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DETERMINATION OF CANRENONE, THE MAJOR METABOLITE OF SPIRONOLACTONE, IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An assay procedure for measuring plasma and urine levels of canrenone is described. The drug is extracted with *n*-hexane—toluene (1:1, v/v) after adding spirorenone as internal standard, and is then separated from plasma constituents and metabolites by high-performance liquid chromatography followed by UV detection at 285 nm. The limit of detection is less than 5 ng/ml. Interference with a series of spironolactone and canrenone metabolites was not observed. Plasma levels and renal excretion of canrenone after oral administration of 200 mg of spironolactone and intravenous injection of 200 mg of potassium canrenoate to a healthy male volunteer were measured.

INTRODUCTION

Spironolactone (7α-acetylthio-17-hydroxy-3-oxo-17α-pregn-4-ene-21-carboxylic acid-γ-lactone) is a synthetic steroid which is widely used in the treatment of oedema, cirrhosis of the liver and hypertension [1]. Its mechanism of action is competitive antagonism of aldosterone [2]. Spironolactone is metabolized in man to a variety of compounds which can be subdivided into two classes [3—5]. In the first group, the sulphur atom of the parent drug is retained in the molecule. The second group is formed by dethioacetylation of spironolactone and subsequent biotransformation steps. The most important compound in this second class is canrenone (Fig. 1). It is pharmacologically active and accounts for the major part of the activity of spironolactone, at least after multiple oral administrations [6]. Potassium canrenoate is used as intravenous formulation due to its higher water solubility in comparison to canrenone and spironolactone. In vivo, potassium canrenoate is rapidly converted to canrenone in an equilibrium reaction [7].

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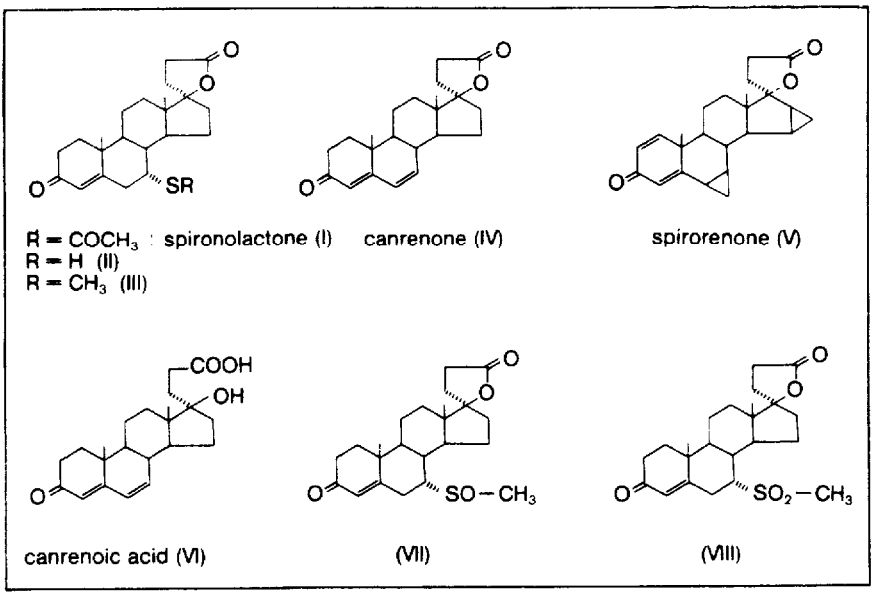

Fig. 1. Structures of spironolactone and some of its metabolites and of the internal standard, spirorenone.

Canrenone is assayed in biological fluids mostly by a fluorimetric method, first described by Gochman and Gantt [8], and modified by Sadee et al. [9] and by Karim et al. [10]. Neubert and Koch automated this procedure [11]. However, this method lacks specificity, which was concluded from a comparison of plasma levels and pharmacologic effects following administration of either spironolactone or potassium canrenoate [12], and therefore other investigators have looked for different methods with higher reliability.

The most selective method at present seems to be a high-performance liquid chromatographic (HPLC) assay described by Neurath and Ambrosius [13]. However, in this procedure normal-phase columns are used which cannot be handled as easily as reversed-phase columns. Furthermore, an internal standard eliminating extraction errors was not used by these authors.

Therefore, the aim of the present study was to establish an assay procedure for canrenone using reversed-phase chromatography, with spirorenone (Fig. 1) as internal standard, and to apply this method to the determination of canrenone levels in the plasma and urine of a healthy male volunteer.

EXPERIMENTAL DESIGN

Subject and medication

A healthy male volunteer (33 years old, 63 kg body weight) was given 200 mg of potassium canrenoate (Sincomen® -pro injectione) intravenously. Blood samples were taken 5, 10, 15, 20, 30, 45, 60, 90 min and 2, 4, 6, 8, 12 and 24 h after drug administration. The samples were immediately centrifuged and the plasma stored at −20°C until analysis. One week later the same test

subject received an oral dose of 200 mg of spironolactone (Sincomen-100). Sampling times were 15, 30, 45, 60, 90 min and 2, 4, 6, 8, 12 and 24 h after drug intake.

Chemicals

Methanol, *n*-hexane and toluene were of analytical-reagent grade (Merck, Darmstadt, F.R.G.) and were used without further purification.

Extraction procedure

A 0.2-ml aliquot of plasma or urine was pipetted into an 8-ml stoppered test tube containing 300 ng of spirorenone (Fig. 1) as internal standard. The samples were extracted two times with 1 ml of toluene—*n*-hexane (1:1, v/v) by vortexing for 1 min and subsequent centrifugation at 1200 *g* for 5 min. The organic layers were combined and taken to dryness under a slight stream of nitrogen. The residue was taken up in 250 μl of the HPLC eluent and 200 μl were injected for analysis.

Alternatively, with low concentration samples, 1 ml of plasma or urine were used with varying amounts of internal standard. Extraction then was performed twice with 3 ml of toluene—*n*-hexane (1:1, v/v).

The extraction efficiencies were determined with 0.2-ml plasma and urine samples containing 31.25, 125 and 300 ng of canrenone. With 1-ml plasma samples recoveries were calculated by comparing two standard curves obtained by plotting peak heights of canrenone before and after extraction versus the amount of drug.

Chromatographic system

The HPLC system consisted of a solvent delivery pump (Waters, Königstein, F.R.G., type 6000 A) and a UV detector with variable wavelength (Schoeffel SF 770) set at 285 nm. The chromatographic columns used were either a Shandon ODS Hypersil (60 × 4.6 mm, 3 μm; Bischoff, Stuttgart, F.R.G.) or a Spherisorb ODS-2 (125 × 4.6 mm, 5 μm; Bischoff) with pre-columns (20 × 4.6 mm). Injection was accomplished with a Rheodyne RH 7120 system or alternatively with an automatic sampling device (WISP, Waters).

The mobile phase was methanol—water in the ratio 60 : 40 (v/v), using the 3-μm column and 65 : 35 (v/v) with the 5-μm column. The flow-rate was 1 ml/min in both cases. The whole system was operated at ambient temperature.

Standard curves were constructed with 0.2-ml plasma and urine samples containing 15.6, 31.25, 125, 250 and 500 ng of canrenone and 300 ng of spirorenone, and 1-ml samples with 10, 20, 40 and 60 ng of drug and 100 or 300 ng of internal standard. These samples were analyzed as described above. Peak areas were calculated from the peak height and the width at half height. Peak area ratios (canrenone/spirorenone) were plotted against the concentration of canrenone and the calibration curves were obtained by regression analysis.

RESULTS

Assay of plasma and urine

Recoveries from 0.2-ml plasma and urine samples were about 85% (Table I).

With 1-ml plasma samples 82% was found. The recovery of the internal standard was 81% as had been investigated earlier [14].

Chromatograms of plasma and urine extracts are shown in Figs. 2 and 3 using the longer column with 5-μm particles. With both columns the limit of detection was less than 5 ng/ml when extracting 1-ml samples. However, for routine analysis the short columns cannot be recommended, because they deteriorate fairly rapidly and lose their resolution.

TABLE I

EXTRACTION RECOVERIES

Recoveries (mean % ± S.D.) were determined by extracting 0.2 ml of plasma or urine (n = 7 each) spiked with different amounts of canrenone and comparing the peak areas measured to those of unextracted drug.

	Amount spiked (ng per 0.2 ml)		
	31.25	125	500
Plasma	85 ± 24	N.D.*	85 ± 7
Urine	88 ± 8	80 ± 6	87 ± 5

*Not determined.

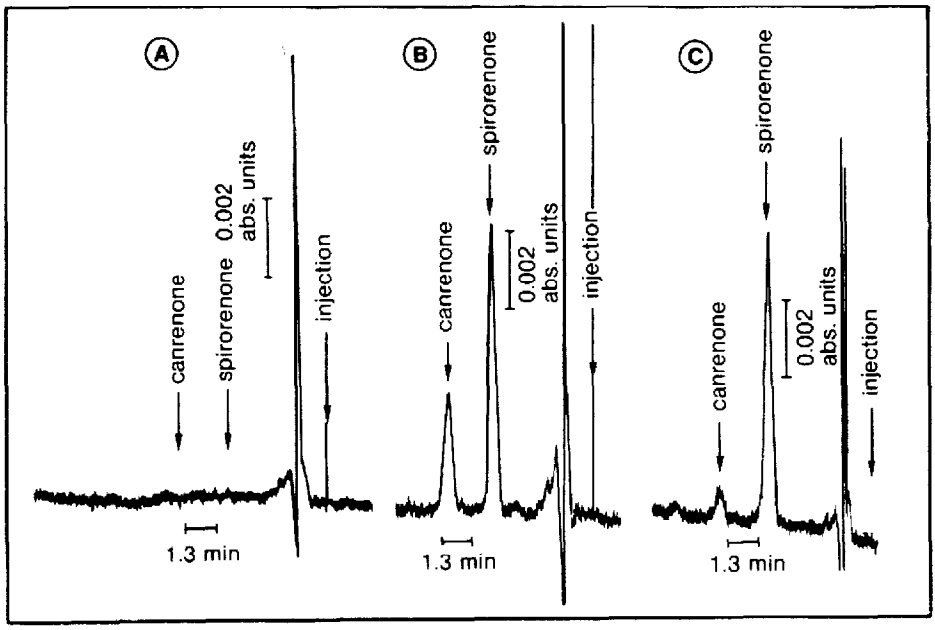

Fig. 2. HPLC chromatograms of 0.2-ml plasma samples obtained with a Spherisorb ODS-2 column (125 × 4.6 mm, 5 μm) before (A), and 4 h (B) and 12 h (C) after oral ingestion of 200 mg of spironolactone and addition of 300 ng of internal standard (B and C).

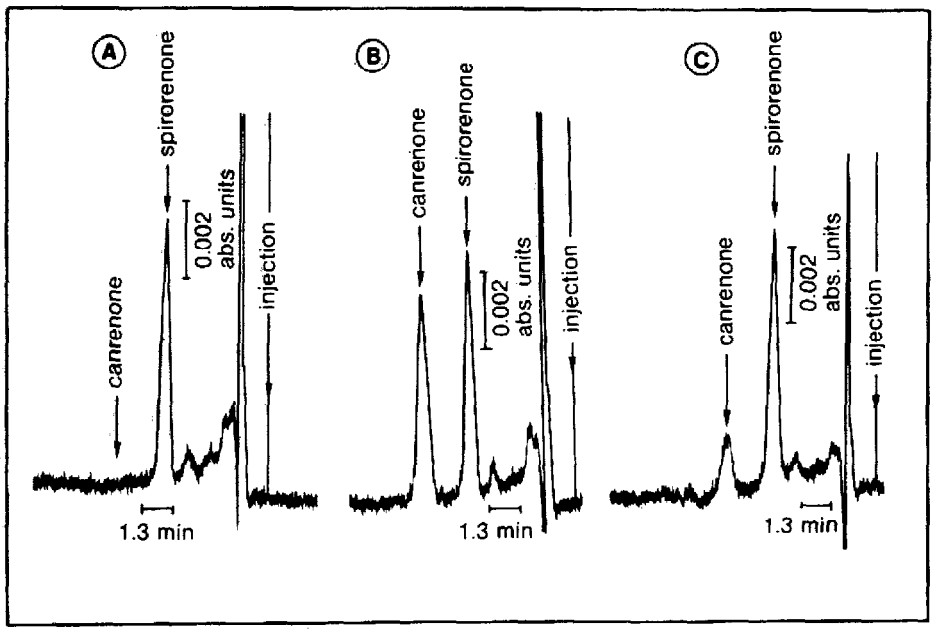

Fig. 3. HPLC chromatograms of 0.2-ml urine samples obtained with a Spherisorb ODS-2 column (125 × 4.6 mm, 5 μm) from blank urine (A), from a 0—2 h fraction (B) and from a 12—24 h fraction after adding 300 ng of internal standard.

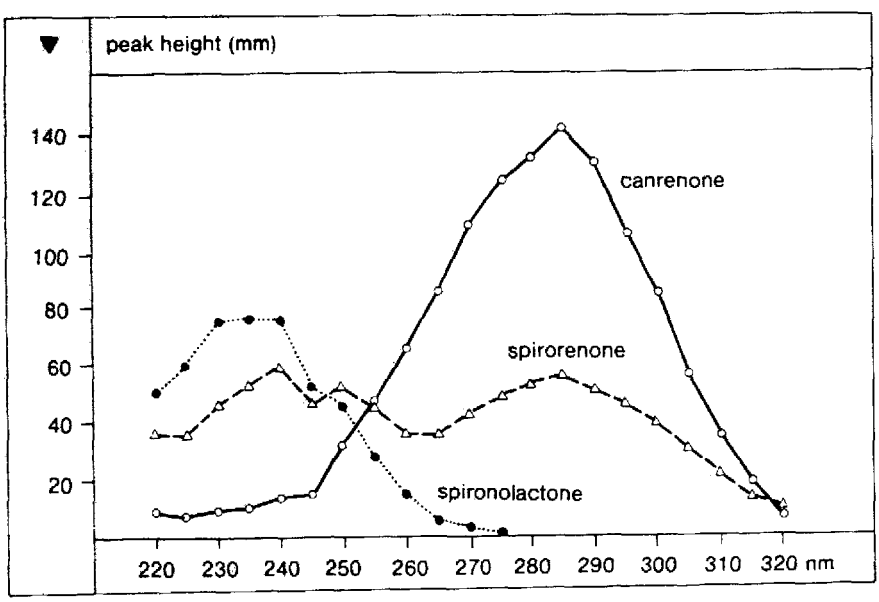

Fig. 4. UV spectra of canrenone, spironolactone and spirorenone obtained by measuring HPLC peak heights as a function of the wavelength of the UV detector.

The retention times of canrenone and of spirorenone were 7.8 and 5.1 min, respectively, with the 125-mm column. Spironolactone was eluted in this system after 5.8 min. However, with the UV detector set at 285 nm, spironolactone was not detected in the concentration range of interest. Sprionolactone has its UV maximum at 235 nm (Fig. 4), while canrenone and spirorenone exhibit a maximum at 285 nm.

This is also the case for those metabolites which are formed after dethioacetylation of the parent drug. The retention time of canrenoic acid, which is one metabolite of this group, was 1.1 min. Compounds of the second series of biodegradation products in which the sulphur atom is retained in the molecule include substances II, III, VII and VIII (Fig. 1). They were eluted after 6.8, 6.5, 2.2 and 2.1 min, respectively. Furthermore, at 285 nm no interference with canrenone or spirorenone was observed.

Calibration curves for the determination of unknown plasma and urine levels are listed in Table II. They were obtained by plotting peak height or peak area ratios of canrenone/spirorenone versus the concentration of the drug. Mean values for the slope and the intercept of the calibration lines obtained on five different days upon extraction of 0.2-ml plasma samples with 300 ng of internal standard and evaluation of peak areas are 0.114 ± 0.087 and 0.008 ± 0.001, respectively. Linearity of the calibration lines was positively tested up to 1500 ng/0.2 ml. The precision of the assay method at different concentration levels is given in Table III.

TABLE II

MATHEMATICAL EQUATIONS OF THE CALIBRATION CURVES FOR THE DETERMINATION OF UNKNOWN CANRENONE CONCENTRATIONS

The curves were obtained by spiking 0.2 ml and 1 ml of plasma or urine with various amounts of drug and 100 ng or 300 ng of internal standard.

Sample	Sample volume extracted (ml)	Amount of standard added (ng)	Column particle size (μm)	Calibration curve	Correlation coefficient
Plasma	0.2	300	5	$Y = 0.045 + 0.010X^{\star}$ ($n = 14$)	0.999
Plasma	1	100	3	$Y = 0.042 + 0.019X^{\star\star}$ ($n = 10$)	0.996
Plasma	1	300	3	$Y = 0.029 + 0.015X^{\star\star}$ ($n = 10$)	0.99
Urine	0.2	300	5	$Y = 0.020 + 0.009X^{\star}$ ($n = 6$)	0.099997

* Y = (peak area of canrenone)/(peak area of spirorenone), X = ng of canrenone.
** Y = (peak height of canrenone)/(peak height of spirorenone), X = ng of canrenone.

Plasma and urine levels

Plasma concentrations measured in the test subject after oral ingestion of 200 mg of spironolactone and intravenous injection of 200 mg of potassium canrenoate are illustrated in Fig. 5. Following oral administration the maxi-

mum concentration was 210 ng/ml at 4 h after ingestion. Intravenous administration resulted in a maximum plasma level of 3136 ng/ml, 20 min post administration.

Renal excretion of unchanged canrenone was less than 0.5% of the dose after both ways of administration (Fig. 6).

TABLE III

PRECISION OF THE ASSAY METHOD

Precision was obtained from consecutive determinations on the same day of canrenone in 0.2 or 1 ml of plasma and urine and calculations of peak area ratios.

Concentration (ng per 0.2 ml)	Plasma (%)	Urine (%)
31.25	6.1 (n = 7)	7.3 (n = 8)
125	4.1 (n = 7)	5.5 (n = 7)
500	2.2 (n = 7)	2.2 (n = 7)
20 (ng/ml)	N.D.*	4.3** (n = 10)

*Not determined.
**Peak height ratio.

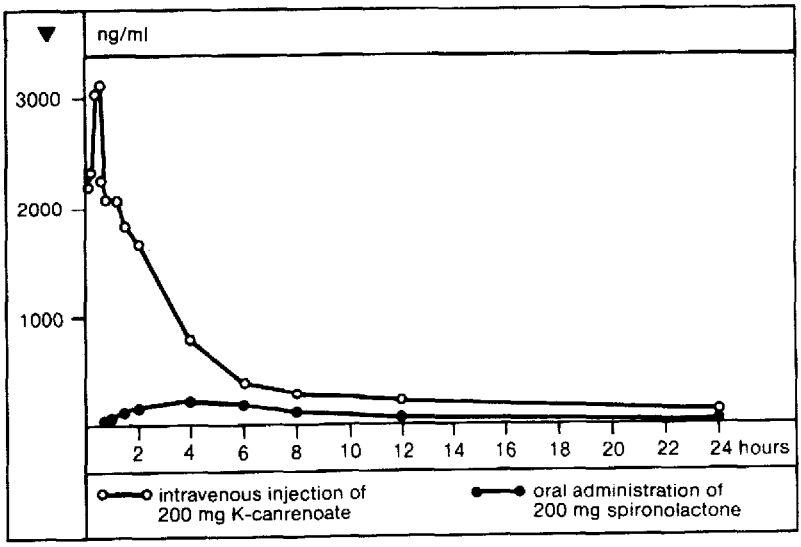

Fig. 5. Plasma level of canrenone in a healthy male volunteer who had received 200 mg of spironolactone orally and 200 mg of potassium canrenoate by intravenous injection.

DISCUSSION

Several methods have been described in the literature for the determination of canrenone in biological fluids. The most widely used procedure is the fluorimetric assay, indicating maximum levels of 400—500 ng/ml after oral

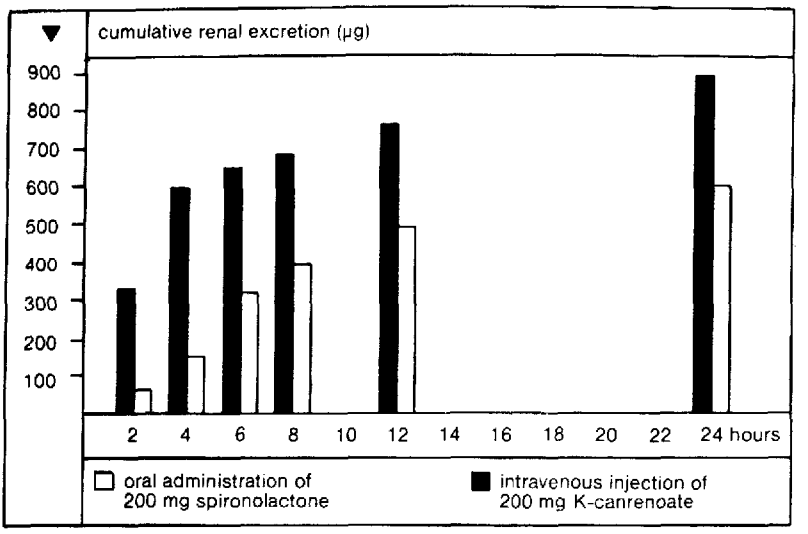

Fig. 6. Cumulative urinary excretion of canrenone in a male test subject after oral administration of 200 mg of spironolactone and intravenous injection of 200 mg of potassium canrenoate.

administration of 100 mg of spironolactone. The HPLC method described by Neurath and Ambrosius [13] yielded peak values of 45 ng/ml after the same dose. This is in the same order of magnitude as the results obtained in the present study considering the higher dose administered and the very small body weight of the test subject studied resulting in a diminished volume of distribution and, therefore, higher plasma levels.

The method of determining canrenone described here differs from the procedure by Neurath and Ambrosius by using *n*-hexane—toluene for extraction, by a reversed-phase column instead of normal-phase HPLC and by the addition of an internal standard.

The advantage of reversed-phase HPLC systems as compared to normal-phase chromatography usually lies in better suitability for routine analysis. Furthermore, an internal standard which is used in the present procedure compensates for losses during the extraction process. Thus the assay procedure described seems to be more suitable for the routine analysis of canrenone in plasma and urine samples. An application of this method in clinical pharmacokinetics will be reported later.

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