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Note

High-performance liquid chromatographic determination of canrenone, a major metabolite of spironolactone, in body fluids

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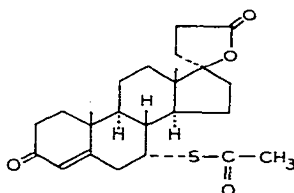
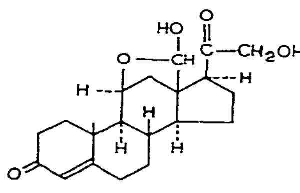
As a closely related compound, spironolactone (I) acts as a competitive inhibitor of aldosterone (II) in the exchange processes of the distal tubules of the kidney. Accordingly, the output of sodium is increased, and the output of potassium is decreased. The importance of spironolactone in the therapy of edematous conditions is thus explained.

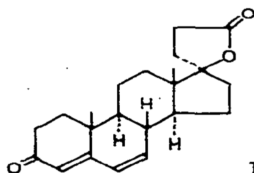


I



II

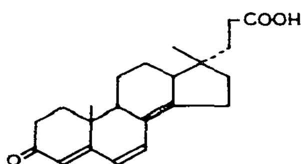
After oral administration, spironolactone is rapidly metabolized. The first step is the elimination of the 7α-acetylthio group. The des-acetylthio-Δ6 derivative formed is canrenone (III), first identified by Gochman and Gantt [1] in 1962, who at the same time described a highly sensitive fluorimetric method for the study of plasma levels of, as claimed by the authors, canrenone.



III

Their method consists of the extraction of the plasma with dichloromethane, re-extraction of the dichloromethane with 65% sulfuric acid, and measurement of the fluorescence (excitation 465 nm, emission at maximum 520 nm). These conditions are very similar to those used for the determination of endogenous cortisol and corticosterone in plasma first described by Sweat [2] and simplified in a large number of publications since then. The specificity of the fluorimetric method for this purpose has been critically examined by Pirke and Stamm [3].

The fluorimetric determination of canrenone has been used by many authors in recent years [4—6]. On reaction with sulfuric acid the fluorophor (IV) can be produced from canrenone and other metabolites, even those having an open ring.



IV

Canrenone is possibly the major effective principle of spironolactone. Funder et al. [7] have proved that the intact γ-lactone ring is necessary for the receptor reaction. Therefore, there exists an important need for a sensitive and specific method for the assay of canrenone.

This paper describes a precise, specific, and sensitive method for the determination of canrenone by means of high-performance liquid chromatography (HPLC) and compares it with the fluorimetric method.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography was performed on a Pye-Unicam LC 20 separator equipped with a Pye-Unicam LC 3 ultraviolet (UV) absorbance detector (Philips, Kassel, G.F.R.) operated at 283 nm. A 25 cm × 0.25 cm I.D. glass column packed with LiChrosorb SI-100, 10 μm (Merck, Darmstadt, G.F.R.) or Nucleosil 100, 10 μm (Macherey-Nagel, Düren, G.F.R.) was used for separation.

Fluorimetric measurements were carried out on a Perkin-Elmer Filter Fluorimeter 1000 M in 3-ml quartz cuvettes (Bodenseewerk Perkin-Elmer, Überlingen, G.F.R.) equipped with a pulsed xenon lamp (6.25 W) and a 450-nm interference filter adjusted to 457 nm.

The mass spectrum of canrenone was measured using a Hewlett-Packard 5992 A computer-controlled gas chromatography—mass spectrometry system.

Materials

Canrenone. A 400-mg amount of potassium canrenoate (Boehringer, Mannheim, G.F.R.) was dissolved in about 5 ml of methanol, and glacial acetic acid

was added until the canrenoic acid had precipitated. The acid was filtered off, washed twice with 5 ml of methanol and dried by washing with 5 ml of *n*-hexane.

The dry acid was heated carefully until the visible evolution of water had ceased. The residue was recrystallized from 150 ml of ethyl acetate and dried (m.p. 161°; UV maximum 283 nm; mass spectrum m/e (%): 340 (M^+, 99), 267 (100), 91 (56), 136 (39), 325 (20), 227 (20)).

Solvents and reagents. All solvents and reagents used for HPLC and fluorescence determinations were of analytical grade. Dichloromethane for fluorescence measurements was of spectroscopic grade (Merck).

Serum. Serum for blanks and recovery studies was obtained from healthy volunteers.

Extraction

Serum. To 1 ml of serum, 200 μl of chloroform were added. The sample was vigorously shaken on a rotary mixer (Cenco, Breda, The Netherlands) for 30 min and then placed in a 3-ml reactivial (Pierce-Eurochemie, Rotterdam, The Netherlands) and centrifuged for 10—15 min at 5500 *g*. Aliquots of 50—100 μl of the chloroform solution were injected for HPLC.

Urine. To a 1-ml sample of urine, 0.5 ml of a β-glucuronidase solution (3000 Fishman units/ml)(Schering AG, Berlin, G.F.R.) in sodium acetate buffer (pH 4.6) was added and the mixture was incubated at 37° for 18 h. Chloroform (200 μl) was added and the sample was moderately shaken for 30 min, placed in a 3-ml reactivial and centrifuged for 10 min at 5500 *g*. 100 μl of the chloroform phase were injected for HPLC.

High-performance liquid chromatography

Serum. The mobile phase, chloroform—*n*-hexane (50:50), was pumped through the column at a flow-rate of 2.0 ml/min at a pressure of 7 MPa. The retention time of canrenone was 4.5 min.

Urine. The mobile phase was chloroform—*n*-hexane (55:45) at a flow-rate of 2.0 ml/min and pressure of 7 MPa. The retention time of canrenone was 4.0 min.

Sensitivity, accuracy, and precision of HPLC

A standard curve for canrenone was prepared by carrying out the analysis with test solutions and 31 repeated injections ranging from 1.25 to 20 ng canrenone. A Nucleosil 100, 10 μm column was used in these experiments.

The over-all average peak area and its standard deviation were found to be 5.53 mm^2/ng of canrenone, $s = \pm\ 0.33$. Linearity was obtained over the entire range.

The recovery rate was determined by carrying out the analysis on serum samples to which canrenone had been added at different concentrations ranging from 5 to 80 ng/ml. The average peak areas after the analytical procedure were 5.41 mm^2/ng canrenone ($s = \pm\ 0.27$), corresponding to a recovery of 97.8%.

The limit of the determination is about 5 ng/ml serum.

Fluorimetric determination

Canrenone standard. For the stock solution, 20 mg of canrenone were dissolved in 10 ml of ethanol and diluted to 1000 ml with water. Working standards were prepared daily by further dilution of the stock with water. Aliquots of the aqueous solution were added to 10 ml of dichloromethane. Four milliliters of 65% sulfuric acid were added, and the sample shaken for 30 sec. The acid phase was separated and kept at room temperature for 1 h.

At sensitivity × 1 the fluorescence values given in Table I were measured (excitation 457 nm, emission 520 nm).

TABLE I

FLUORESCENCE OF CANRENONE STANDARDS

Canrenone (ng)	7.5	15	30	60	90	120	150
Fluorescence	4	8	16	32	49	61	92

Spiked serum. To 0.2 ml serum and 5 ml water, aliquots of the standard canrenone solution were added. Dichloromethane (15 ml) was added and the sample shaken for 30 sec. The mixture was centrifuged, and the aqueous phase removed. To the dichloromethane, 1 ml of 0.1 *N* sodium hydroxide solution was added, the mixture shaken for 15 sec, and the aqueous phase discarded. Four milliliters of 65% sulfuric acid were added to 10 ml of the dichloromethane solution and shaken for 30 sec. The acid phase was separated and the fluorescence measured as described for the standard. The results are given in Table II.

TABLE II

FLUORESCENCE OF CANRENONE-SPIKED SERUM

Canrenone (ng) added to 0.2 ml serum	0	5	10	20	40	60	80
Fluorescence	0*	2	5	10	22	33	43

*Adjusted to zero.

Serum samples after administration of spironolactone. The samples were treated for fluorescence measurement as described for the spiked samples. A blank serum sample of each subject was spiked for comparison (subject A: 8 ng canrenone added to 0.2 ml serum — fluorescence value 3; B: 5 ng canrenone added to 0.2 ml serum — fluorescence value 2).

RESULTS

The high-performance liquid chromatography of canrenone gives precise, sensitive and reproducible determinations of this compound, which probably

represents the major active principle of spironolactone, without interference by inactive metabolites and endogenous corticosteroids. The method thus forms a useful tool for studies of the pharmacokinetics and bio-availability of spironolactone.

As a specific method, HPLC produces lower values than the fluorimetric determinations utilizing a dichloromethane-soluble fraction isolated from body fluids without further separation steps.

Peak serum values of canrenone determined by HPLC after oral administration of 100 mg spironolactone in different preparations assayed were of the order of magnitude of 45 ng/ml serum, as shown in Fig. 1.

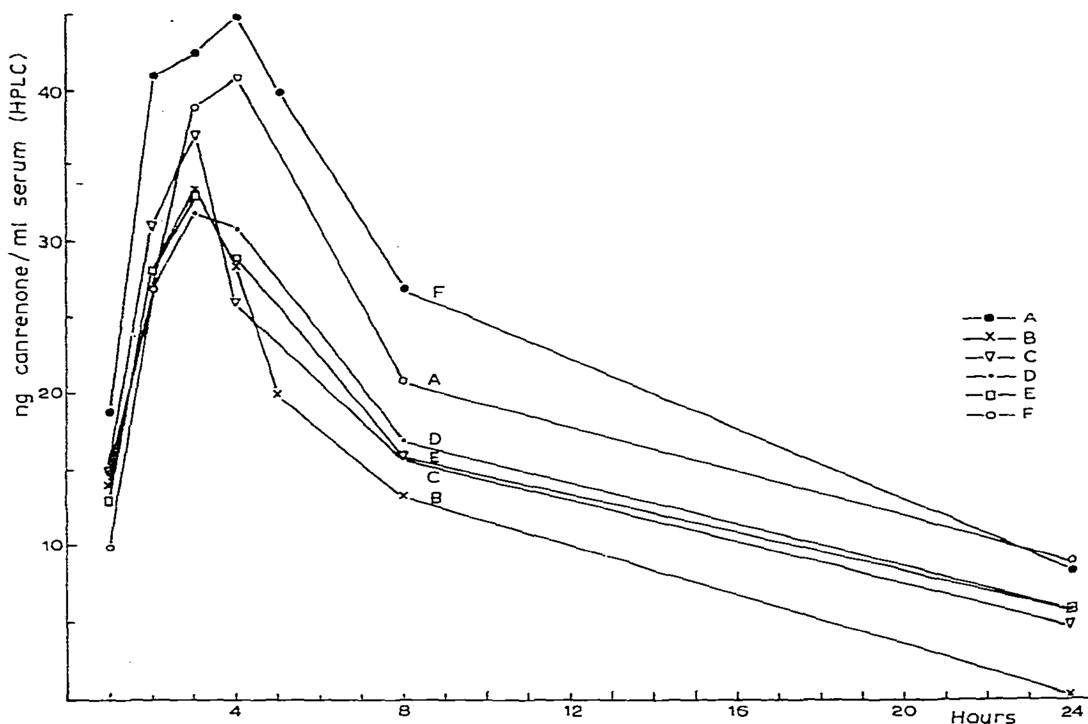


Fig. 1. Serum levels of canrenone after oral administration of 100 mg spironolactone in different preparations.

Literature data on the fluorimetric assay indicate peak values of 400—500 ng/ml serum for comparison. A comparison of HPLC and fluorimetric values collected on a small number of serum samples from two male subjects documents the difference, as shown in Table III.

TABLE III

COMPARISON OF SERUM LEVELS OF CANRENONE DETERMINED BY MEANS OF HPLC AND FLUORIMETRY

	Hours after oral administration of spironolactone	HPLC (ng canrenone per ml serum)	Fluorimetry* (ng fluorigenic compounds as "canrenone" per ml serum)
Male subject A	blank	0	+
	1	10	72.5
	2	31	320.0
	3	39	422.5
	4	45	282.5
	6	34	207.5
	8	24	80.0
Male subject B	blank	0	+
	1	8	10.0
	2	11	125.0
	3	18	217.5
	4	21	282.5
	6	17	150.0
	8	14	132.5

*+ = Blank of the order of magnitude of 25 ng "canrenone" per ml serum, adjusted to zero.

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