Journal of Chromatography, 575 (1992) 269–274
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6226

Simultaneous determination of furosemide and amiloride in plasma using high-performance liquid chromatography with fluorescence detection

H. J. E. M. Reeuwijk, U. R. Tjaden* and J. van der Greef

Division of Analytical Chemistry, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden (Netherlands)

(First received October 21st, 1991; revised manuscript received November 26th, 1991)

ABSTRACT

A high-performance liquid chromatographic method using fluorescence detection for the simultaneous determination of furosemide and amiloride is described. The chromatographic system is based on reversed-phase ion-pair chromatography with sodium dodecylsulphate as ion-pairing agent. The same counter-ion is used for the ion-pair liquid–liquid extraction to ethyl acetate. The minimum detectable concentration amounts to 0.3 ng of furosemide and 0.03 ng of amiloride per ml of plasma. The applicability of the method is demonstrated by the analysis of plasma samples taken from volunteers receiving both drugs.

INTRODUCTION

Furosemide is an anthranilic acid derivative. It chemically resembles the thiazide diuretics, but has a much stronger diuretic potential. Furosemide exerts its action on the luminal side of the thick ascending limb of Henle's loop by inhibiting chloride transport or by inhibiting sodium chloride cotransport. This may provoke excessive cation loss of magnesium and particularly potassium, thus causing hypokalaemia. Low plasma potassium levels may cause ventricular ectopic activity, ventricular fibrillation and sudden death.

Potassium-sparing diuretics have been widely described for prophylaxis and treatment of hypokalaemia. Amiloride is such a drug; it acts on the distal tubule to promote sodium excretion and potassium reabsorption. The drug is only weakly diuretic and its potassium-sparing action predominates. The combined administration of furosemide and amiloride was introduced in 1984 [1]. It has been proved to be effective in patients with congestive heart failure and in patients with mild to moderate essential hypertension [2–4]. This combination has also proved to be more effective during long-term treatment than the combination of amiloride and a thiazide diuretic [4].

In the literature, almost all high-performance liquid chromatographic (HPLC) methods for amiloride [5–10] and furosemide [11–20] apply reversed-phase (RP) systems using methanol or acetonitrile as modifier, with the pH of the mobile phase in the range 2.5–5. Only one reversed-phase ion-pair system has been applied for the determination of amiloride [21]. The pretreatment of amiloride-containing plasma samples has been described for either protein precipitation [7,10], alkaline liquid–liquid extraction [5,8,21], solid-phase extraction [6] or precolumn concentration [9]. Sample pretreatment for furosemide has been described for protein precipita-

0378-4347/92/$05.00

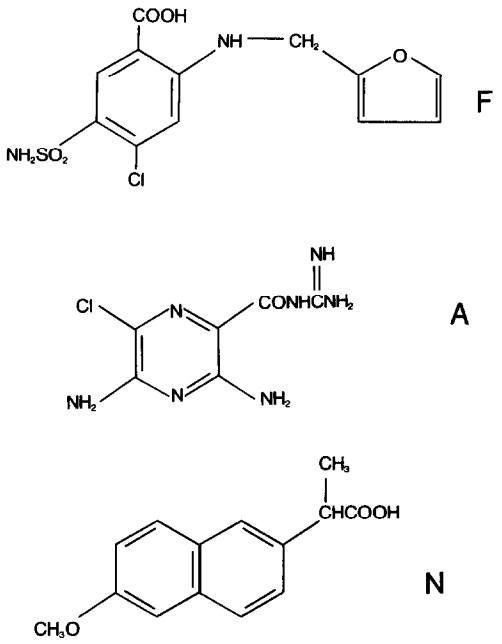

Fig. 1. Structures of furosemide (F), amiloride (A) and naproxen (N).

tion [16,18,19], liquid–liquid extraction in acidic medium [13–15,17,20], solid-phase extraction [11,12], and direct plasma injection [22].

From their molecular structures (Fig. 1), it is clear why different separation mechanisms have to be combined in order to separate both substances within an acceptable total analysis time. The method described in this paper uses ion-pair liquid–liquid extraction of furosemide and amiloride as the pretreatment step. The developed method enables the simultaneous determination of both compounds in plasma taken from volunteers administered orally with 40 mg of furosemide and 5 mg of amiloride concomitantly.

EXPERIMENTAL

Chemicals

Furosemide and naproxen, the internal standard, were obtained from Aldrich (Milwaukee, WI, USA) and amiloride hydrochloride from Sigma (St. Louis, MO, USA). Ethyl acetate (Baker Chemicals, Deventer, Netherlands) was saturated with water before use. Sodium dodecylsulphate (SDS) was of high-purity grade (Serva, Heidelberg, Germany). Perchloric acid solution for the mobile phase was prepared from concentrated perchloric acid (80%) by diluting with deionized water (Milli-Q water purification system, Millipore, Bedford, MA, USA) until pH 2.0 was reached. All other reagents were of analytical grade and were used as such. The stock solutions of the three compounds, containing 1 mg/ml methanol, were made freshly every month and protected from daylight with aluminium foil and stored at 4°C. Dilutions of the stock solutions for calibration curves were made freshly every day.

Apparatus

The LC system consisted of a Jasco Model 880-PU isocratic pump (Japan Spectroscopic, Tokyo, Japan) and a Promis autosampler (Spark Holland, Emmen, Netherlands). Fluorescence detection was performed with a Perkin Elmer LS-4 detector (Beaconsfield, UK) operated at an excitation wavelength of 360 nm and an emission wavelength of 413 nm, with an excitation slit width of 15 nm and an emission slit width of 20 nm. The detector signal was monitored by an Axxi-Chrom 727 chromatography data station (Axxiom Chromatography, Calabasas, CA, USA).

Chromatography

Chromatography was performed on a glass Nucleosil 100 C_{18} 5-μm column (100 mm × 3 mm I.D.) (Chrompack, Bergen op Zoom, Netherlands), equipped with a hand-packed guard column (10 mm × 2 mm I.D.), which was packed with Nucleosil 100 C_{18} 5-μm stationary phase (Macherey Nagel, Düren, Germany).

The eluent was acetonitrile–0.125 *M* SDS–0.01 *M* perchloric acid (pH 2.0) (234.6:35:665, w/w). Chromatography was performed at ambient temperature at a flow-rate of 0.6 ml/min.

Sample preparation and storage

Blood samples of 5 ml were taken over defined time intervals during the 24 h after co-administration of one tablet of Lasix containing 40 mg of furosemide and one tablet of Midamor containing 5 mg of amiloride. The blood samples were

collected in lithium-heparinized tubes, and plasma was separated by centrifugation and stored in polypropylene tubes at −40°C until analysis.

Sample pretreatment

Aliquots of 1.00 ml of defrosted plasma samples were mixed with 0.25 ml of acetic acid (8.5 *M*) in a polypropylene tube. After the addition of 0.25 ml of SDS solution (0.125 *M*) and mixing for 5 s, 100 μl (corresponding to 4 μg of naproxen) of a methanolic internal standard solution were added. This mixture was extracted for 30 min on a laboratory-made rotating mixing device (60 rpm) with 7 ml of ethyl acetate saturated with water, and subsequently centrifuged for 10 min at 5200 *g*. The resulting organic phase was evaporated in a vortex evaporator (Haake Büchler Instruments, Lenexa, KS, USA) at 35°C. The residue was dissolved in 0.25 ml of mobile phase, and 100 μl were analysed on the chromatographic system.

Calibration standards were obtained by adding known amounts of the analytes to blank plasma samples. The sample pretreatment was as described above.

In order to avoid artefact formation by degradation as a consequence of the photosensitivity of furosemide, both the sample pretreatment and the analysis were executed in a darkened room illuminated by a sodium lamp. Under these conditions no degradation peaks were observed.

RESULTS AND DISCUSSION

Chromatography

The separation of furosemide and amiloride is strongly dependent on the choice of stationary phase, pH and ionic strength. The shape of the amiloride peak, in particular, is poor when another type (Phase Sep ODS 3) column is used, possibly due to the interaction of amiloride with free silanol groups.

After injection of *ca.* 200–300 samples a slight loss of retention may occur, which can be resolved by washing the column with *ca.* 50 ml of pure methanol and re-equilibration with the mobile phase.

Detection

In the literature both UV absorbance [5,6,18,21] and fluorescence detection [7–17,19–21] have been described for both analytes. Using fluorescence detection lower concentrations of the compounds of interest can be detected. The two analytes have different excitation and emission wavelengths. Furosemide has an excitation wavelength of 268 nm. Depending on the pH, different emission wavelengths can be applied. In the pH range between 2 and 5 emission can be observed at 410 nm, whereas at pH 6–9 an emission wavelength of 385 nm is more favourable. Amiloride can be excited at 285 nm as well as at 360 nm, and the emission wavelength for both excitation wavelengths is 413 nm.

The fluorescence in pure acetonitrile for furosemide and amiloride, respectively, is *ca.* 3.5 and 1.3 times higher than in pure methanol.

Sample pretreatment

Different sample pretreatment methods can be applied, but for the simultaneous determinations of amiloride and furosemide only a few are suitable, such as injection of the supernatant after deproteination and liquid–liquid extraction using ion-pair formation. In our hands, the direct precipitation of proteins did not give satisfactory results for furosemide: the recoveries of furosemide and amiloride were 10–40 and 50–70%, respectively, depending on the method of deproteination.

The sample pretreatment of plasma is a modification of a liquid–liquid extraction described in literature [13]. Instead of dichloromethane, ethyl acetate was used because, with respect to the recovery and interferences, ethyl acetate appeared to be easier to handle in routine bioanalysis. Liquid–liquid extraction with ethyl acetate under acidic conditions delivered only furosemide, and under alkaline conditions only the amiloride was extracted. Use of a cationic ion-pairing agent, *i.e.* tetrabutylammonium bromide, decreased the stability of the chromatographic system. Therefore the extraction under acidic conditions with an anionic ion-pairing agent, such as hexane sulphonate or sodium dodecylsulphate, was investigat-

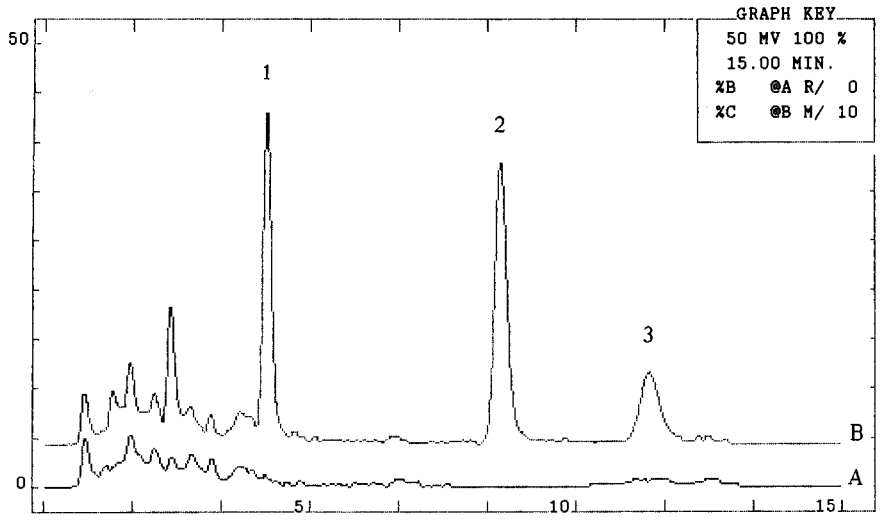

Fig. 2. Chromatograms of an extract of plasma from a volunteer receiving an oral dose of 40 mg of furosemide and 5 mg of amiloride, (A) before administration and (B) 360 min after administration. Peaks: 1 = furosemide (46 ng/ml); 2 = amiloride (4.0 ng/ml); 3 = naproxen (internal standard). Conditions as in Experimental.

ed. The recovery obtained with SDS appeared to be more favourable than that with hexane sulphonate. The remaining SDS in the residue of the liquid–liquid extraction did not disturb the HPLC system, because of the presence of SDS in the mobile phase. Optimization of the concentration of SDS added to the plasma mixture with respect to the retention in the chromatographic system and the recovery in the sample pretreatment resulted in the sample pretreatment described. An example of a chromatogram of an extract of a real plasma sample is given in Fig. 2. No metabolites could be observed. No data are given in the literature on the presence of metabolites.

TABLE I

VALIDATION OF THE METHOD

Concentration (ng/ml)	Recovery (mean ± C.V., n = 3) (%)	Precision (%)		Accuracy (%)
		Day-to-day (n = 3)	Within-day (n = 3)	
Furosemide				
10	70.4 ± 17.8	0.98	2.21	98.0
100	84.2 ± 4.1	2.46	3.46	97.7
1000	82.1 ± 4.8	1.58	3.02	98.2
Mean	80.7 ± 5.0			
Amiloride				
0.5	52.6 ± 10.8	2.69	4.08	99.2
5	55.8 ± 12.9	1.49	2.66	97.4
25	62.5 ± 5.0	2.54	1.80	98.9
Mean	60.7 ± 5.5			

Validation

The method was validated by the three-fold assay on three consecutive days of blank plasma samples to which different amounts of both compounds had been added. Eight plasma samples of 1.0 ml were spiked with concentrations ranging from 10 to 1000 ng of furosemide per ml and from 0.5 to 50 ng of amiloride per ml, covering the relevant concentration ranges.

Typical regression lines with the standard deviation of slope and intercept are: $y = (0.0441 \pm 0.0001)x - (0.0479 \pm 0.0141)$ and $y = (0.4011 \pm 0.0068)x + (0.0950 \pm 0.0082)$ for furosemide and amiloride, respectively, where y represents the peak-area ratio and x the concentration in ng/ml. The results of the validation are summarized in Table I.

All calculations were performed using weighted regression, because of the wide range of concentrations. The method is linear for furosemide up to 1 μg/ml and for amiloride up to at least 25 ng/ml. Samples with concentrations of furosemide above 1 μg/ml were diluted with blank plasma.

The detection limits, based on a signal-to-noise ratio of 3, amounted to 100 and 7.5 pg for furosemide and amiloride, respectively. The corresponding minimal detectable concentrations in plasma for furosemide and amiloride were 0.3 and 0.03 ng/ml, respectively.

Drug monitoring

An example of mean plasma concentration–time curves after oral administration of furosemide and amiloride is presented in Fig. 3. The concentration of amiloride is considerably lower than that of furosemide, which is why the detection has been optimized for amiloride. The large standard deviation in the furosemide concentrations is a well known phenomenon [22,23]; the mean bioavailability calculated from plasma concentrations after oral administration ranges from 43 to 71%.

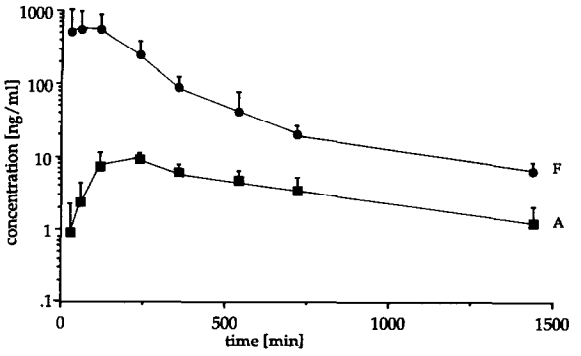

Fig. 3. Mean plasma concentration–time curves (+S.D.) for four volunteers after oral administration of 40 mg of furosemide (F) and 5 mg of amiloride (A).

CONCLUSIONS

The method is based on liquid–liquid ion-pair extraction and reversed-phase ion-pair HPLC separation with fluorescence detection and permits the measurement of plasma samples when 40 mg of furosemide and 5 mg of amiloride are administered orally. The assay provides good sensitivity, linearity and reproducibility. The advantage is a simultaneous determination of furosemide and amiloride in plasma, with respect to the plasma volume, needed for the analysis of low plasma levels of amiloride.

ACKNOWLEDGEMENTS

The authors thank Dr. A. F. Cohen, Drs. J. M. T. van Griensven and Ms. R. Kroon of the Centre for Human Drug Research (Leiden, Netherlands) for kindly supplying the plasma samples.

REFERENCES

1 S. G. Brooks, R. B. Christie, J. Roche, A. P. Fairhead, D. Muirhead, H. A. Townsend and H. L. Shaw, *Curr. Med. Res. Opin.* 9 (1984) 141.
2 R. J. Crawford, S. Allman, S. P. Kitchen and H. H. Richards, *Curr. Med. Res. Opin.*, 10 (1988) 675.
3 H. A. Townsend, A. L. Waddy, C. T. Eason and H. H. Richards, *Curr. Med. Res. Opin.*, 9 (1984) 132.
4 F. Ramsey, R. J. Crawford, S. Allman, R. Bailey and A. Martin, *Curr. Med. Res. Opin.*, 10 (1988) 682.
5 Q. C. Meng, Y. F. Chen and S. Oparil, *J. Chromatogr.*, 529 (1990) 201.
6 G. Forrest, G. T. McInnes, A. P. Fairhead, G. G. Thompson and M. J. Brodie, *J. Chromatogr.*, 428 (1988) 123.
7 R. J. Y. Shi, L. Benet and E. T. Lin, *J. Chromatogr.*, 377 (1986) 399.

8 M. S. Yip, P. E. Coates and J. J. Thiessen, *J. Chromatogr.*, 307 (1984) 343.
9 E. Bechgaard, *J. Chromatogr.*, 490 (1989) 219.
10 D. Xu, J. Zhou, Y. Yuan, X. Liu and S. Huang, *J. Chromatogr.*, 567 (1991) 451.
11 W. Radeck and M. Heller, *J. Chromatogr.*, 497 (1989) 367.
12 F. G. M. Russel, Y. Tan, J. J. M. van Meyel, F. W. J. Gribnau and C. A. M. van Ginneken, *J. Chromatogr.*, 496 (1989) 234.
13 L. J. Lovett, G. Nygard, P. Dura and S. K. W. Khalil, *J. Liq. Chromatogr.*, 8 (1985) 1611.
14 K. Uchino, S. Isozaki, Y. Saitoh, F. Nakagawa, Z. Tamura and N. Tanaka, *J. Chromatogr.*, 308 (1984) 241.
15 A. L. M. Kerremans, Y. Tan, C. A. M. van Ginneken and F. W. J. Gribnau, *J. Chromatogr.*, 229 (1982) 129.
16 R. S. Rapaka, J. Roth, C. T. Viswanathan, T. J. Goehl, V. K. Prasad and B. E. Cabana, *J. Chromatogr.*, 227 (1982) 463.
17 S. E. Szwezey, P. J. Meffin and T. F. Blaschke, *J. Chromatogr.*, 174 (1979) 469.
18 E. T. Lin, D. E. Smith, L. Z. Benet and B. Hoener, *J. Chromatogr.*, 163 (1979) 315.
19 R. L. Nation, G. W. Peng and W. L. Chiou, *J. Chromatogr.*, 162 (1979) 88.
20 K. Carr, A. Rane and J. C. Fröhlich, *J. Chromatogr.*, 145 (1978) 421.
21 M. J. van der Meer and L. W. Brown, *J. Chromatogr.*, 423 (1987) 351.
22 C. T. Santasania, *J. Liq. Chromatogr.*, 13 (1990) 2605.
23 M. Saugy, P. Meuwly, A. Munafo and L. Rivier, *J. Chromatogr.*, 564 (1991) 567.