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Short communication

Simultaneous determination of spironolactone and its metabolites in human plasma[1]

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Abstract

This study describes a specific, precise, sensitive and accurate method for determination of unchanged spironolactone and its major active metabolites in human plasma. After one-step liquid–liquid extraction, analysis of the parent drug and its metabolites was performed in one chromatographic run, using a high performance liquid chromatography (HPLC) method with a programmed switchover of the UV wavelength. Spironolactone and 7α-thiomethyl-spironolactone were detected at 245 nm, while canrenone and internal standard were detected at 280 nm. The column used was an S5 ODS2 (500 mm × 4.6 mm i.d.). The mobile phase was a mixture of acetonitrile–aqueous orthophosphoric acid (pH 3.4). Chromatographic separations were performed at 5°C. The standard curves were linear over the range 10–400 ng ml^{-1} for spironolactone and 10–600 ng ml^{-1} for 7α-thiomethyl-spironolactone and canrenone. The precision and accuracy of the method were confirmed by relative standard deviations below 10% for different concentrations, except for the concentration equal to the quantitation limit, where these parameters ranged from 12–15%. The recovery was above 80% for all investigated compounds and for the internal standard. The assay proved to be suitable for pharmacokinetic studies of spironolactone.

Keywords: Canrenone; Human plasma; Spironolactone; 7α-Thiomethyl-spironolactone; UV–HPLC detection

1. Introduction

Spironolactone (SL), a synthetic steroid, is a competitive antagonist of aldosterone, which has been in clinical use as an effective diuretic agent, especially in patients with oedema or ascites from heart failure or liver cirrhosis. After oral administration, SL is extensively metabolised into a large number of metabolites [1–7]. Since the clearance of SL is extremely rapid, the resulting diuresis with sodium loss and potassium retention has been attributed to its metabolites. Early non-specific assays were based on the assumption that canrenone, the dethioacetylated derivative of SL, was the major active circulating metabolite [8–10]. However, with the development of a specific high

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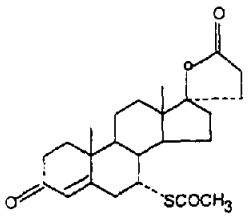
performance liquid chromatography (HPLC) assay for determination of canrenone, it became clear that these methods were not specific for canrenone but were measuring other metabolites as well [11,12]. Hence, canrenone level was previously overestimated and its true concentration was only one-third of the concentration measured with non-specific methods [13,14].

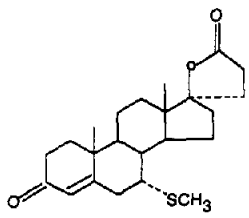
Biotransformation of SL produces ten metabolites, but so far only three of them have been isolated from human plasma. The main quantitative and physiologically-active metabolites are 7α-thiomethyl-spironolactone (7α-thiomethyl-SL) and canrenone (see Fig. 1) [4–7], [15]. Hence, in pharmacokinetic studies of SL, the parent drug as well as its two major metabolites should be measured.

Previously described HPLC studies of SL and the determination of its metabolites were based on liquid–liquid extraction [4,16] or two sequential solid-phase extractions [5]. However, in the first group of methods [4,16], only one wavelength (240 nm) was used, although the UV absorption maximum for canrenone appears at 280 nm. The second method [5] is an expensive one and time-consuming.

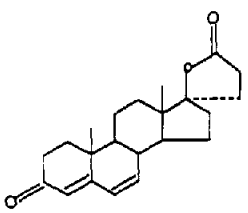
The aim of this study was to develop a rapid and sensitive HPLC assay with liquid–liquid extraction for the simultaneous determination of SL and its two major metabolites in human plasma.



SPIRONOLACTONE



7α-THIOMETHYL-SPIRONOLACTONE



CANRENONE

Fig. 1. Chemical structures of investigated compounds.

2. Experimental

2.1. Materials and reagents

SL, 7α-thiomethyl-SL and canrenone were kindly supplied by Rosemont Pharmaceuticals Ltd. (Leeds, UK). Diazepam used as internal standard was supplied by Pharmaceutical Enterprise (Polfa, Poland). Other reagents and solvents were of analytical grade and were obtained from Fluka (Switzerland) with the exception of acetonitrile, orthophosphoric acid and tetrachloromethane (CCl_4) which were from POCh (Gliwice, Poland). In all experiments doubly-distilled water was used.

2.2. Sample preparation

To 1 ml of plasma in a 10 ml centrifuge tube 100 μl of internal standard solution (diazepam, 10 μg ml^{-1}) was added. Each tube was vortexed to mix the plasma with the internal standard solution. Then 5 ml of tetrachloromethane was added and the tubes were shaken mechanically for 15 min. After centrifugation for 10 min at 700*g*, the aqueous layer was discarded and the organic layer was evaporated to dryness under nitrogen at 40°C. The residue was reconstitued in 200 μl of the mobile phase and 20 μl was injected onto the chromatographic column.

2.3. Chromatography

The HPLC system used was from Kontron Instruments (Zurich, Switzerland) equipped with a computer system for acquisition and integration of data (D 450, v. 3.3). The apparatus consisted of a solvent pump (model 420) and a UV detector (model 432). The separation system used two S5 ODS 2 Kontron columns with a total length of 500 mm (2 × 250 mm) and 4.6 mm i.d. These two columns were protected with a C-18 pre-column. The column temperature was kept at 5°C by submersion of the columns in a stirred constant-temperature water bath, which was connected to a thermostat. The temperature was controlled with an accuracy of ± 0.5°C. Mobile phase containing acetonitrile–orthophosphoric acid, pH 3.4 (89:11, v/v), was used. The analysis of the parent drug and its metabolites was carried out in one chromatographic run, using a programmed switchover of the detection wavelength. The UV absorbance was firstly monitored at 245 nm (SL and 7α-thiomethyl-SL) and then at 280 nm (canrenone and internal standard). Retention times of SL, 7α-thiomethyl-SL, canrenone and internal standard were 6.4, 7.2, 7.9 and 8.4 min respectively. The total chromatographic run was set for 10 min.

2.4. Validation of analytical method

The extraction yield of SL and its metabolites from plasma samples was assessed with spiked samples at three different levels over the therapeutic concentration ranges, i.e. lower in the case of SL (40, 80, 160 ng ml^{-1}) and higher for canrenone and 7α-thiomethyl-SL (50, 100, 200 ng ml^{-1}). Recovery calculations were based on comparison of the analyte/internal standard peak-height ratios obtained after the extraction of spiked samples to the peak-height ratio of an equal amount of the analytes added directly to the tube before the last evaporation. The same procedure for recovery calculation of internal standard at a concentration of 1 μg ml^{-1} was used. The results are listed in Table 1.

Linear calibration plots of SL and its metabolites were generated by leasts-square regression of the analyte/internal standard peak-height ratios against their respective concentration in plasma: 10–200 ng ml^{-1} for SL or 10-300 ng ml^{-1} for 7α-thiomethyl-SL and canrenone.

Table 1
Recoveries of SL (I) and its metabolites (II, III) from human plasma[a]

Concentration I/II/III (ng ml^{-1})	Recovery (mean ± %RSD)		
	SL (I)	7α-thiomethyl-SL (II)	Canrenone (III)
40/50/50	88.5 ± 8.5	86.0 ± 8.0	90.6 ± 6.5
80/100/100	87.9 ± 7.4	85.4 ± 7.6	86.1 ± 6.8
160/200/200	81.4 ± 6.6	85.9 ± 8.4	81.2 ± 4.4

[a] Recovery of internal standard at concentration 1 μg ml^{-1} was equal to 87.0 ± 4.2%.

Intra-assay variabilities were determined by replicate measurements of SL and its metabolites in plasma, at six inependent concentrations on the day of preparation (Table 2). Inter–day variabilities were determined at three concentrations of all investigated compounds, which correspond to quality control samples: 40, 80, 160 ng ml^{-1} for SL and 50, 100, 200 ng ml^{-1} for 7α-thiomethyl-SL and canrenone (Table 2). All data were derived from the same standards used in the calibration curves.

3. Results and discussion

Simultaneous determination of the investigated compounds encountered two problems: firstly, the selection of separation conditions for each compound on the column, and secondly monitoring of effluent at two UV wavelengths. Since the UV switchover occured between 7α-thiomethyl-SL and canrenone, good separation between these two compounds was particularly important.

At ambient temperature the separation of the investigated steroids was not complete. Therefore, column cooling was applied, which significantly improved the separation and quantitative determination of all investigated compounds.

Typical chromatograms of blank plasma (A) and plasma obtained from a volunteer who had

Table 2
Precision and accuracy of the assay of SL and its metabolites in plasma

(a)	SL					
Parameters	Concentration (ng ml^{-1})					
	10	20	40	80	160	200
Within-assay						
n	6	6	6	6	6	6
Mean	9.7	20.6	41.8	84.8	151.9	209.3
SD	1.40	2.08	3.56	3.99	12.77	18.86
RSD (%)	14.5	10.1	8.5	4.7	8.4	9.0
Acc. (%)	11.0	8.8	8.5	6.2	7.5	8.6
Between-assay						
n	–	–	11	12	12	–
Mean	–	–	44.0	84.2	164.5	–
SD (%)	–	–	1.96	7.31	19.42	–
RSD (%)	–	–	4.4	8.7	11.8	–
Acc. (%)	–	–	10.1	9.1	11.0	–
(b)	7α-thiomethyl-SL					
Parameter	Concentration (ng ml^{-1})					
	10	20	50	100	200	300
Within-assay						
n	6	6	6	6	6	6
Mean	10.5	20.0	47.6	103.1	194.6	308.8
SD	1.57	1.73	4.71	9.08	18.58	19.75
RSD (%)	15.0	7.8	9.9	8.8	9.6	6.4
Acc. (%)	13.8	10.1	8.5	7.9	7.6	7.1
Between-assay						
n	–	–	11	12	12	–
Mean	–	–	50.8	99.2	212.6	–
SD (%)	–	–	6.21	10.4	17.84	–
RSD (%)	–	–	12.2	10.5	8.4	–
Acc. (%)	–	–	10.7	8.9	9.8	–
(c)	Canrenone					
Parameters	Concentration (ng ml^{-1})					
	10	20	50	100	200	300
Within-assay						
n	6	6	6	6	6	6
Mean	10.3	19.9	48.3	98.6	207.2	295.4
SD	1.54	1.96	4.95	5.64	9.52	18.02
RSD (%)	14.9	9.9	10.2	7.5	4.6	6.1
Acc. (%)	13.9	8.5	8.4	4.1	4.9	5.0
Between-assay						
n	–	–	13	13	13	–
Mean	–	–	52.5	104.6	215.7	–
SD (%)	–	–	5.39	10.05	14.11	–
RSD (%)	–	–	10.3	9.6	6.5	–
Acc. (%)	–	–	9.7	9.8	8.3	–

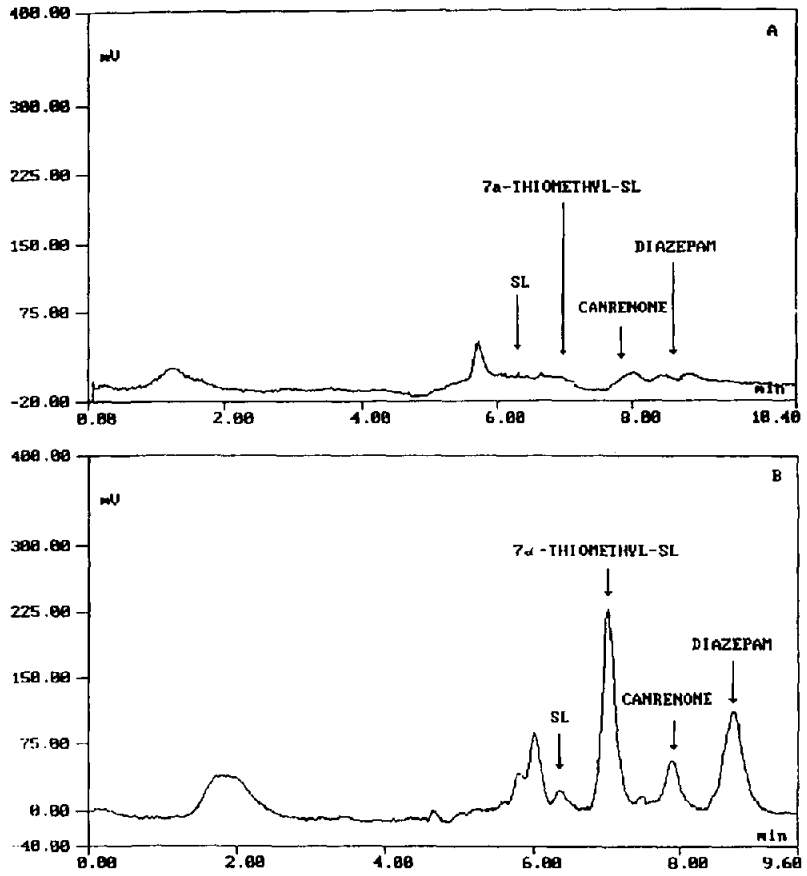


Fig. 2. Chromatograms of blank plasma (A) and plasma obtained from a volunteer who had received a single dose of 100 mg of the drug (B). (Concentrations: 46, 278, 105, 1000 ng ml^{-1} for SL, 7α-thiomethyl-SL, canrenone and internal standard respectively.)

received a single dose of 100 mg of SL (B) are shown in Fig. 2 (the concentrations found are 46, 278, 105, and 1 μg ml^{-1} for SL, 7α-thiomethyl-SL, canrenone and internal standard respectively).

Under optimised conditions, the linearity of the standard curves, with accuracy and precision below 10% was confirmed experimentally in the range 20–200 ng ml^{-1} for SL and 20–300 ng ml^{-1} for 7α-thiomethyl-SL and canrenone. These ranges correspond well with the concentrations obtained in pharmacokinetic profiles of SL and its metabolites after dosage commonly used. Only at the point of the quantitative limit (10 ng ml^{-1}) did the accuracy and precision fall below 15% for all compounds. Relative standard deviations (RSDs) for slopes of six independent calibration curves, made between-days, correspond to 5.0% for canrenone, 7.8% for 7α-thiomethyl-SL and 12.6% for SL. Correlation coefficients were greater than 0.0991 (Table 3).

For inter-assay validation, precision and accuracy obtained at three different concentrations, corresponding to quality control samples, below 13% has been found (Table 2).

Table 3
Linear regression parameters obtained from the calibration curves of SL and its metabolites in plasma

Compound	*n*	Slope (mean ± %RSD)	Intercept	Correlation coefficient
SL	6	0.0043 ± 12.6	0.0209	0.991
7α-thiomethyl-SL	6	0.0041 ± 7.8	0.0361	0.994
Canrenone	6	0.0068 ± 5.0	0.0336	0.994

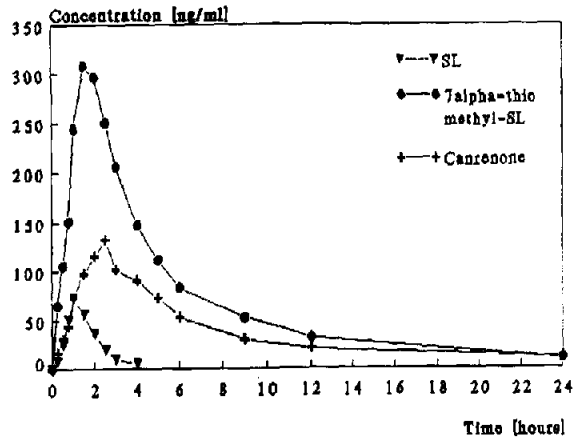


Fig. 3. Concentration–time curves of SL and its two metabolites after an oral dose of 100 mg of the drug.

Extraction yields (verified at three different concentrations for all compounds including internal standard) are above 80% (Table 1).

Additionally, a freeze–thaw stability test for each compound at three different concentrations (50, 100, 200 ng ml^{-1}), made three times, was carried out. Average recoveries of 92.2%, 90.9% and 94.3% after the third thaw for SL, 7α-thiomethyl-SL and canrenone respectively, were found.

The optimised method was applied successfully for the determination of SL and its metabolites in human plasma after oral administration of 100 mg of the drug. Examples of the concentration profiles for each compound are shown in Fig. 3. As can be seen, the method enables one to find whole profiles for each investigated compound.

The role of canrenone, as the main active metabolite, is less important than had previously been assumed [11,12,14,15]. 7α-thiomethyl-SL and SL itself must contribute considerably to the pharmacological effects of SL [3,15–17]. Until now, however, only a few specific methods for simultaneous determination of SL and its major metabolites have been described [4,5,16]. Therefore, these methods should be used for comparison. Excellent results have been obtained in a method with two-step solid-phase extraction, where the quantitative limit of determination of all compounds was found to be of the level of 6.25 ng ml^{-1} [5]. With liquid–liquid extraction methods, the limit of quantitation of investigated compounds, as derived from their calibration curves, was 12.5 ng ml^{-1} in guinea pig plasma [16], but only 50 ng ml^{-1} in human plasma [4]. Hence, there is obviously a need for a simple, and reproducible, assay for simultanous determination of SL and its metabolites with simple one-step preparation of the sample, a lower quantitation limit, and a short total time for the whole analysis.

The method described in a combination of a simple one-step liquid–liquid extraction and chromatographic separation on two columns, at subambient temperature, which provides excellent selectivity for the investigated compounds. The applied extraction conditions give high enough recovery for all investigated compounds and the biological impurities in chromatograms are negligible. Moreover, the statistical results are approximately comparable with those obtained by Varin et al. [5]. Therefore, this method should be particularly suitable for pharmacokinetic studies and routine therapeutic drug monitoring.

4. Conclusions

In order to achive satisfactory separation and simultaneous determination of spironolactone and its main active metabolites in human plasma, an HPLC method was developed. The important features of the introduced method are the application of a wavelength switchover technique and the development of the chromatograms at subambient temperature.

Acknowledgements

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