37
	90.59	101.38	101.22
	98.15	102.28	101.61
	92.34	98.97	99.69
	95.85	95.56	100.85
Cycle 2	94.96	101.14	100.51
	94.84	102.43	100.10
	97.47	102.88	100.41
	84.99	98.77	106.12
	90.57	102.15	101.62
Cycle 3	91.32	91.18	95.87
	91.29	91.31	105.23
	91.32	90.61	104.81
	95.32	96.45	101.47
	90.63	93.44	102.76
Mean value	93.48	97.83	101.64
SD	4.2	4.4	2.5
RSD (%)	4.5	4.5	2.5

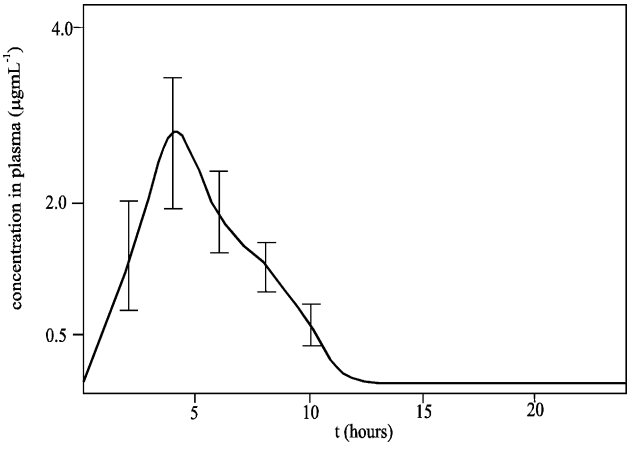

Fig. 3. Representative data showing mean plasma concentration–time profiles of seven patients on haemodialysis after the administration of an oral single dose of 10 mg tablets of fosinopril. The error bars represents ±standard deviation.

bioanalytical method validation guidance [15]. The results from seven patients are presented by graph (Fig. 3).

The obtained results shown that concentration of fosinoprilat in plasma reached the maximum after 4 h of administration.

Some other drugs which are usually applied (Gliclazide, Pentaerythritol Tetranitrate, Pentoxifylline, Amiodarone, Digoxine, Warfarin sodium and Acetylsalicylic acid) in therapy of those patients did not interfere to the determination of fosinoprilat. The retention times are in a first 10 min.

4. Conclusion

The HPLC method, using microemulsion as eluent, for the analysis of fosinoprilat in plasma samples obtained from patients with total renal disfunction was developed, validated and applied. The novelty of method was the possibility of direct analysis of biological samples after proper dilution with microemulsion eluent. The use of a microemulsion mobile phase enabled analysis of plasma samples without prior extraction so the thereby obviating the possibility of any loses was excluded during sample processing. The method was validated in accordance with FDA and ICH regulatives and obtained results are within acceptance criteria. The proposed method proved to be is simpler, easier and less time consuming comparing to previously reported bioanalytical methods.

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