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High-performance liquid chromatography–mass spectrometric analysis of furosemide in plasma and its use in pharmacokinetic studies

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Abstract

This study presents a rapid, specific and sensitive high-performance liquid chromatography–mass spectrometric (LC–MS) assay for the determination of furosemide in human plasma using diclofenac as an internal standard (IS). Both compounds were extracted from human plasma with ethyl acetate at pH 1 and were chromatographed using Shim-Pack GLC-CN column and a mobile phase consisting of acetonitrile and 20 mM ammonium acetate buffer solution pH 7, 4:1 (v/v) at a flow rate 1 ml min^{-1}. Furosemide and IS were detected by mass spectrometer operated in the negative single ion monitoring mode using APCI as an ionization process at m/z 329.2 and 294.1, respectively. The assay linearity of furosemide was confirmed over the range 50–2000 ng ml^{-1}. Detection limit for furosemide in plasma was 10 ng ml^{-1}. The selected concentration range corresponds well with the plasma concentrations of furosemide for pharmacokinetic study. Intraday and interday relative standard deviations were 1.3–4.7 and 2.7–11.5%, respectively. The extraction recovery percentages of furosemide and IS from plasma were in the range 89.3–97.1%. The developed LC–MS procedure was applied for the determination of the pharmacokinetic parameters of furosemide after an oral administration of tablet formulation (40 mg) to two healthy male volunteers. The calculated parameters were in good agreement with the reported values.

Keywords: Furosemide; LC–MS; Pharmacokinetics

1. Introduction

Furosemide is a relatively fast absorbed and rapidly eliminated diuretic which is very useful in clinical practice as a once-daily adult dosage regimen [1]. Although many diuretics are now available, furosemide is still used as a potent diuretic in the treatment of edema associated with pulmonary [2], cardiac and renal failure [3,4] and to control hypertension [5,6]. The pharmacokinetic/pharmacodynamic behavior of furosemide is well documented in healthy individuals [7]. The onset of diuresis following oral administration is within 1 h. The peak effect occurs within 1–2 h and the duration extends for 6–8 h. The drug shows high plasma protein binding and ~3% of the plasma drug concentration remains in the unbound state. These parameters are varied in geriatics [8]. For clinical studies, it is necessary to establish an accurate and specific analytical technique which permits measurement of furosemide in biological specimens at different therapeutic levels. HPLC was extensively applied for the determination of furosemide and its 1-*O*-acyl glucuronide in biological fluids using UV [9,10], fluorescence [11] and electrochemical [12] detectors. Sample preparation for HPLC analysis was done by applying liquid–liquid extraction [13], protein precipitation [14] and solid-phase extraction [15] procedures. In the majority of HPLC methods, a complete resolution of furosemide from the endogenous plasma constituents is necessary to avoid erroneous results. Recently, LC–MS has been used as an alternative of HPLC in many clinical investigations such as metabolic and pharmacokinetic studies [16]. The method provides better efficiency in the detection and quantitation processes, particularly when the ana-

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lytes have poor UV absorption properties and when the analytes are not completely resolved or contaminated with endogenous plasma constituents. The high specificity and sensitivity with marked short-time of analysis are prominent advantages of LC–MS.

The purpose of the present investigation is to elucidate the potential of LC–MS in the determination of furosemide in plasma using one-step extraction procedure. The availability of the developed method for measurement of the pharmacokinetic parameters of furosemide following an oral administration of 40-mg tablet of furosemide to two healthy male volunteers is elucidated.

2. Experimental

2.1. Chemicals and reagents

Furosemide and diclofenac sodium (IS) were purchased from Sigma Co. (St Louis, MO, USA). HPLC grades of acetonitrile and ethyl acetate (APS Ajax, Finechem, Australia) were used. Water was purified by Milli-Q-System from Millipore Corp. (Milford, MA, USA). Other chemicals were of analytical grade.

2.2. LC–MS

The LC–MS system used was from Finnigan Mat (USA) and was composed of high-performance liquid chromatograph (Spectra P 2000) and mass spectrometer detector operating in the negative Atmospheric Pressure Chemical Ionization (APCI) mode at vaporization temperature 450°C. The MS detector was programmed to permit detection and quantitation of the deprotonated molecules $[M–H]^-$ of furosemide and diclofenac in single ion monitoring (SIM) mode at m/z 329.2 and 294.1, respectively. Analytical data were acquired using LCQ software.

Chromatographic analysis of furosemide and diclofenac (IS) were performed on a Shim-pack GLC-CN, 5 μm, column (150 × 6 mm) using a mobile phase composed of acetonitrile and 20 mM ammonium acetate solution (pH ~ 7) in a ratio 4:1 (v/v). Measurements were made at a flow rate 1 ml min^{-1} at ambient temperature using a 10 μl loop.

2.3. Assay procedure

To 1 ml of plasma sample in 10-ml screw-capped tube, a 100 μl aliquot of the internal standard solution (diclofenac sodium, 10 ng μl^{-1} in acetonitrile) and 1 ml

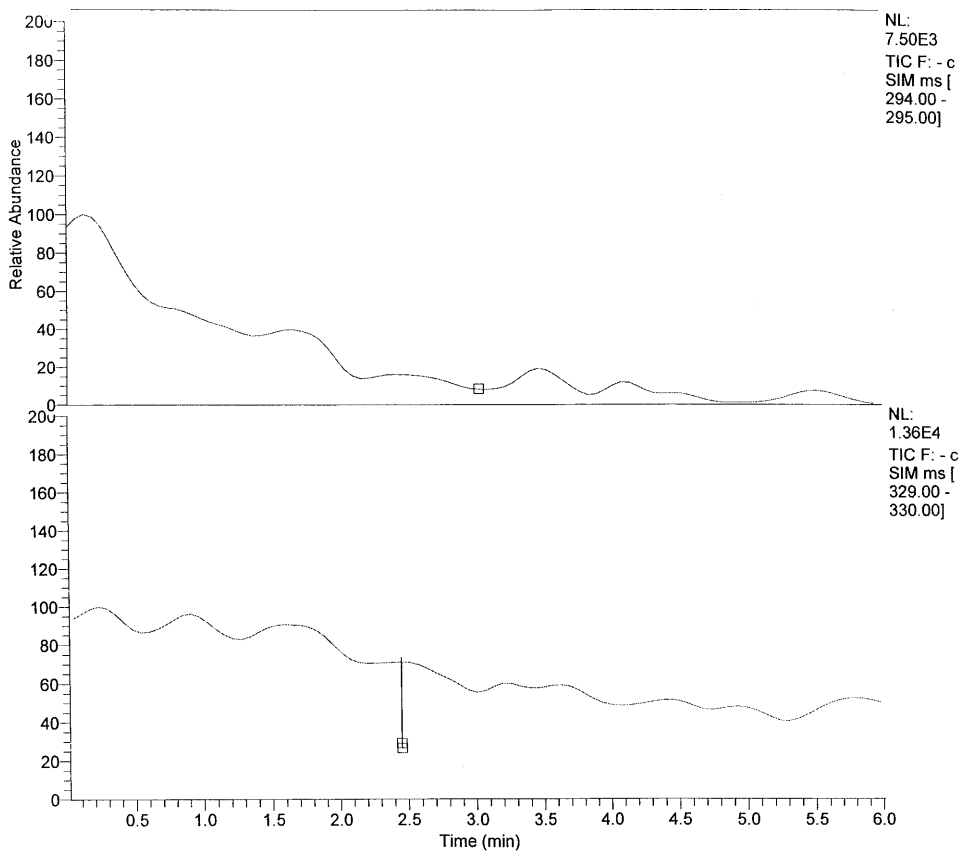

Fig. 1. Representative HPLC–MS chromatograms from blank human plasma sample monitored at m/z 329.2 and 294.1, respectively.

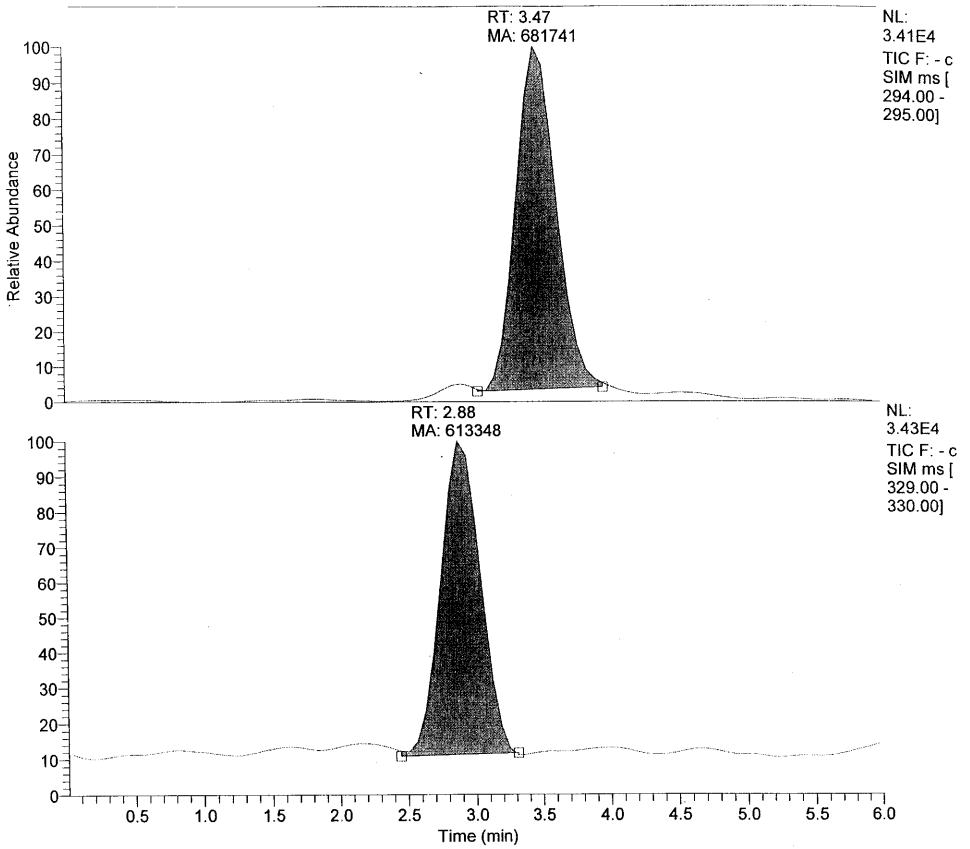

Fig. 2. Representative HPLC–MS chromatograms from extracted human plasma spiked with 500 ng ml^{-1} furosemide, 1000 ng ml^{-1} IS and monitored at m/z 329.2 and 294.1, respectively.

1 M HCl solution were added. The tube was vortexed and the plasma was extracted with 6 ml of ethyl acetate on orbital shaker for 10 min at moderate shaking rate. After centrifugation of the sample at 2000 rpm for 10 min, the organic layer was carefully separated, transferred to 10-ml centrifuge tube and evaporated to dryness under nitrogen gas at 50°C. The residue was reconstituted in the appropriate volume of acetonitrile (100–200 μl) and a 10-μl aliquot was injected into HPLC–MS system. The concentration of furosemide in the sample was calculated from the calibration curve run out simultaneously. Calibration curves of furosemide in plasma were established by spiking 1 ml-plasma samples with 5–200 μl aliquots of furosemide, 10 ng μl^{-1}, in acetonitrile and 100 μl aliquot of IS, 10 ng μl^{-1}, in acetonitrile. The samples were similarly treated as mentioned above. A linear regression equation representing the calibration curve was established by plotting the peak area ratio of furosemide–diclofenac versus furosemide concentration. Peak area values were automatically calculated by LCQ software. Representative chromatograms from blank human plasma, spiked and collected plasma samples, monitored at m/z 329.2 and 294.1 are shown in Figs. 1–3.

2.4. Pharmacokinetic studies

Two healthy male volunteers participated in the study. After fasting overnight, each volunteer was given an oral dose of furosemide (single tablet, 40 mg). Blood samples were collected at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 8.0, 10.0 and 12.0 h. The samples were centrifuged and plasma fractions were separated and stored at −20°C until analysis. The pharmacokinetic parameters, C_{max}, t_{max}, AUC, K_e, $t_{1/2}$, Vd and CL, were calculated using PK-Solutions 2.0-Pharmacokinetics Data Analysis Software (Summit-Research Services).

3. Results and discussion

3.1. Development of LC–MS method

For optimum detection and quantitation of furosemide in plasma by mass spectrometry, it was necessary to adjust the chromatographic and mass spectrometric conditions. A mobile phase composed of acetonitrile and 20 mM ammonium acetate solution, pH 7 in a ratio 4:1 (v/v) and flow rate 1 ml min^{-1} was suitable to ensure complete ionization and detection of furosemide and IS by a MS detector at relatively low concentrations. A high percentage of acetonitrile in the

mobile phase permitted rapid detection of the examined compounds at low retention times (<3.5 min). Furosemide and diclofenac were detected by a MS detector, applying ion trapping technology, as negative molecular ions $[M–H]^-$ at m/z 329.2 and 294.1, respectively. Typical chromatograms of an extracted plasma sample containing furosemide and IS showed peak signals at 2.88 and 3.47 min, respectively (Figs. 2 and 3). The rapid elution of drugs permitted the determination of furosemide concentrations within 3.5 min. The retention times are reproducible as indicated from RSD% values of 0.27 and 0.15% for furosemide and IS, respectively.

3.2. Quantitation

The concentration of furosemide in the plasma samples was calculated from the peak area ratios of furosemide to the internal standard, using the slope and the intercept calculated by the linear regression analysis of the calibration curve data, made on each day of the analysis. A representative calibration curve of furosemide–diclofenac peak area ratio over the concentration range 50–2000 ng ml^{-1} resulted in the following linear regression equation: $Y = 0.0281 + 0.00176X$ ($r = 0.9996$).

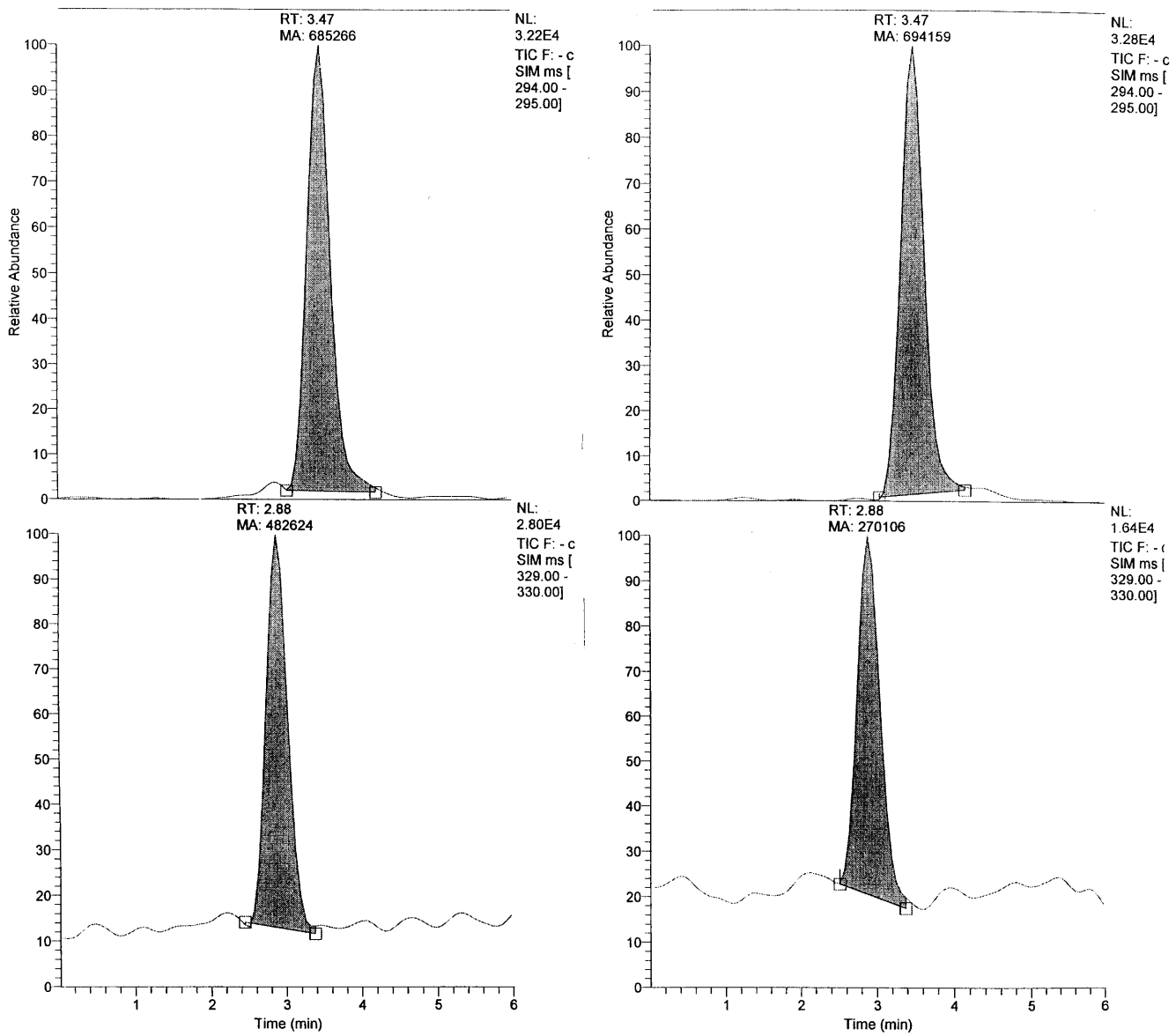

Fig. 3. Representative HPLC–MS chromatograms from extracted human plasma collected 0.5 and 4 h after dosing with furosemide tablets (40 mg) to a healthy male volunteer.

Table 1
Intraday precision and accuracy of LC–MS for determination of furosemide in plasma[a]

	Nominal concentration (ng ml^{-1})		
	100	500	1000
	Calculated concentration (ng ml^{-1})		
	95.0	497.9	1002.7
	97.0	521.0	1014.0
	102.9	504.7	997.1
	105.7	501.9	1019.7
	96.1	510.4	1031.0
Mean	99.3	507.2	1012.9
SD	4.7	8.9	13.5
RSD%	4.7	1.8	1.3
DEV% (bias)	−0.7	+1.4	+1.3

[a] Calibration curve: $a = 0.0281$; $b = 0.00176$; $r = 0.9996$.

Table 2
Interday precision and accuracy of LC–MS for determination of furosemide in plasma

	Nominal concentration (ng ml^{-1})		
Day	100	500	1000
	Calculated concentration		
1	91.6	493.4	991.4
	89.9	486.6	985.8
2	97.2	516.0	1019.7
	102.9	521.7	1031.1
	107.7	492.9	1004.7
3	109.1	538.7	985.8
	114.8	527.4	997.1
	119.9	550.0	1031.1
4	95.1	516.6	993.0
	107.3	510.5	1035.8
	89.0	498.3	1047.9
5	84.7	514.8	975.9
	90.5	518.8	1044.9
	113.5	527.5	1062.2
	119.2	510.2	987.5
n	15	15	15
Mean	102.2	514.9	1012.9
SD	11.8	17.4	27.5
RSD%	11.5	3.4	2.7
DEV% (bias)	+2.2	+3.0	+1.3

Table 3
Recovery percentages of furosemide and IS from human plasma ($n = 10$)

	Recovery (%) ± SD		
	100 (ng ml^{-1})	1000 (ng ml^{-1})	2000 (ng ml^{-1})
Furosemide	97.1 ± 9.3	92.5 ± 1.7	89.3 ± 2.6
Diclofenac (IS)		95.3 ± 3.5	

3.3. Validation of LC − MS method

3.3.1. Linearity

The linearity of LC–MS method for the determination of furosemide was checked over the concentration range 50–2000 ng ml^{-1}. These concentrations used were based on the range expected during pharmacokinetic studies. The variability (mean ± SD) of the slopes, intercepts and regression coefficients of calibration curves constructed in different days were (0.00188 ± 0.00024), (0.0571 ± 0.0186) and (0.9979 ± 0.0028), respectively.

3.3.2. Sensitivity

The limit of quantitation of LC–MS was attained with plasma samples containing 10 ng ml^{-1} of furosemide. The absolute detection limit was 0.1 ng of furosemide.

3.3.3. Specificity

Plasma samples from different sources were found to be free from interfering molecular ions at the retention times of furosemide and internal standard (Fig. 1). Furthermore, no interference was observed from drugs that may be co-administered with furosemide such as hydrochlorothiazide, captopril, enalapril, lisinopril, atenolol and propranolol as these compounds have different molecular ions and can be only measured by monitoring their positive rather than their negative molecular ions.

3.3.4. Precision

The intraday precision of LC–MS was evaluated by replicate ($N = 5$) analysis of plasma samples containing furosemide at three different concentrations 100, 500 and 1000 ng ml^{-1}. The intraday precision showed a relative standard deviation (RSD%) of 1.3–4.7%. The percentage deviation (DEV%) from nominal concentrations was −0.7 + 1.4 (Table 1). The interday precision was evaluated at the above concentration levels for 5 days. The interday RSD% and DEV% were 2.7–11.5 and 1.3–3.0, respectively (Table 2). The data proved good precision and accuracy of the developed LC–MS method.

3.3.5. Recovery

Extraction recoveries of furosemide and IS were quantified at concentrations of 100, 1000, 2000 ng ml^{-1} (furosemide) and 1000 ng ml^{-1} (internal standard). Recoveries of (89.3–97.1%) and 95.3% were calculated for furosemide and diclofenac, respectively (Table 3). The data proved the suitability of ethyl acetate for the extraction of furosemide and IS from acidified plasma samples. The stability of furosemide in spiked plasma samples prepared at concentrations 100, 500 and 1000 ng ml^{-1} and stored at −20°C for 5 days was evaluated

using the proposed LC–MS method. As shown in Table 4, the calculated recovery percentages confirmed good stability of drug in plasma. The data suggest that the plasma samples of furosemide collected from human volunteers and kept frozen at −20°C, can be safely analyzed at least within 5 days from the date of sample collection.

3.4. Clinical application

Fig. 4 shows mean plasma concentration-time profile of furosemide after an oral administration of furosemide tablets (40 mg) to two healthy male volunteers. Pharmacokinetic analysis of the plasma concentration data using PK-Solutions software gave comparable results for both volunteers. Mean values of the different pharmacokinetic parameters were listed in Table 5. As shown, furosemide attained peak concentration (1056 ng ml^{-1}) after a relatively short time (t_{max}, 1.5 h). The area under the plasma concentration-time curve was 3345 ng h ml^{-1} for both AUC_{0-t} and $AUC_{0-\infty}$. The volume of distribution Vd and drug clearance CL were 0.6 l kg^{-1} and 11.9 l h^{-1}, respectively. The pharmacokinetic results confirmed the rapid diuretic action and fast elimination of furosemide as indicated from the small values of t_{max}, $t_{1/2}$, and Vd and the large values of K_e and CL. The calculated data were in good agreement with the reported values [13].

Table 4
Effect of frozen storage at −20°C on the stability of furosemide in spiked plasma samples[a]

Concentration added (ng ml^{-1})	Recovery (%) ± SD
100	102.2 ± 11.8
500	102.9 ± 3.5
1000	101.3 ± 2.7

[a] $n = 15$.

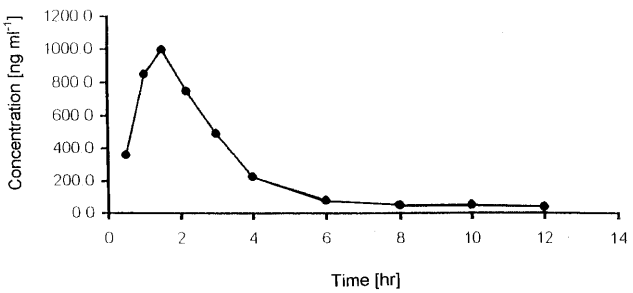

Fig. 4. Mean plasma concentration-time profile of furosemide after oral administration of tablet formulation (40 mg) to two healthy male volunteers.

Table 5
Mean of the pharmacokinetic parameters of furosemide after oral administration of 40-mg oral dose to two healthy male volunteers

Peak plasma concentration (C_{max})	1056.0 ng ml^{-1}
Time of peak concentration (t_{max})	1.5 h
Area under the curve (AUC_{0-t})	3345 ng h ml^{-1}
AUC $_{0-\infty}$	3345 ng h ml^{-1}
Elimination rate constant (K_e)	0.348 h^{-1}
Elimination half life ($t_{1/2}$)	1.99 h
Volume of distribution (Vd)	0.6 l kg^{-1}
Clearance (CL)	11.9 l h^{-1}

4. Conclusions

Robust and reliable APCI LC–MS method for the quantification of furosemide in human plasma has been developed. Method validation has been demonstrated by a variety of tests for accuracy, precision, sensitivity and specificity. The developed LC–MS has several advantages compared to the reported HPLC methods, as it provides lower levels of detection and quantitation of furosemide than UV detection, very low degree of biological matrix interference, unnecessary complete resolution of the analytes and obvious short run-cycle per sample (< 3.5 min). LC–MS method has been successfully applied to evaluate the stability of furosemide in collected human plasma samples as well to establish the pharmacokinetic parameters of drug in human volunteers after single oral dose.

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