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Short Communication

Liquid chromatographic determination of canrenone in human serum using solid-phase extraction*

HAILANG ZHANG and JAMES T. STEWART†

Department of Medicinal Chemistry, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA

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Introduction

Spironolactone is a steroidal compound which has properties consistent with specific competitive antagonism of aldosterone and other mineralocorticoids. It is used clinically for the treatment of essential hypertension, primary aldosteronism and various edematous states. After oral administration, spironolactone is rapidly and extensively metabolized. Canrenone, the dethioacetylated derivative of spironolactone, is not the principal metabolite of spironolactone. However, it appears to play a very important role with respect to the pharmacological activity of spironolactone at steady state concentration. Canrenone has a relatively long half-life in humans which leads to considerable accumulation during long term treatment [1]. The chemical structures of canrenone and spironolactone are shown in Fig. 1.

Early fluorometric assays for canrenone in biological fluids lack specificity due to cross interference with other fluorigenic metabolites of spironolactone [2–5]. Other analytical methods to measure canrenone in biological fluids by liquid chromatography (LC) have been reported [5–8]. Most of these methods are based on time-consuming liquid–liquid extraction steps. One of them reported the use of tandem solid-phase extraction columns. In this paper, 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. It exhibits excellent sensitivity, specificity, precision and accuracy for canrenone.

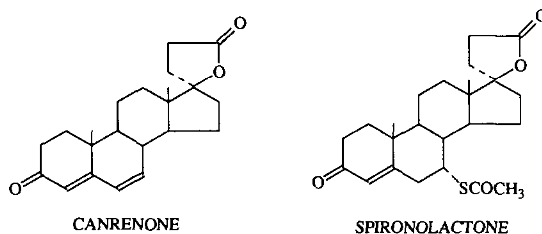

Figure 1
Chemical structures of canrenone and spironolactone.

Experimental

Reagent and chemicals

Powdered samples of canrenone, spironolactone, 7α-thiospironolactone, 7α-thiomethylspironolactone and 6-β-hydroxy-7-α-thiomethylspironolactone were supplied by G.D. Searle (Chicago, IL, USA). The internal standard, 5α-androstan-17β-ol-3-one was obtained from Sigma (St Louis, MO). Pooled drug-free human serum (Biological Specialty, Lansdale, PA) was stored at −20°C and allowed to thaw at ambient temperature (23 ± 1°C) before use. Disposal 1-ml octadecylsilane (C18) solid-phase extraction columns (Varian Sample Preparation Products, Harbor City, CA) were used for

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† Author to whom correspondence should be addressed.

sample cleanup. All other chemicals and solvents were the highest grade of commercially available materials.

LC parameters

The chromatograph consisted of a Beckman Model 110B solvent delivery module (Beckman, San Ramon, CA) an Alcott Model 738 autosampler (Alcott Chromatography, Norcross, GA), and an ABI Model 759A Absorbance Detector (Applied Biosystems, Foster City, CA). The detector wavelength was set at 280 nm. Data acquisition and manipulation were performed on a HP model 3394A integrator (Hewlett–Packard, Avondale, PA). Quantitation was based on linear regression analysis of peak height ratios of canrenone to internal standard versus canrenone concentration.

Separation was accomplished on a Spherisorb ODS-1 (5-μm, 250 mm × 4.6 mm i.d.) column (Alltech, Deerfield, IL) equipped with a direct-connect ODS guard column. The column was maintained at ambient temperature (23 ± 1°C). The isocratic mobile phase consisted of acetonitrile–water (50:50, v/v) and was deaerated by sonication before use. The flow rate was set at 1.0 ml min^{-1}.

Preparation of stock solutions

A 2 μg ml^{-1} stock solution of canrenone was prepared by dissolving a weighed amount of a powder in the mobile phase. An internal standard stock solution containing 2 mg ml^{-1} of 5α-androstan-7β-ol-3-one was also prepared in the mobile phase.

Preparation of standard solutions

Standard solutions of canrenone for a calibration curve were prepared from the canrenone stock solution by transferring accurately measured volumes of 5, 40 and 170 μl into individual 1-ml volumetric tubes. Internal standard stock solution (50 μl) was added followed by mobile phase to a volume of 300 μl. Aliquots were placed into autosampler vials, and a 100 μl sample was injected into the liquid chromatograph.

Extraction of serum samples

To a 1-ml human serum sample was added 50 μl of the internal standard stock solution. The sample was vortexed for 30 s and subjected to solid-phase extraction (SPE) for sample clean-up. A C_{18} extraction column was attached to the Vac-Elut apparatus (Varian Sample Preparation Products) and the column conditioned with four column volumes each of acetonitrile followed by four column volumes each of water. The serum sample was added to the SPE column and a vacuum was applied to draw the sample through the column. Then the SPE column was washed with one column volume of water. A 1 ml volumetric tube was inserted into the collection manifold and the drug and internal standard were eluted from the column using 2–500 μl aliquots of acetonitrile. The eluent was collected and evaporated to dryness under a gentle stream of nitrogen. A 300-μl portion of the mobile phase was added to reconstitute the residue, and duplicate 100-μl aliquots were injected into the liquid chromatograph.

Results and Discussion

Typical chromatograms of a blank human serum and a serum spiked with 195 ng ml^{-1} canrenone are shown in Fig. 2. 5α-Androstan-7β-ol-3-one was chosen as the internal standard since it eluted without interference from canrenone and endogenous serum components. Retention times of canrenone and 5α-androstan-7β-ol-3-one were approximately 10.9 and 11.9 min, respectively. The method

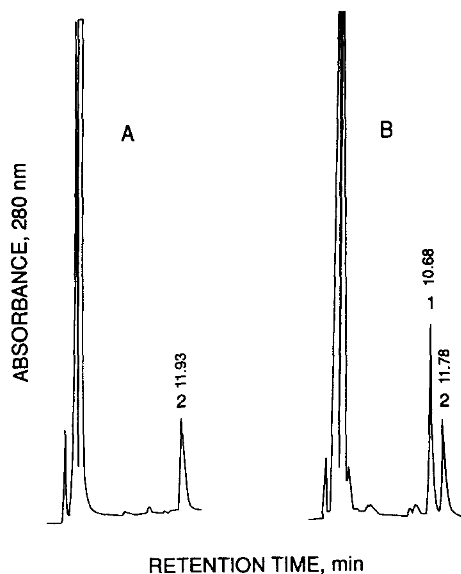

Figure 2
Typical chromatograms of blank serum (A) and serum spiked with 195 ng ml^{-1} of canrenone (B). Peak 1 is canrenone and Peak 2 is the internal standard at 100 μg ml^{-1}. Chromatographic parameters are listed in the Experimental section.

Table 1
Recovery of canrenone from spiked human serum

Concentration added ($ng\ ml^{-1}$)	Concentration found ($ng\ ml^{-1}$)	Recovery (%)
10.73	11.07 ± 0.33*	103.2 ± 3.0
85.86	87.52 ± 1.90	101.9 ± 2.2
322.0	321.9 ± 4.66	100.0 ± 1.4

*Mean ± standard deviation based on $n = 3$.

Table 2
Precision and accuracy of canrenone in human serum

Concentration added ($ng\ ml^{-1}$)	Concentration found ($ng\ ml^{-1}$)	Error (%)	RSD (%)
Intra-day			
21.46	22.30 ± 0.89*	3.9	4.0
214.6	219.4 ± 1.30	2.2	0.6
Inter-day			
21.46	22.30 ± 1.32*	0.2	5.9
214.6	219.9 ± 9.70	2.4	4.4

*Mean ± standard deviation based on $n = 5$.

was shown to be linear for canrenone in the 10–340 ng ml^{-1} range in serum. Linear regression analysis of canrenone/internal standard peak height ratios versus canrenone concentration in serum gave a correlation coefficient of 0.9999 ($n = 6$). The minimum quantifiable level was determined to be 10 ng ml^{-1}. The limit of detection ($S/N = 2$) for canrenone in serum was 3 ng ml^{-1}.

The recovery of canrenone from human serum was assessed using spiked samples at three levels over the 10–340 ng ml^{-1} calibration range. The samples were subjected to identical SPE extraction and assay procedures as serum samples (see Experimental section). The recovery data are shown in Table 1. The intra-day and inter-day precision and per cent error of the method were determined by replicate measures of serum spiked with two different canrenone concentrations on the initial day of preparation and on five subsequent days, respectively. The data are listed in Table 2.

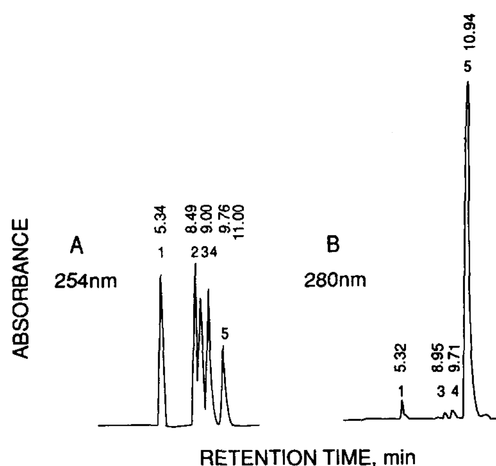

Figure 3
HPLC separation of 6β-hydroxy-7-α-thiomethylspironolactone (1), spironolactone (2), 7-α-thiospironolactone (3), 7-α-thiomethylspironolactone (4) and canrenone (5) at 254 nm (A) and 280 nm (B). Chromatographic conditions are listed in the Experimental section.

Canrenone was well separated from spironolactone and its other metabolites using the chromatographic conditions described herein. Setting the detector wavelength at 280 nm not only utilizes the absorption maximum of canrenone in the mobile phase, but it is also the wavelength at which the absorbancies of spironolactone and its metabolites are less interfering. Figure 3(a) shows a typical LC separation of canrenone, spironolactone, 7α-thiospironolactone, 7α-thiomethylspirolactone and 6β-hydroxy-7α-thiomethylspironolactone at 10 μg ml^{-1} concentrations measured at 254 nm. The same mixture measured at 280 nm [Fig. 3(b)] shows a 4-fold increase in the canrenone peak with concomitant decreases in the sizes of the remaining analytes, thus not interfering with the measurement of the canrenone peak.

The method developed herein was applied to the assay of canrenone levels in the serum of a human subject administered a single oral dose

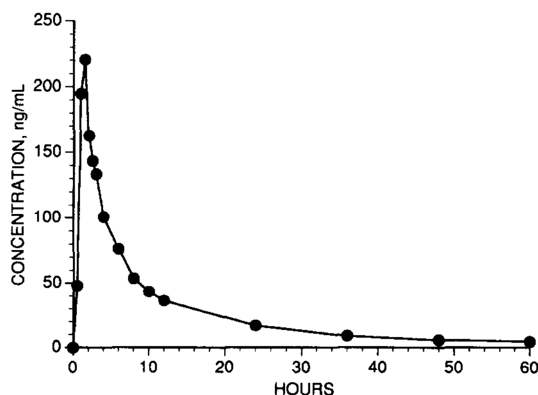

Figure 4
Serum concentration–time curve for canrenone in human subject administered a single oral dose of spironolactone.

of spironolactone. Figure 4 shows a concentration–time curve for canrenone in that subject. The assay procedure should be useful in measuring canrenone levels in human serum for bioavailability and/or pharmacokinetic studies.

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