ELSEVIER

Journal of Chromatography B, 693 (1997) 383–391

JOURNAL OF CHROMATOGRAPHY B

Determination and pharmacokinetics of a furosemide–amiloride drug combination[1]

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Received 16 July 1996; revised 7 November 1996; accepted 30 December 1996

Abstract

The study presents an accurate and precise HPLC assay for the determination of furosemide and amiloride in human specimens. Both drugs were extracted from human plasma with ethyl acetate; furosemide was extracted at pH 1 and amiloride at pH 12. While chromatographic separation conditions, i.e., column, mobile phase and flow-rate were the same for both investigated drugs, furosemide was detected using a UV absorbance detector, whereas amiloride, because of its very low therapeutic range, was detected with a spectrofluorimetric detector. The linearity of the furosemide and amiloride assays were confirmed over the range of 30–3000 ng/ml and 0.5–30 ng/ml, respectively. These concentrations correspond well with the therapeutic ranges of both drugs. The extraction recoveries, depending on concentration, exceed 80% for furosemide and 74% for amiloride. The reported methods were applied to pharmacokinetic investigations of the two compounds taken in form of a drug combination.

Keywords: Furosemide; Amiloride

1. Introduction

A combination of relatively fast absorbed and eliminated diuretics, such as furosemide and mild, potassium-sparing, with a longer action diuretic, such as amiloride is very useful in clinical practice as a once-daily adult dosage regimen [1–3]. Both drugs are potent diuretics used in the treatment of oedematous status associated with cardiac, renal and hepatic failure, and in the treatment of hypertension [1,4]. The furosemide–amiloride preparation provides a balanced diuretic, reducing potassium loss while avoiding the possible gastro-intestinal disturbances associated with potassium supplements.

The pharmacokinetics of furosemide alone are well documented in healthy subjects. Absorption is rapid and peak plasma levels occur at 60–90 min post-dose. Although, the drug is insoluble in water and favours partitioning into fatty tissue, the high degree of plasma protein binding (97–98%) restricts the volume of distribution, which is relatively low (0.11 l/kg), whereas the elimination is fast ($t_{0.5}$ about 1 h) [1,2,5–7].

Several methods for the assay of furosemide, administered alone, in plasma have been described, including spectrofluorometry [8], gas chromatography [9,10], high-performance liquid-chromatography with liquid–liquid extraction [11–15] and solid-phase extraction [16].

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[1]Presented at the 10th International Symposium on Advances and Applications of Chromatography in Industry, Bratislava, June 30–July 4, 1996.

PII S0378-4347(97)00055-8

Amiloride kinetics are rather poorly documented, and the paucity of data on disposition of this drug is mainly due to the lack of sensitive and specific analytical methods for its measurement in biological fluids. From the literature it appears, that peak plasma levels occur 4 h after oral administration, absorption ranges from 90% to 95% of the dose and half-life ranges from 10–14 h [1,17–19].

Amiloride exhibits an extremely low therapeutic range (0.5–25 ng ml^{-1}), and therefore only a few sufficiently specific and sensitive HPLC methods for pharmacokinetic studies have been described [20–25].

The purpose of the present investigation was to develop assays for furosemide and amiloride and to perform a pharmacokinetic study of both drugs taken concomitantly in one formulation.

2. Experimental

2.1. Apparatus and reagents

The high-performance liquid chromatographic system used was from Kontron Instruments (Zurich, Switzerland, Model 400), and was equipped with a computer system for aquisition and integration of the data (D 450, v. 3.3). Other apparatus consisted of a solvent pump (Model 420), syringe loading sample injector (Model 7125), Rheodyne, Cotati, CA, USA), variable-wavelength UV–Vis absorbance detector (Model 432) as well as an spectrofluorimetric detector (Model SFM 25).

Chromatographic conditions for separation are the same for both investigated drugs. A Nucleosil C_{18}, 250×4.6 mm I.D., 5 μm, column (Macherey Nagel, Duren, Germany) was used. A mobile phase of acetonitrile–0.3 *M* sodium acetate, pH 5.0 (69:31, v/v) was used. This phase was prepared by mixing 690 ml of acetonitrile with 310 ml of water, adding 9.0 ml of glacial acetic acid and adjusting the pH of the solution to 5.0 with 5 *M* NaOH. Measurements were carried out at a flow-rate of 1.5 ml/min in ambient laboratory temperature.

Furosemide and amiloride were kindly supplied by DDSA Pharmaceuticals, London, UK. Nitrazepam and prazosine were obtained from Pharmaceutical Enterprise "Polpharma", Starogard Gdański, Poland. Doubly distilled water was used throughout. Other reagents and solvents were of HPLC grade, obtained from POCh, Gliwice, Poland, except acetonitrile which was purchased from Merck, Darmstadt, Germany. Methanol was additionally double distilled.

2.2. Assay procedure

2.2.1. Furosemide

Plasma concentrations of furosemide were analysed by high-performance liquid chromatography with UV absorbance detection.

To 1.0 ml of plasma in a 10-ml centrifuge tube, 100 μl of internal standard solution (nitrazepam, 10 μg ml^{-1}, in methanol) was added. Each tube was vortexed and the sample buffered with 0.5 ml of 0.2 *M* glycine buffer (pH 0.9) and extracted with 5.0 ml of ethyl acetate. After centrifugation for 10 min at 700 *g*, the aqueous layer was discarded and 4.0 ml of the organic phase was transferred to the conical tube and evaporated to dryness under nitrogen at 60°C. The residue was reconstituted in 200 μl of the mobile phase and a 20-μl aliquot was injected into the chromatographic column. The detection wavelength was set at 280 nm. Typical chromatograms of blank plasma (A) and plasma spiked with 792 ng/ml of furosemide and 1000 ng/ml of internal standard (B) are shown in Fig. 1.

2.2.2. Amiloride

Plasma concentration of amiloride was analysed by high-performance liquid chromatography with spectrofluorimetric detection.

To 1.0 ml of plasma in a 10-ml centrifuge tube, 50 μl of internal standard solution (prazosine, 1 μg ml^{-1} in methanol) was added. The sample was alkalized with 0.5 ml 5 *M* NaOH and extracted with 5.0 ml of ethyl acetate. After centrifugation, 4.0 ml of organic phase was transferred to the conical tube and evaporated to dryness in a water bath at 60°C under a stream of nitrogen. The residue was redissolved in 200 μl of mobile phase and a 20-μl aliquot was injected into the chromatographic column. Excitation wavelength was set at 362 nm, emission wavelength at 414 nm. Typical chromatograms of blank plasma (A) and plasma spiked with 22.3 ng/ml of amiloride and 50 ng/ml of internal standard (B) are shown in Fig. 2.

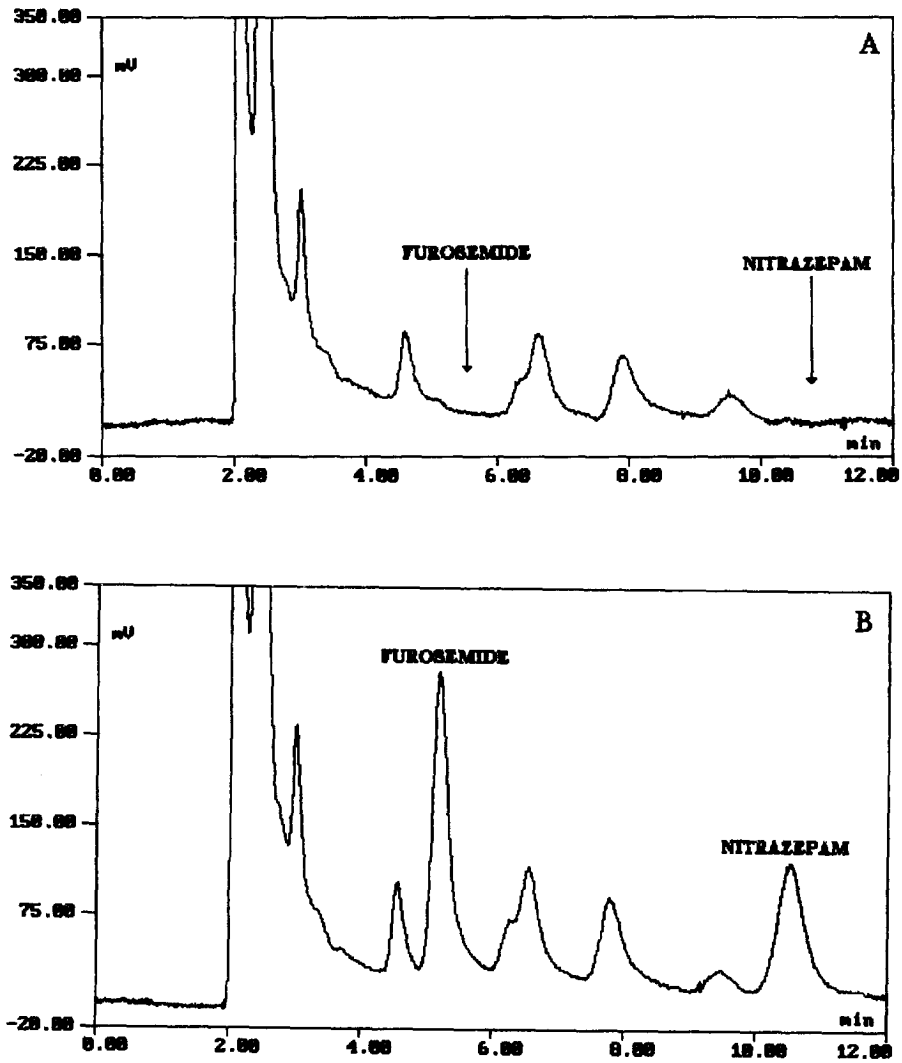

Fig. 1. Chromatograms of blank plasma (A) and plasma obtained from volunteer with concentration of 792 ng/ml of furosemide and 1000 ng/ml of internal standard (B).

The concentrations of both furosemide and amiloride in the samples were calculated from peak height ratios of each compound to its internal standard, using the slope and intercept calculated by linear regression analysis of the calibration curve data, made on each day of analysis.

Calibration curves were made on each day of analysis, using seven concentrations: 50, 100, 300, 1000, 1500, 2000 and 3000 ng/ml for furosemide, and 1, 2, 5, 10, 15, 20 and 30 ng/ml for amiloride. Calibration curves were constructed from one set of spiked plasma samples by plotting the ratio of peak heights for furosemide or amiloride and internal standards, nitrazepam and prazosine respectively, against known concentrations of those compounds.

2.3. Subjects and procedure

Pharmacokinetic studies were carried out on a group of sixteen healthy volunteers, aged 20–26 years, who were taking no concurrent medications or alcohol. All gave prior informed written consent.

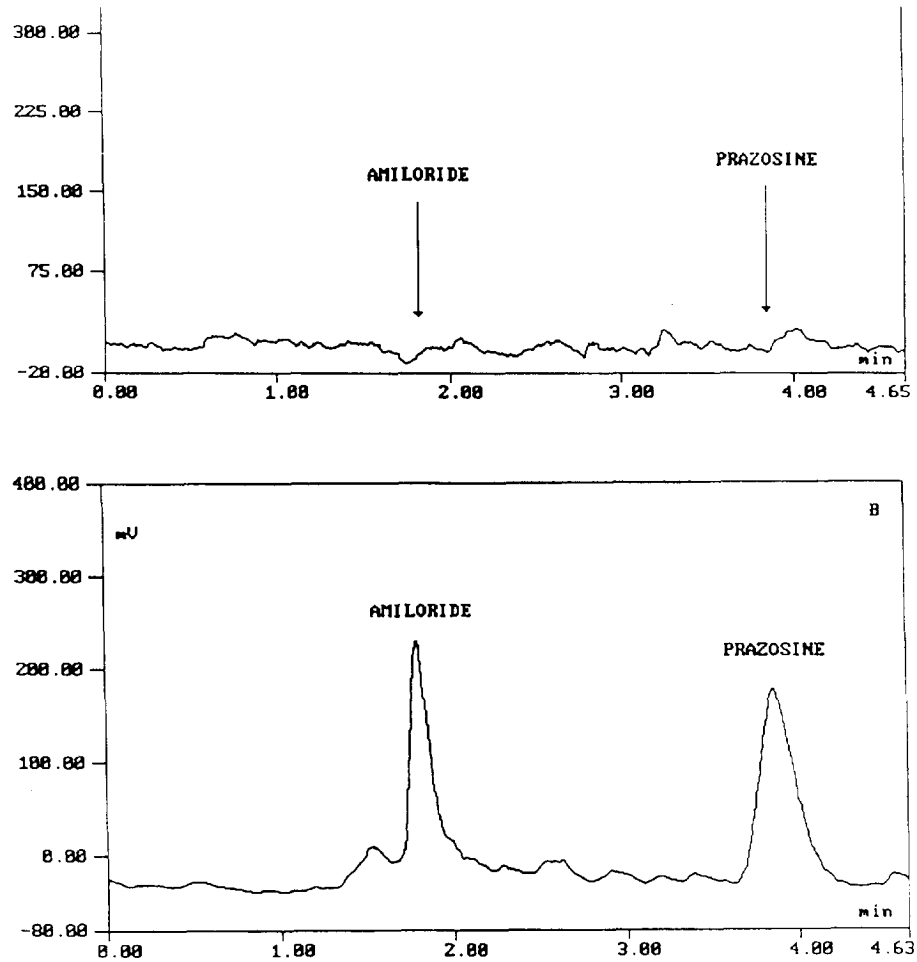

Fig. 2. Chromatogram of blank plasma (A) and plasma obtained from volunteer with concentration of 22.3 ng/ml of amiloride and 50 ng/ml of internal standard (B).

After fasting overnight, each volunteer was administered an oral dose of 80 mg of furosemide and 10 mg of amiloride in form of a complex preparation (two tablets 40 mg/5 mg of Frumil). Subjects received their first meal 3 h after drug administration. Blood samples were withdrawn into heparinised glass tubes prior to the dose and again at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h after drug ingestion. The samples were centrifugated immediately and plasma harvested was stored at −20°C until assay.

The pharmacokinetic parameters – such as maximum of plasma concentration (C_{max}), time to peak (t_{max}) total area under the plasma concentration–time curve (AUC) and half life ($t_{0.5}$) – were obtained for each subject separately and then the average values were calculated.

2.4. Validation of analytical methods

Linearity of the methods were checked in the range of plasma concentration 30–3000 ng/ml and 0.5–30 ng/ml for furosemide and amiloride, respectively. The concentrations used were based on the range expected during pharmacokinetic investigation. Precision and inaccuracy (bias) were estimated on the base of four concentrations of each compound (see Tables 2 and 4). For the within-day precision

Table 1
Precision and accuracy for furosemide during one analytical run

No.	Nominal concentration of furosemide (ng/ml)			
	100	300	1000	3000
	Back calculated concentrations			
1	114	294	992	3198
2	100	303	1050	3063
3	102	303	1005	3037
4	95	293	1020	3247
5	96	307	1005	3228
6	95	340	1057	3389
Mean	100.3	306.5	1021.3	3193.8
S.D.	7.3	17.3	26.4	129.3
C.V. (%)	7.3	5.6	2.6	4.0
Bias (%)	0.3	2.1	2.2	6.5

Calibration curve: $r=0.9997$, $a=0.0020$, $b=-0.019$.

and inaccuracy, pool of 6 ml of plasma was spiked with furosemide (methanol solution) and amiloride (aqueous solution) working standards to obtain concentration of: 100, 300, 1000, 3000 ng/ml for furosemide and 1, 2, 10, 20 ng/ml for amiloride. Between-day validation was calculated for three concentrations (100, 1000, 2000 ng/ml for furosemide and 2, 10, 20 ng/ml for amiloride, Tables 3 and 5, respectively) corresponding to quality control samples of the methods in low, medium and high concentrations. These samples were prepared before experiments, stored in the some conditions as samples taken from volunteers

Table 2
Precision and accuracy for amiloride during one analytical run

No.	Nominal concentration of amiloride (ng/ml)			
	1.0	2.0	10.0	20.0
	Back calculated concentrations			
1	1.05	2.2	8.8	19.4
2	0.97	2.3	10.4	19.4
3	1.10	1.8	11.3	22.6
4	0.85	2.0	8.9	20.9
5	0.92	2.1	8.9	19.6
6	0.89	1.8	9.4	18.1
Mean	0.96	2.03	9.62	19.99
S.D.	0.10	0.21	1.04	1.57
C.V. (%)	10.0	10.2	10.8	7.9
Bias (%)	−3.7	1.7	−3.8	0

Calibration curve: $r=0.9996$, $a=0.0072$, $b=0.0031$.

and were assessed over the current made calibration curve. Limits of quantitation for furosemide and amiloride were calculated from the six independent replications of extraction. Specificity of the assay was determined on the basis of different plasma samples.

Extraction recoveries were quantified at three different concentrations in six replications, of each investigated compound (100, 1000, 2000 ng/ml for furosemide and 1, 10, 20 ng/ml for amiloride). The recoveries of internal standards in concentrations used in the experimental procedure have also been carried out (Tables 6 and 7). They were carried out by comparing peak heights from extracted plasma samples at each concentration with the peak heights obtained from direct injected working standard on chromatography column. The same method was used to assess the recovery of internal standards. The stability of compounds stored in three concentrations (100, 1000, 2000 ng/ml of furosemide and 1, 10, 20 ng/ml of amiloride) after each of the 3 freeze–thaw cycles during one month have also been investigated.

3. Results and discussion

The linearity of the methods was confirmed with precision and inaccuracy below 10%, over a range of 30–3000 ng/ml for furosemide and 0.5–30.0 ng/ml for amiloride. These concentrations cover a whole range of bioanalysis parameters including C_{max}. No interference was found in the region of investigated compounds (Figs. 1 and 2). This confirms specificity of the methods.

The limit of detection (the lowest measurable concentration which can be distinguished from zero, defined as the peak three times that of the baseline noise) was 10 ng/ml for furosemide and 0.25 ng/ml for amiloride. The limit of quantitation (lowest concentration level, which is measured precisely and accurately with %C.V. and %bias less than 15%) was 30 ng/ml for furosemide and 0.5 ng/ml for amiloride (see Table 1). Precision of the assay, calculated as a coefficient of variance for within-day variability, ranged from 2.6% for 1000 ng/ml to 7.3% for 100 ng/ml for furosemide and from 7.9% for 20 ng/ml to 10.8% for 10 ng/ml for amiloride,

Table 3
Precision and accuracy between-days for furosemide

No.	Parameters of calibration curve			Nominal concentrations of furosemide (ng/ml)		
	b	*a*	*r*	100	1000	2000
				Back calculated concentrations		
1	0.0167	0.0019	0.9996	88.1	999.3	2061.1
				88.4	993.6	NR
2	−0.0140	0.0021	0.9994	102.6	860.9	1956.4
				111.5	916.1	1871.7
3	−0.0261	0.0021	0.9997	109.1	1042.1	1964.5
				107.2	1072.9	1978.4
				106.3	1064.5	2194.5
4	0.0128	0.0019	0.9988	102.3	1016.0	2075.7
				NR	1038.3	2077.9
5	−0.0451	0.0017	0.9892	106.1	1093.4	2073.7
				111.1	1127.6	2224.6
				103.8	1084.4	2284.1
				95.8	NR	NR
6	−0.0396	0.0021	0.9987	115.4	1030.6	2113.4
				112.6	855.7	1833.2
				NR	NR	1765.6
n				14	14	14
Mean				104.3	1014.0	2033.9
S.D.				8.4	83.5	149.3
C.V. (%)				8.1	8.2	7.4
Bias (%)				4.3	1.4	1.7

NR: not reported.

respectively (see Tables 3 and 5) and between-day variability ranged from 7.4% for 2000 ng/ml to 8.2% for 1000 ng/ml for furosemide and from 10.4% for 20 ng/ml to 8.1% for 2 ng/ml for amiloride, respectively. Inaccuracy of the methods for within-day variability ranged from 6.5% for 3000 ng/ml to 0.3% for 100 ng/ml for furosemide and 3.8% for 10 ng/ml to 1.7% for 2 ng/ml for amiloride, respectively (Tables 2 and 4).

Mean recoveries of extraction for three different concentrations of furosemide (100, 1000, 3000 ng/ml), as well as internal standard (1000 ng/ml) in human plasma exceeded 80% (Table 6). Extraction yield of amiloride for three different concentrations (2, 10, 20 ng/ml) were 78.8%, 74.7% and 74.0%, respectively, whereas mean recovery of internal standard at concentration 50 ng/ml was 80.2% (Table 7).

Freeze–thaw stability test made three times during one month in three different concentrations confirms the stability of both compounds during these operations. The average recovery after the third thaw was 95.8% for furosemide and 95.4% for amiloride.

The reported methods were applied to human pharmacokinetic investigations of these drugs in combined preparation.

Pharmacokinetic parameters after oral administration of two tablets of 40 mg/5 mg (furosemide–amiloride) are listed in Table 8. Furosemide has been shown to be rapidly absorbed, reaching peak concentration (C_{max}) on the level of 2.373±1124 mg/ml after relatively short time (t_{max}=1.50±0.71 h). Amiloride absorbs more slowly, reaching peak concentration (C_{max}=26.7±9.07 mg/ml), after longer time (t_{max}=2.97±0.74). Elimination of furosemide is faster ($t_{0.5}$=1.91±0.55 h) and is five times shorter in comparison with amiloride ($t_{0.5}$=9.47±1.64 h). The combination of these two diuretics, taken concomitantly in one combined tablet, provides a long acting preparation with two mechanisms of action.

Average serum profiles of furosemide and amiloride using two scales of concentration are

Table 4
Precision and accuracy between-days for amiloride

No.	Parameters of calibration curve			Nominal concentrations of amiloride ng/ml		
	b	*a*	*r*	2.0	10.0	20.0
				Back calculated concentrations		
1	0.0310	0.1240	0.9999	1.8	10.2	17.5
				1.9	11.2	18.7
2	−0.0211	0.1196	0.9990	2.3	11.0	20.3
				1.8	10.7	21.9
				1.8	11.4	17.1
				NR	NR	20.1
3	0.0217	0.1397	0.9904	1.9	8.4	16.4
				1.9	9.2	18.9
				1.8	9.7	NR
4	−0.0030	0.1209	0.9990	2.3	11.2	19.7
				1.9	11.1	19.3
5	−0.0068	0.1100	0.9984	1.8	11.0	20.3
				1.9	9.2	21.6
				1.8	9.4	21.2
6	0.0014	0.1172	0.9997	1.7	10.6	19.6
				2.0	8.8	17.0
				2.0	8.6	18.7
n				16	16	16
Mean				1.91	10.11	19.27
S.D.				0.17	1.05	1.66
C.V. (%)				8.1	10.4	8.6
Bias (%)				−4.4	1.1	−3.7

NR: not reported.

Table 5
Recovery of furosemide and internal standard from human plasma (mean±C.V., $n=6$)

Compound	Recovery (%)		
	100 (ng/ml)	1000 (ng/ml)	3000 (ng/ml)
Furosemide	80.3±11.3	80.1±3.0	80.2±4.9
Internal standard	NC	81.8±3.9	NC

NC: not calculated.

shown in Fig. 3. This figure confirms, rapid diuretic action caused by furosemide and long diuretic action maintained by amiloride.

4. Conclusions

Satisfactory qualitative separation and quantitative determination of furosemide and amiloride in human

Table 6
Recovery of amiloride and internal standard from human plasma (mean±C.V., $n=6$)

Compound	Recovery (%)			
	2.0 (ng/ml)	10.0 (ng/ml)	20.0 (ng/ml)	50.0 (ng/ml)
Amiloride	78.8±13.0	74.7±1.4	73.9±6.8	NC
Internal standard	NC	NC	NC	80.2±5.1

NC: not calculated.

Table 7
Limit of quantitaton for furosemide and amiloride

No.	Limit of quantitation (ng/ml)	
	Nominal concentration of furosemide 30 ng/ml	Nominal concentration of amiloride 0.5 ng/ml
1	32.1	0.55
2	33.1	0.47
3	33.8	0.56
4	27.0	0.53
5	29.4	0.57
6	34.0	0.45
Mean	31.6	0.52
S.D.	2.8	0.05
C.V. (%)	8.9	9.6
Bias (%)	5.2	4.3

Calibration curve for furosemide: $r=0.9997$, $a=0.0020$, $b=-0.019$.
Calibration curve for amiloride: $r=0.9996$, $a=0.0072$, $b=0.0031$.

Table 8
Pharmacokinetic parameters of furosemide and amiloride

Compound	C_{max} (ng/ml)	t_{max} (h)	AUC (ng×h/ml)	$t_{0.5}$ (h)
Furosemide	2373±1124	1.50±0.71	5971±2303	1.91±0.55
Amiloride	26.7±9.07	2.97±0.74	287.4±88.7	9.47±1.64

plasma, within their therapeutic ranges, has been described. This method has been successfully applied to pharmacokinetic investigations after ingestion of combined preparation. Pharmacokinetic results obtained confirm a rapid start of diuretic action caused by furosemide and maintenance of this action by amiloride.

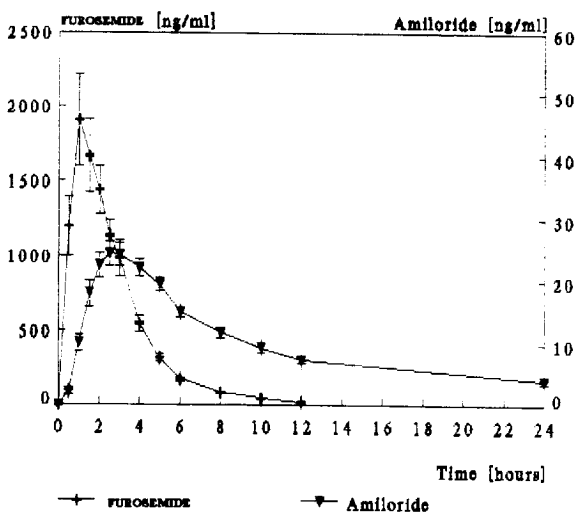

Fig. 3. Average serum concentration–time profiles of furosemide and amiloride after oral administration of two complex tablets of furosemide–amiloride (40 mg:5 mg).

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