ELSEVIER

Journal of Chromatography B, 658 (1994) 303–310

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic determination of spironolactone and its major metabolite canrenone in urine using ultraviolet detection and column-switching

Rosa Herráez-Hernández, Eva Soriano-Vega, Pilar Campíns-Falcó*

Departamento de Química Analítica, Facultad de Química, Universidad de Valencia, Dr. Moliner 50, 46100 Burjassot (Valencia), Spain

First received 20 January 1994; revised manuscript received 2 May 1994

Abstract

A rapid and simple column liquid chromatographic method involving a column-switching system for the determination of spironolactone and its main metabolite canrenone in urine is described. Purification and concentration was performed using an Hypersil ODS-C_{18}, 30 μm (20 × 2.1 mm I.D.) pre-column. The most polar urinary compounds were removed by washing the pre-column with water, and the analytes were subsequently switched to a LiChrospher RP C_{18}, 5 μm (125 × 4 mm I.D.) analytical column and separated by means of an acetonitrile–water mobile-phase. Under the proposed conditions, the extraction efficiency was approximately 100% over the 0.5–10.0 μg/ml concentration range. The limits of detection were 20 ng/ml for both compounds. The proposed method has been applied to urine samples obtained after the oral administration of spironolactone.

1. Introduction

Spironolactone (Fig. 1a) is a competitive inhibitor of aldosterone, which is widely used in the treatment of edematous states, as an adjunct to thiazide diuretics in the treatment of essential hypertension, and in several pathological conditions associated with hyperaldosteronism [1–5]. Spironolactone is extensively metabolized to different metabolites. It is generally accepted that the metabolite canrenone (Fig. 1b) is the major circulating form of the drug; the sulphur-containing metabolites 7α-thio-spironolactone and 7α-thiomethyl-spironolactone are also important in mediating the biological effects of the parent drug [6,7].

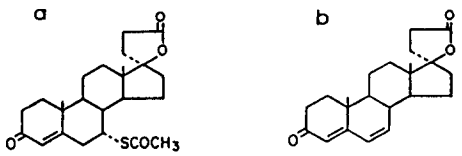

Fig. 1. Chemical structure of spironolactone (a) and canrenone (b).

The quantification of spironolactone in urine has been found to be essential in patients on diuretic therapy, but most of the reported procedures are dealing with the determination of spironolactone and metabolites in plasma or serum samples [8–11]. In addition, the sample

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00241-V

treatments used in such procedures are based on liquid–liquid extraction of the analytes from the samples; however, liquid–liquid extraction techniques are particularly problematic in the analysis of spironolactone and metabolites owing to the low stability of these compounds in several organic solvents [10,12]. The solvent-induced degradation of spironolactone and metabolites may explain the poor recoveries, and low reproducibility obtained by different authors [9].

Recently, Varin et al. [12] have proposed an assay for the determination of spironolactone and its main metabolites in plasma and urine, based on solid-pase extraction columns. The sequential utilization of C_{18} and CN columns prior to separation on a reversed-phase column, provided adequate selectivity and a recovery of the important compounds higher than 80% in plasma, and higher than 72% in urine. However, since two consecutive extraction steps are necessary, numerous handling steps are involved (conditioning of the columns, elution and washing of the samples, evaporation and redissolution of the extracts, etc.) resulting in a very time-consuming procedure. In addition, the evaporated residues must be stored at −20°C until HPLC analysis to prevent their degradation.

A reversed-phase LC method using solid-phase extraction and UV detection at 280 nm for the determination of canrenone in human serum is reported by Zhang and Stewart [13]. The C_{18} extraction column used was washed with one column volume of water and the drug was eluted using two 500-μl aliquots of acetonitrile. The recoveries of the drug were ca. 100%. Canrenone was well separated from spironolactone and its other metabolites. At 280 nm, i.e. the wavelength at which canrenone has its absorption maximum, the absorbancies of spironolactone and its metabolites are less interfering.

Since solid-phase extraction by column-switching eliminates intermediate off-line steps (such as evaporation or redissolution), we have evaluated the usefulness of switching techniques in the analysis of spironolactone in urine. This study also includes canrenone, which is the predominant form of the drug found in urine.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a quaternary pump (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA), an automatic sample injector (Hewlett-Packard, 1050 series) and a high-pressure six-port valve (Rheodyne Model 7000). A diode-array detector (Hewlett-Packard, 1040 series) linked to a data system (Hewlett-Packard HPLC Chem Station, Dos Series) was used for data acquisition and storage. The detector was set to collect a spectrum every 640 ms, over the range 200–400 nm. The chromatographic signal was monitored at 230 and 300 nm for the quantification of spironolactone and canrenone, respectively. All the assays were carried out at ambient temperature.

2.2. Reagents

All the reagents were of analytical grade. Acetonitrile was of HPLC grade (Scharlau, Barcelona, Spain). Water was distilled, deionized and filtered over 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain). Spironolactone and canrenone were provided by Searle Ibérica S.A. (Madrid, Spain).

2.3. Standard solutions

Standard solutions of the analytes were prepared by dissolving the pure compounds in methanol (100 μg/ml). These solutions were stored in the dark at 2°C; under such conditions, the standard solutions are stable at least for a month and no degradation products were observed.

Working solutions were prepared daily by dilution of the stock solutions with the appropriate volumes of water.

2.4. Columns and mobile-phases

The pre-column (20 × 2.1 mm I.D.) was dry-packed with Hypersil ODS-C_{18}, 30 μm (Hew-

lett-Packard, Germany) stationary-phase. The analytical column was an HP-LiChrospher 100 RP 18, 125 × 4 mm I.D., 5 μm, column (Merck, Darmstadt, Germany) or an HP-Hypersyl ODS-C_{18}, 250 × 4 mm I.D., 5 μm (Hewlett-Packard, Germany).

Purified water was used for washing the pre-column, and acetonitrile–water in gradient elution mode was used for the analytical separation. The mobile-phases were prepared daily, filtered over a 0.45-μm nylon membrane (Teknokroma) and degassed with helium before use.

2.5. Column-switching operation

At the beginning of each assay, 50 μl of sample were injected from the injector to the pre-column. By pumping water, the most polar components of the matrix were directly eluted to waste, whereas the analytes were retained on the pre-column. At $t = 1.5$ min, the valve was rotated, and the fraction of eluate containing the analytes was directly transferred from the pre-column to the analytical column, by increasing the acetonitrile content in the mobile-phase (from 0% at $t = 0$–1.5 min, to 60% at $t = 4.5$ min. After $t = 4.5$ min, the mobile-phase composition was kept constant (see Table 1)); At $t = 8$ min the switching valve was turned back to the original position and another cycle was started, again starting at $t = 0$ for the new sample.

2.6. Urine samples

Volumes of 1 ml of untreated urine samples were placed into glass injection vials, and 50 μl were directly injected onto the chromatographic system.

2.7. Recovery studies

Blank urine samples of 10 ml were spiked with spironolactone or canrenone standard solutions giving different concentrations in the 0.5–10 μg/

Table 1
Analytical conditions for the determination of spironolactone and canrenone

Sample pretreatment	None
Injection volume	50 μl
Chromatographic conditions	
Sample clean-up	Pre-column: Hypersil Mobile phase: water Flow-rate: 1 ml/min Duration of the washing: 1.5 min 0–1.5 min: water 100% (valve rotation)
Analytical separation	Column: LiChrospher RP C_{18} Mobile phase: acetonitrile–water 1.5–4.5 min: water 100% to acetonitrile–water 60:40, v/v 4.5–8 min: acetonitrile–water, 60:40, v/v Flow-rate: 1 ml/min
Detection wavelengths	Spironolactone: 238 nm Canrenone: 300 nm
Total run-time: 8 min	

ml range. The percentage of analyte recovered for a particular injection was calculated by comparing the peak areas obtained for the spiked samples with the values obtained for direct injections onto the analytical column of 50 μl of aqueous solutions containing the same concentrations of analyte. Each concentration was assayed in triplicate.

2.8. Preparation of standards for calibration

Standards for calibration were prepared by spiking 10 ml of urine samples with the appropriate volumes of spironolactone or canrenone aqueous solutions giving different concentrations in the 0.5–10.0 μg/ml range. These samples were processed in triplicate as described above. Peak areas were plotted versus the analyte concentration, and the resulting calibration curves were used to calculate the spironolactone or canrenone concentrations of the unknown samples. Calibration curves from aqueous solutions were also investigated. Each concentration was assayed in triplicate.

2.9. Human studies

Urinary excretion studies were performed with a healthy human volunteer after administration of a single dose of spironolactone (25 mg). Urine samples were collected at appropriate time intervals post-dose, and processed as described above.

2.10. Detection and quantification limits

The detection and quantification limits were estimated for each analyte by analysis of standard solutions of decreasing concentration. They were established as the concentrations required to generate a signal-to-noise ratios of 3 and 10, respectively. The values obtained were confirmed by analysis of spiked urine samples at concentrations equivalent to the estimated limits.

3. Results and discussion

According with previous results [14,15], we have selected a standard Hypersil C_{18} stationary phase for the pre-column. A vast majority of the urinary endogenous compounds can be directly eliminated from this pre-column by washing with water for 1.5 min. Extension of the flushing time does not significantly improve the selectivity, and unnecessarily increases the total analysis time.

For the analytical separation, different mobile-phases and columns were tested. As we have previously indicated, the stability of the analytes can be affected in several organic solvents. Since spironolactone and metabolites are stable in acetonitrile, this solvent was selected as the organic component of the mobile-phase. Control of the pH has been recommended for chromatography of spironolactone and canrenone [16]. Therefore, a phosphate buffer (pH 3) was initially tested as the aqueous component of the mobile-phase. With this mobile-phase, an adequate resolution of the analytes was obtained. However, we have noticed that substitution of the phosphate buffer with water did not significantly modify either the resolution or the retention times. Consequently, acetonitrile–water was chosen as mobile-phase for the analytical separation. Using this mobile-phase, satisfactory resolution of a standard mixture of spironolactone and canrenone was obtained with the two tested analytical columns. However for a given mobile-phase composition, the resolution was always better for the LiChrospher RP C_{18}, 5 μm (125 × 4 mm I.D.) analytical column, and the time needed for analysis was shorter. Therefore, this latter column was selected for subsequent studies.

On the other hand, several authors have recommended a back-flush configuration for the sample clean-up process, because in that way band broadening is minimized [17–19]. This configuration reverses the flow on the pre-column, and thus the analytes retained at the top of the pre-column are transferred to the analytical column by means of a secondary pumping system. However, we evaluated the potential of a

straight-flush elution, which only requires a binary pump (although in this work a quaternary pump was used) to achieve both, the clean-up and the analytical separation. We tested mobile-phases with different final elution strengths, as well as different gradient profiles, in order to achieve chromatographic responses for spironolactone and canrenone comparable to those obtained when the samples are directly injected onto the analytical column. The best results were obtained with the elution conditions summarized in Table 1. Under the proposed conditions the loss of efficiency is negligible.

Fig. 2 shows the chromatograms obtained for a standard solution containing spironolactone and canrenone with those obtained for a blank urine sample. Canrenone and spironolactone are eluted at 4.9 and 7.2 min, respectively. Most of the urinary endogenous compounds are eluted at retention times lower than 5 min. Several compounds are eluted at retention times slightly lower than that of canrenone; however, the absorbance of these compounds at 300 nm is negligible (canrenone exhibits its maximum in the 280–290 nm interval); therefore under the proposed conditions the matrix components do not interfere with the determination of canrenone. Spironolactone can be determined with satisfactory selectivity at 238 nm, where it presents an absorbance maximum.

3.1. Recovery

The efficiency and precision obtained in the sample clean-up process is excellent, and do not depend on the drug concentration in the studied interval. The mean recoveries were $(104 \pm 6)\%$ and $(101 \pm 6)\%$ ($n = 12$) for spironolactone and canrenone respectively, indicating a complete retention of the analytes on the pre-column. These values are comparable or even higher than those reported for conventional liquid extraction procedures [8–11]. The results are clearly better than those obtained with off-line solid-phase extraction of the same kind of samples, where the recoveries are lower than 85% [12].

Although in our study rotation of the valve was manually effected, the reproducibility is greatly improved, due to the lack of any off-line operation, compared to methods where liquid–liquid or solid-phase extraction on disposable

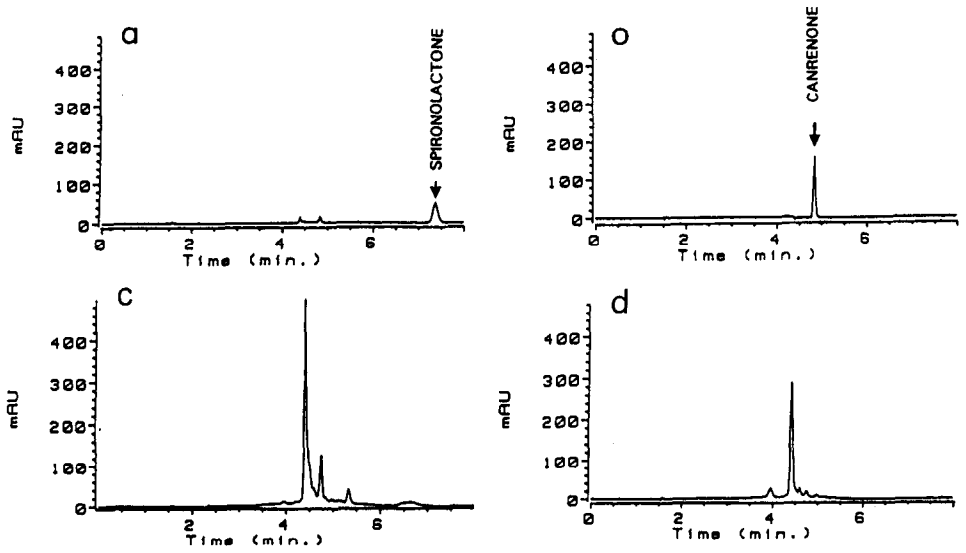

Fig. 2. Chromatograms obtained from a standard solution containing 2.0 μg/ml of spironolactone (a) and canrenone (b), and from a blank urine sample at 238 nm (c) and 300 nm (d). For elution conditions, see Table 1.

cartridges is used. Therefore, the addition of a standard can be avoided.

3.2. Precision and accuracy

The calibration graphs [area (a) versus concentration (c)] for spironolactone and canrenone from standard solutions are: $a = 79.778c - 4.887$ ($r = 0.9998$) and $a = 100.022c + 2.109$ ($r = 0.9997$), respectively. These relationships obtained for spiked urine samples are: $a = 78.963c - 3.730$ ($r = 0.9991$) for spironolactone and $a = 104.726c - 8.215$ ($r = 0.9991$) for canrenone. In all cases a good linearity over the working concentration interval is observed. The slopes of the calibration graphs for standards in water and in urine are statistically equivalent, which indicates that the matrix of the samples does not affect the determination of the compounds of interest.

The accuracy and precision of the assay were evaluated by analyzing control urine samples obtained from different subjects. The control samples were spiked with three different concentrations of spironolactone and canrenone. Each urine sample (1, 2, or 3) was assayed on the initial day of preparation and the results reported in Table 2 (%C.V.) for each urine sample are an estimation of the intra-day precision. Meanwhile, samples 1, 2, and 3 were processed on different days. Since the matrix of the samples does not disturb the determinations, an estimation of the inter-day precision can be obtained from these data. The coefficient of variation (%) fro the added concentrations 2.55, 4.08 and 7.65 μg/ml are 4, 1.5, and 1.2 respectively for spironolactone and 8.4, 5.1, and 2.7

Table 2
Precision and accuracy for spironolactone and canrenone in urine (three replicates were assayed in each case)

Sample	C_{added} (μg/ml)	$C_{calculated}$ (μg/ml)	C.V. (%)	E_r (%)
Spironolactone				
1	2.55	2.55 ± 0.04	2	0.0
	4.08	4.03 ± 0.01	0.25	−1.2
	7.65	7.6 ± 0.1	2	−0.7
2	2.55	2.45 ± 0.08	3	−3.9
	4.08	4.1 ± 0.1	3	0.5
	7.65	7.46 ± 0.04	1	−2.5
3	2.55	2.6 ± 0.1	6	2.0
	4.08	4.05 ± 0.02	0.5	−0.7
	7.65	7.62 ± 0.02	0.3	−0.4
Canrenone				
1	2.55	2.100 ± 0.008	0.4	−17.6
	4.08	3.9 ± 0.3	8	−4.4
	7.65	7.67 ± 0.08	1	0.3
2	2.55	2.52 ± 0.01	0.4	−1.2
	4.08	3.9 ± 0.1	4	−4.4
	7.65	7.70 ± 0.05	0.6	0.7
3	2.55	2.47 ± 0.01	0.4	−3.3
	4.08	4.1 ± 0.15	4	0.5
	7.65	7.7 ± 0.4	5	0.7

respectively for canrenone. The concentrations found were close to the actual concentrations in all cases tested, as can be seen from the relative errors (%) shown in Table 2. From these results it can be established that the accuracy and precision of the method are satisfactory.

3.3. Detection and quantification limits

Under the proposed conditions, the limits of detection correspond to spironolactone and canrenone concentrations in urine of 20 ng/ml. The quantification limits are 70 ng/ml for both drugs.

3.4. Human studies

The described assay has been applied to the measurement of urinary levels of spironolactone and canrenone after a single dose administration of 25 mg of spironolactone to a human volunteer. Fig. 3 shows chromatograms obtained 15 h post-dose. The estimated concentrations of spironolactone and canrenone in this sample were 7.1 and 0.31 μg/ml, respectively. As mentioned before, canrenone is the predominant form of the drug in urine.

The urinary excretion–time profiles of spironolactone and canrenone are in agreement with previously reported pharmacokinetic data [6,12]. The urinary concentration of spironolactone can be determined within a 40-h period after administration of the drug, although it can be detected even at 50 h after dosing. The range of concentrations found in urine was 0.020–0.387 μg/ml. Canrenone can be well determined even at 70 h after dosing. At that time the amount determined was 3.07 μg/ml. The minimum amount was found at 47 h, which corresponded with the limit of detection. The maximum concentrations are found between 15 and 35 h, and are higher than 4 μg/ml.

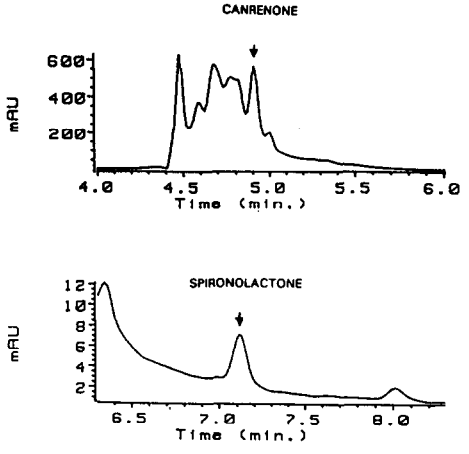

Fig. 3. Chromatogram of a urine sample obtained 15 h after a single oral dose of 25 mg of spironolactone. For elution conditions, see Table 1.

3.5. Utility

The main advantage over previously published procedures probably is the considerable simplification of the clean-up process. Although untreated samples were directly injected, more than 50 samples can be processed without precolumn replacement. In principle, the assay described can be adapted to any liquid biological fluid with minimum sample conditioning [19]. In addition, omission of intermediate steps (extraction, redissolution or storage of the analytes) avoids stability problems. During this study, no decomposition products have been observed.

It should be noted that the diuretics bendroflumethiazide, chlorthalidone and hydrochlorothiazide which are commonly coadministered with spironolactone, are eluted at 6.6, 5.7 and 5.1 min respectively (hydrochlorothiazide is almost not retained on the pre-column), and therefore do not interfere with the assay.

4. Conclusions

We have illustrated the usefulness of column-switching in the analysis of spironolactone and its main metabolite canrenone. These compounds can be determined with satisfactory precision, accuracy and sensitivity at therapeutic levels. The proposed assay is very simple and rapid, with analysis times shorter than 8 min.

Acknowledgement

The authors are grateful to the CICyT for financial support received for the realization of Project SAF 92-0655.

References

[1] L.E. Ramsay, in M.K. Agarwal (Editor), *Hormone Antagonists*, Walter de Gruyter, Berlin, New York, 1982, p. 335.
[2] J. Muller, *Clin. Ther.*, 9 (1986) 63.
[3] V.K. Rocco and A.J. Ware, *Ann. Intern. Med.*, 105 (1986) 573.
[4] H.A. Skluth and J.G. Gums, *Drug Intell. Clin. Pharm.*, 24 (1990) 52.
[5] V. Rimbau, *Offarm*, 10 (1991)58.
[6] J.H. Sherry, J.P. O'Donnel and H.D. Colby, *Life Sci.*, 29 (1981) 2727.
[7] J.W.P.M. Overdiek, W.A.J.J. Hermens and F.W.H.M. Merkus, *Clin. Pharmacol. Ther.*, 38 (1985) 569.
[8] G.B. Neurath and D. Ambrosius, *J. Chromatogr.*, 163 (1979) 230.
[9] E. Besenfelder and R. Endele, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 419.
[10] J.W.P.M. Overdiek , W.A.J.J. Hermens and F.W.H.M. Merkus, *J. Chromatogr.*, 341 (1985) 279.
[11] J.H. Sherry, J.P. O'Donnell and H.D. Colby, *J. Chromatogr.*, 374 (1986) 183.
[12] F. Varin, T.M. Tu, F. Bonoît, J.P. Villeneuve and Y. Theóreî, *J. Chromatogr.*, 574 (1992) 57.
[13] H. Zhang and J.T. Stewart, *J. Pharmaceut. Biomed. Anal.*, 11 (1993) 1341.
[14] P. Campíns-Falcó, R. Herráez-Hernández and A. Sevillano-Cabeza, *J. Liq. Chromatogr.*, 14 (1991) 3575.
[15] P. Campíns-Falcó, R. Herráez-Hernández and A. Sevillano-Cabeza, *Anal. Chem.*, 66 (1994) 244.
[16] F. De Croo,.W. Van den Bossche and P. De Moerloose, *J. Chromatogr.*, 325 (1985) 395.
[17] W. Roth, K. Beschke, R. Jauch, A. Zimmer and F.W. Koss, *J. Chromatogr.*, 222 (1981) 13.
[18] E.M. Bargar, *J. Chromatogr.*, 417 (1987) 143.
[19] D. Wasterlund, *Chromatographia*, 24 (1987) 155.