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# DETERMINATION OF THE SERUM CONCENTRATION OF SPIRONOLACTONE AND ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A simple and rapid high-performance liquid chromatographic assay is described for the simultaneous determination in serum of the aldosterone antagonist spironolactone and its metabolites 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone. Ultraviolet absorption at 240 nm was used to detect the different compounds after elution on a normal-phase column. Endogenous serum substances did not interfere with the assay. This method provides a convenient tool in pharmacokinetic studies of spironolactone, in contrast to previously reported aspecific fluorimetric assays or time-consuming thin-layer chromatographic analyses of radioactive biological material.

## INTRODUCTION

Spironolactone, a synthetic steroid, has been used for more than twenty years

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in the therapy of oedematous conditions, as an adjunct to thiazide diuretics in the treatment of essential hypertension and in all pathological conditions associated with hyperaldosteronism [1, 2]. It is extensively metabolized in the body into a large number of metabolites (Fig. 1) [3—6].

For a long time, canrenone was thought by many authors to be the major metabolite [7, 8], and its serum levels were most commonly measured by a fluorimetric assay developed by Gochman and Gantt [9], which was modified by other investigators [10].

With the introduction of high-performance liquid chromatographic (HPLC) methods to measure canrenone concentrations, it became clear that the fluorimetric method was not specific for canrenone but measured other fluorigenic metabolites as well [11—13]. True canrenone concentrations (i.e. measured by HPLC) were only ca. one third of the concentrations measured with the fluorimetric method [14, 15]. This finding gave support to the thought that canrenone was not the major metabolite [16]. Other metabolites,

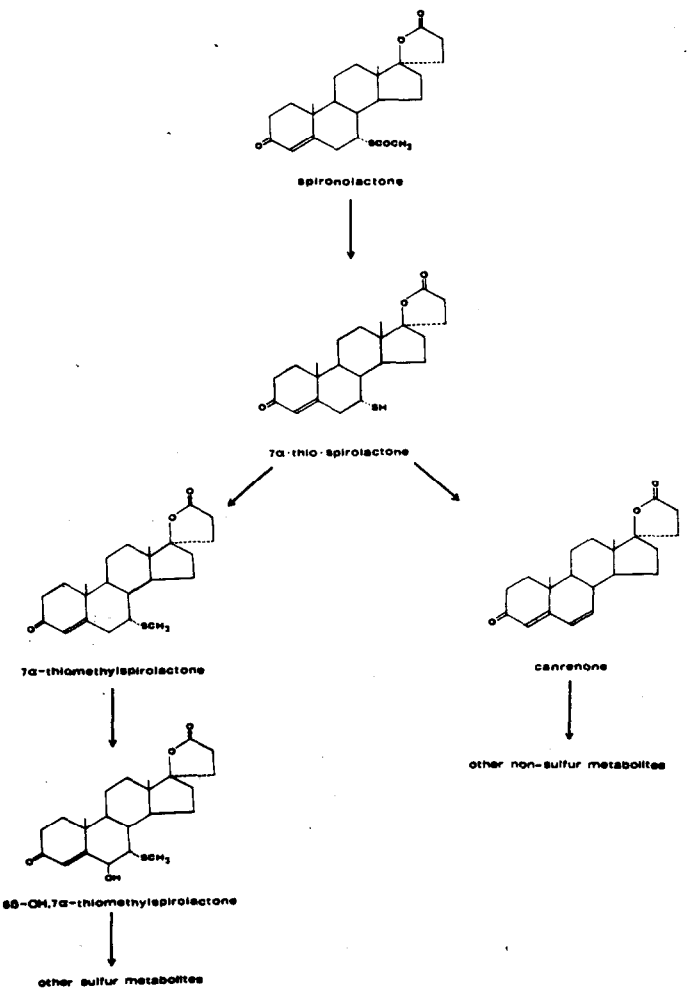


Fig. 1. Routes of metabolism of spironolactone.

or perhaps spironolactone itself, might also be responsible for the activity of spironolactone [17, 18].

The aim of this study was to develop a rapid and sensitive HPLC assay for the simultaneous determination in serum of spironolactone and its metabolites 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone. These are the compounds we found in serum of human volunteers who had taken 200 mg of spironolactone orally.

## EXPERIMENTAL

### *Reagents and standards*

Spironolactone, 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone were kindly supplied by Searle (Chicago, IL, U.S.A.). Serum standards were prepared by spiking blank serum samples with these compounds.

As the internal standard we used megestrolacetate (Novo, Copenhagen, Denmark). Diisopropyl ether, methanol, ethanol and tetrachloromethane (Merck, Darmstadt, F.R.G.) were of analytical-reagent grade. Methanol and diisopropyl ether were filtered through a Sartorius filter (0.2 μm pore size, Type 11687; Göttingen, F.R.G.) before serving as the mobile phase.

### *HPLC instrumentation and conditions*

The HPLC system consisted of a solvent-delivery pump (Waters Assoc., Milford, MA, U.S.A., Model 510) and a variable-wavelength UV detector (Waters, Model 481), set at 240 nm. A Partisil column (particle size 5 μm, 150 x 4.6 mm I.D., Chrompack, Middelburg, The Netherlands) was used for the separation. To protect the column a silica precolumn (Guard-Pak Precolumn Module, Waters) was installed. The mobile phase consisted of diisopropyl ether—methanol (98.25:1.75) at a flow-rate of 2.2 ml/min. Chromatography was carried out at ambient temperature.

### *Sample preparation*

To 1 ml of serum in a 10-ml centrifuge tube were added 100 μl of internal standard solution (megestrolacetate 2 μg/ml) and 100 μl of demineralised water. For the preparation of the serum standard samples, instead of 100 μl of demineralised water, 100 μl of a standard solution were added. This solution contained spironolactone, 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone in demineralised water, each in a concentration of 2 μg/ml.

Each tube was vortexed to mix the serum with these solutions. Then 5 ml of tetrachloromethane were added and the tubes were shaken mechanically for 10 min. After centrifugation for 5 min at 700 *g*, the aqueous layer was discarded and the organic layer evaporated to dryness under a mild nitrogen stream at 40°C. The residue was reconstituted in 200 μl of the mobile phase and 100 μl were injected onto the column.

Serum samples were spiked with different amounts of spironolactone and its metabolites. Final concentrations of spiked serum samples were 0, 50, 100, 200 and 400 ng/ml.

*Reproducibility and recovery studies*

The reproducibility of each compound was determined in eight-fold at the concentrations 50 and 200 ng/ml. Blank human serum was spiked with the compounds to obtain these concentrations. Recovery studies were performed for the internal standard and for each of the compounds. Six replicate analyses for each compound and the internal standard were carried out at a concentration of 400 ng/ml. Peak height ratios of the compounds concerned and the internal standard were plotted against the concentrations of the compounds.

## RESULTS

Under the conditions described, 7α-thiomethylspirolactone, spironolactone, canrenone and 6β-hydroxy-7α-thiomethylspirolactone are eluted after ca. 7.1, 8.4, 9.3 and 10.1 min, respectively. The internal standard has a retention time of 3.3 min. Fig. 2A shows a representative chromatogram of blank human serum spiked with the above-mentioned compounds. Chromatograms of non-spiked serum samples showed no interference from endogenous serum substances (Fig. 2B).

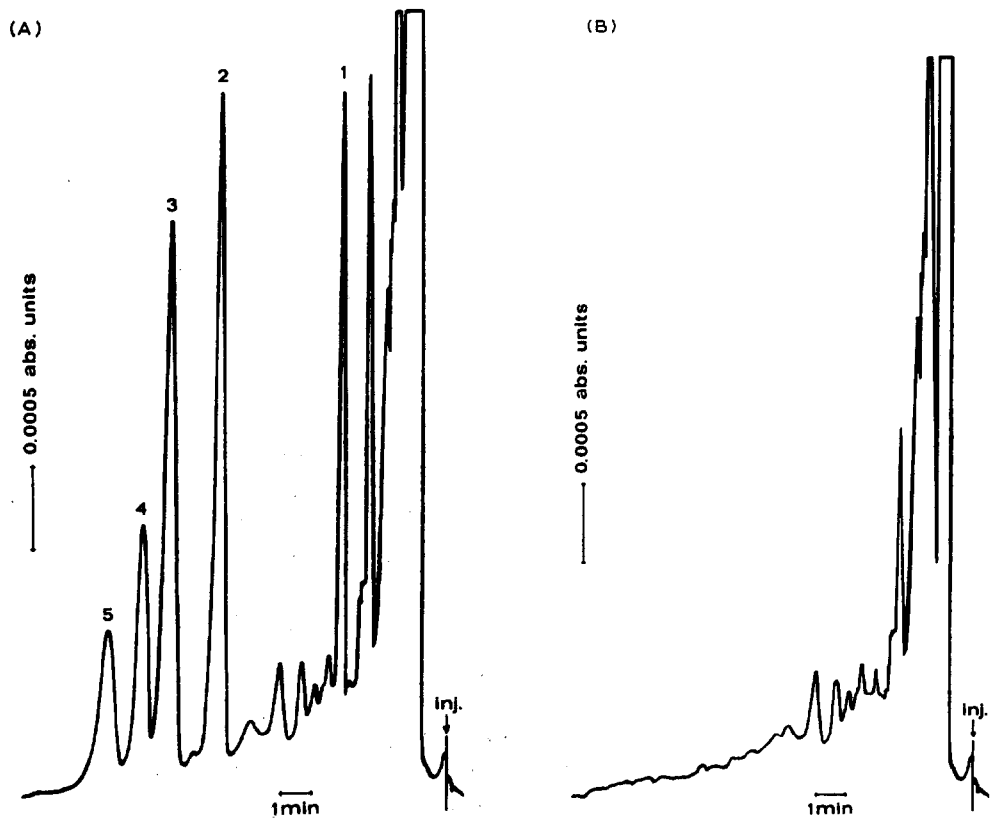


Fig. 2. (A) Chromatogram obtained after extraction from 1 ml of blank human serum spiked with 200 ng of the internal standard megestrolacetate (1), 7α-thiomethylspirolactone (2), spironolactone (3), canrenone (4) and 6β-hydroxy-7α-thiomethylspirolactone (5). (B) Chromatogram obtained after extraction from 1 ml of non-spiked blank human serum.

Methanol in a concentration of 1.75% in diisopropyl ether, at a flow-rate of 2.2 ml/min, was found to give the best resolution and reasonable elution times. In preliminary studies, small differences in the methanol concentration of the mobile phase greatly altered the retention times and the resolution of all compounds.

The detection wavelength was set at 240 nm, at which spironolactone and the thiomethyl- and the hydroxythiomethyl compounds exhibit their absorption maxima. Canrenone has its maximum at 280 nm but, because of a plateau-like absorption curve, it still shows sufficient absorption at 240 nm to be measured accurately. Of the many steroid compounds tested, megestrolacetate was selected as the internal standard because it has a retention time that did not interfere with spironolactone and its metabolites and with endogenous serum compounds.

Serum standard curves for all compounds were linear over the range 50—400 ng/ml. The coefficients of correlation of the standard curves were 0.9994, 0.9998, 0.999 and 0.998 for 7α-thiomethylspirolactone, spironolactone, canrenone and 6β-hydroxy-7α-thiomethylspirolactone, respectively.

The coefficients of variation at two concentrations and the limits of detection for each compound are listed in Table I. The detection limit was defined as the concentration of the compound yielding a signal-to-noise ratio of 4.

TABLE I

COEFFICIENTS OF VARIATION AND LIMITS OF DETECTION OF THE ASSAY METHOD

Coefficients of variation were obtained at two concentrations for each compound by spiking human blank serum with the appropriate amount of each compound. Analyses were performed in eight-fold. Limits of detection are based on extracting 1-ml samples of serum.

| Compound | Coefficient of variation (%) | | Limit of detection (ng/ml) |
|---|---|---|---|
| | 50 ng/ml | 200 ng/ml | |
| 7α-Thiomethylspirolactone | 8.7 | 4.5 | 5 |
| Spironolactone | 7.0 | 3.6 | 5 |
| Canrenone | 6.8 | 4.7 | 10 |
| 6β-Hydroxy-7α-thiomethylspirolactone | 6.6 | 5.7 | 20 |

TABLE II

EXTRACTION RECOVERIES

Recoveries (mean percentage ± S.D.) were determined by extracting 1 ml of serum with a concentration of 400 ng/ml of each compound ($n = 6$ in all cases) and comparing the peak heights measured with those of unextracted compounds.

| Compound | Recovery |
|---|---|
| Internal standard (megestrolacetate) | 94 ± 3 |
| 7α-Thiomethylspirolactone | 83 ± 3 |
| Spironolactone | 89 ± 5 |
| Canrenone | 95 ± 2 |
| 6β-Hydroxy-7α-thiomethylspirolactone | 57 ± 3 |

The recoveries from serum of each of the compounds tested can be found in Table II.

## DISCUSSION

The role of the metabolite canrenone in the metabolism and activity of spironolactone is less important than had previously been assumed [11—16]. Other metabolites, or perhaps spironolactone itself, must contribute considerably to the pharmacological effects of spironolactone [16—18]. The sulphur-containing metabolites of spironolactone have been mentioned particularly as possibly active metabolites [2, 16, 18]. Until now, however, no simple and specific assay for the determination of sulphur-containing metabolites and spironolactone in humans has been described.

Two studies described a quantitative thin-layer chromatographic (TLC) analysis of serum after administration of radioactive labelled spironolactone to volunteers [1, 6]. However, contrasting results were found, which were attributed to methodological differences in work-up procedures. Also, in order to obtain sufficient radioactivity to carry out both extraction and TLC analysis, either serum samples had to be pooled [1], or a relatively high dose of radioactivity had to be administered to volunteers [6].

There is obviously a need for a simple, specific and reproducible assay for the determination of spironolactone and its metabolites in biological material. The HPLC method described in this paper permits a rapid simultaneous determination of serum concentrations of spironolactone and its metabolites 7$\alpha$-thiomethylspirolactone, 6$\beta$-hydroxy-7$\alpha$-thiomethylspirolactone and canrenone.

Preparation of serum samples prior to chromatography is simple. The analysis time for a single sample is ca. 40 min, but this is shortened considerably if a number of samples are extracted and analysed simultaneously. The sensitivity, reproducibility and limits of detection are satisfactory and no interference of endogenous serum compounds is observed. The method will be valuable in clarifying many unknown pharmacokinetic aspects of spironolactone.

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