Journal of Chromatography, 230 (1982) 37—45
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1234

DETERMINATION OF PLASMA LEVELS OF SPIRORENONE, A NEW ALDOSTERONE ANTAGONIST, AND ONE OF ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

WERNER KRAUSE* and UWE JAKOBS

Department of Pharmacokinetics, Schering Berlin/Bergkamen, Müllerstrasse 170—178, 1000 Berlin 65 (G.F.R.)

(First received November 16th, 1981; revised manuscript received January 11th, 1982)

SUMMARY

A method for the determination of plasma concentrations of spirorenone, a new aldosterone antagonist, and one of its metabolites, chromatographically characterized as 1,2-dihydro-spirorenone, is described. The assay utilizes high-performance liquid chromatography with UV detection. Reproducible results can be obtained with standard deviations of about 5% and the limit of detection is less than 5 ng/ml. Plasma levels of drug and metabolite have been measured after oral doses of 10 and 40 mg, respectively, administered to two male volunteers.

INTRODUCTION

Spirorenone (6β,7β,15β,16β-dimethylene-1,4-androstadiene-[17(β-1′)-spiro-5′]perhydrofuran-2′,3-dione) is a newly synthesized aldosterone antagonist which is reported to be more than five times as potent in rats than spironolactone [1]. Currently the drug is under investigation in man using an aldosterone infusion model with constant oral water load of the test subjects. During these studies blood samples were drawn for the analysis of spirorenone levels. Another point of investigation was the possible appearance of the 17(α-1′) compound (Fig. 1) in blood, which was known from in vitro studies to be the acid-catalyzed rearrangement product of spirorenone [2] and therefore might have been formed in the stomach. The 17(α-1′) form is pharmacologically inactive and so, if substantial amounts of this compound are to be detectable in blood, a pharmaceutical formulation resistant to gastic juice must be developed.

Therefore the aim of the present study was to establish an assay procedure capable of detecting low plasma concentrations and which was able to separate the 17(α-1′) and 17(β-1′) forms described above.

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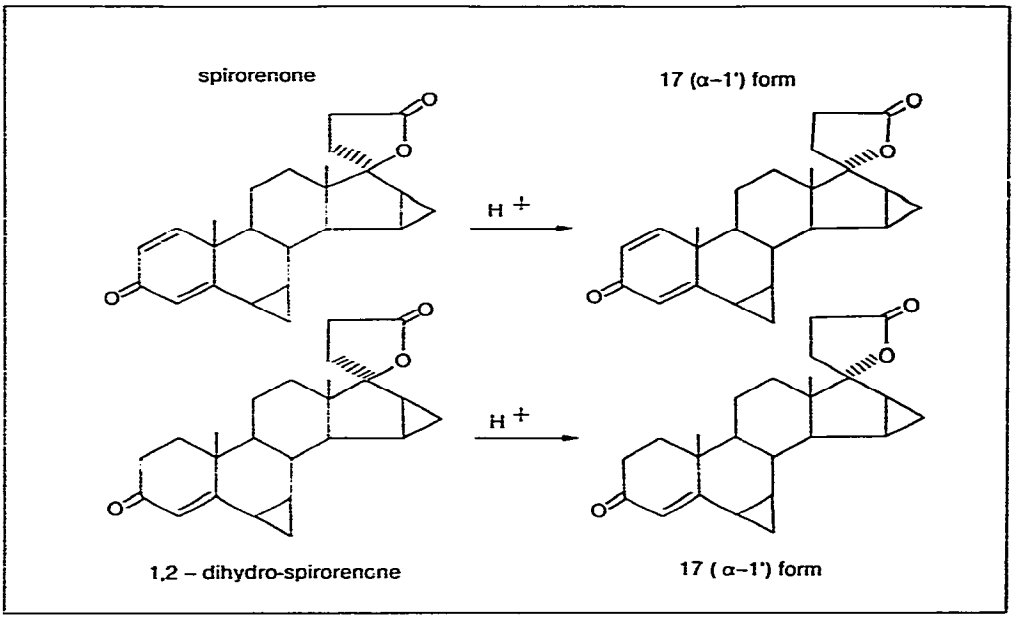

Fig. 1. Acid-catalyzed rearrangement of spirorenone and its 1,2-dihydro derivative.

EXPERIMENTAL

Subjects and medication

Two healthy male volunteers (24 and 21 years of age, 67 and 74 kg body weight, respectively) were given 10 mg of spirorenone in tablet form during constant aldosterone infusion (1 mg per 12 h) and oral water load (3250 ml per 12 h). Blood samples were taken at 0, 0.5, 1, 1.5, 2, 3, 6 and 11.5 h after drug administration. The samples were immediately centrifuged and the plasma kept frozen until analysis. One week later the same subjects were given 40 mg of spirorenone under the same experimental conditions.

Chemicals

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

Spirorenone and 1,2-dihydro-spirorenone were stored dissolved in methanol in concentrations of 10 μg/ml.

Extraction procedure

Three millilitres of plasma were pipetted into a 10-ml stoppered test tube and 3 ml of *n*-hexane—toluene (1:1, v/v) were added. After thorough mixing on a Vortex mixer for 1 min and centrifugation at 1200 *g* for 5 min, the organic phase was removed. The residue was extracted once again with 3 ml of the solvent described above and the two organic phases were combined and taken to dryness under a slight stream of nitrogen. The dry extract was taken up in 200 μl of the high-performance liquid chromatography (HPLC) mobile phase; 150 μl were injected for analysis. Alternatively, for the low-dose study, 5 ml of plasma were extracted three times each with 2 ml of solvent.

The extraction efficiencies were determined with 3-ml plasma samples containing 200 ng/ml spirorenone and 30 ng/ml 1,2-dihydro-spirorenone, and with 5-ml plasma samples containing 20 ng/ml and 10 ng/ml, respectively.

Chromatographic systems

High-performance liquid chromatography. The HPLC system consisted of a solvent delivery pump (Waters, Königstein, G.F.R.; type 6000 A), a LiChrosorb RP-18 chromatographic column (10 μm particle size, 250 × 4.6 mm; Knauer, Berlin, G.F.R.) and a UV detector with fixed wavelength (254 nm; Knauer). Alternatively, a UV detector with variable wavelength (Schoeffel SF 770) was used. The detector signals were converted to a chromatographic trace by a W + W recorder (Basle, Switzerland). Injection was accomplished with a Rheodyne RH 7120 system. The mobile phase consisted of methanol—water (60:40, v/v). The eluent was degassed at reduced pressure before use. The chromatographic system was operated at ambient temperature with an eluent flow-rate of 2 ml/min.

For in vitro acid-catalyzed rearrangement studies of spirorenone two LiChrosorb RP-18 columns (10 μm particle size, 250 × 4.6 mm) in series were used; the mobile phase consisted of methanol—water (60:40, v/v) with 0.01 *M* sodium dodecyl sulphate and 2 ml of acetic acid per litre. Injection was accomplished with an automatic sampling device (WISP, Waters).

Standard curves were constructed with 3-ml blank plasma samples containing 0, 25, 50, 100, 200, 400 and 800 ng of spirorenone and 20, 40, 80, 160 and 320 ng of 1,2-dihydro-spirorenone. These samples were extracted by the method described above. Peak heights as determined by HPLC analysis were measured and plotted against the amount of the compound studied. Five-millilitre samples were handled likewise, since it had been ascertained previously that there was no difference in recoveries.

The overall accuracy of the HPLC assay was calculated from five consecutive determinations of two concentrations (see Table I).

Thin-layer chromatography. For thin-layer chromatography (TLC) silica gel precoated plates (Merck 60 F_{254}, 20 × 20 cm, layer thickness 0.25 cm) were used. Plasma extracts obtained by the procedure described above were dissolved in chloroform and spotted onto the plates. Development was performed twice with chloroform—methanol (96:4, v/v). Thereafter the upper half of each plate was cut off and run again in toluene—*n*-hexane (50:50, v/v). TLC spots were analyzed with a Zeiss scanner (KM 3) in the remission mode.

In vitro rearrangement

Two millilitres of 0.1 *N* aqueous hydrochloric acid solution were added to 500 μg of spirorenone and its 1,2-dihydro derivative in a sampling vial of the WISP. After short ultrasonic treatment 20 μl of the solution were repetitively injected into the HPLC system.

RESULTS

Assay of plasma levels

A highly sensitive and selective method for the determination of the new aldosterone antagonist spirorenone and one of its metabolites in plasma is described utilizing HPLC with UV detection (Fig. 2). Extraction from biological samples is performed with *n*-hexane—toluene (1:1). The recovery using this

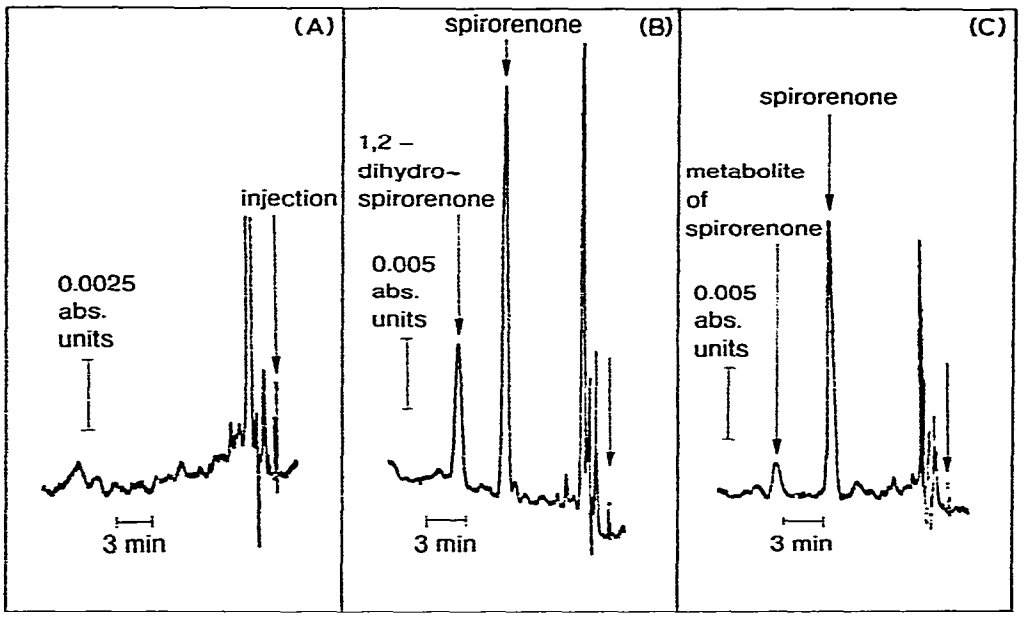

Fig. 2. HPLC chromatograms of (A) blank plasma samples, (B) plasma spiked with 133 ng/ml spirorenone and 53 ng/ml 1,2-dihydro-spirorenone, and (C) a plasma sample obtained from subject No. 2, 3 h after an oral dose of 40 mg of spirorenone.

TABLE I

EXTRACTION RECOVERIES OF DRUG AND METABOLITE

Recoveries were determined by extracting 3 or 5 ml of plasma spiked with different amounts of spirorenone and 1,2-dihydro-spirorenone, and comparing the peak heights measured to those of non-extracted material.

Spirorenone				1,2-Dihydro spirorenone			
200 ng/ml (3 ml)		20 ng/ml (5 ml)		30 ng/ml (3 ml)		10 ng/ml (5 ml)	
Peak height* (mm)	Recovery (%)	Peak height** (mm)	Recovery (%)	Peak height* (mm)	Recovery (%)	Peak height** (mm)	Recovery (%)
47	73	36	76	10	73	23	83
52	81	37	78	10	73	25	89
52	81	38	80	11	79	25	89
54	85	39	83	11	79	25	89
53	83	42	90	11	79	26	91
52 ± 3	81 ± 4	38 ± 2	82 ± 6	11 ± 1	76 ± 4	25 ± 1	88 ± 3

* 0.04 absorbance units.
** 0.01 absorbance units.

procedure was found to be about 80% independent of the volume of the test samples (Table I).

Matrix constituents and metabolites of spirorenone are then separated from the drug by HPLC using a reversed-phase system. The retention times of spirorenone, its 1,2-dihydro derivative and of the corresponding α forms were 9.2 min, 13.4 min, 10.5 min and 15.9 min corresponding to k' values of 9.2, 13.9, 10.7 and 16.7, respectively. Spirorenonic acid, the compound obtained after hydrolyzing the lactone ring, had a retention time of 2.7 min, corresponding to $k' = 2.0$.

Unknown concentrations of spirorenone and of its metabolite, tentatively characterized as 1,2-dihydro-spirorenone (cf. below), were determined by comparing their peak heights with those of spiked plasma samples. Linear calibration curves corresponding to the following equations were obtained:

spirorenone: peak height (mm) = 0.896 + 0.567 drug amount (ng)

1,2-dihydro-spirorenone: peak height (mm) = 1.871 + 0.462 drug amount (ng)

Correlation coefficients were calculated to be $r = 0.995$ and $r = 0.990$, respectively.

The overall accuracy of the assay expressed as standard deviation of five consecutive determinations of 200 and 20 ng/ml of drug were 5.2% and 6.0%, respectively; 20 and 10 ng/ml of the metabolite were determined with an accuracy of 5.2% and 4.4%, respectively. The detection limit of the assay is less than 5 ng/ml for both compounds of interest.

In vitro rearrangement

Spirorenone and its 1,2-dihydro derivative are unstable towards acid-catalyzed lactone ring isomerization (Fig. 3). On incubating the two compounds with 0.1 *N* hydrochloric acid, about 80% is converted into the α forms reaching a plateau at about 400 min after beginning (Fig. 4). However, the process of rearrangement was relatively slow compared to possible absorption rates in the stomach (cf. below).

Study of plasma levels

Spirorenone was absorbed with a half-life of 0.5—0.7 h (Table II) and reached its maximum plasma concentration 3 h after administration at a level of 41 ± 1 ng/ml (10 mg dose) and 105 ± 9 ng/ml (40 mg), respectively (Fig. 5). Until 11.5 h after administration the concentration of the drug diminished with half-lives of 6—9 h. The area under the plasma concentration—time curve (AUC) was 290 ± 16 ng h ml^{-1} (10 mg dose) and 851 ± 102 ng h ml^{-1} (40 mg dose), respectively.

The lactone rearrangement product of spirorenone was not detectable in the plasma, suggesting that the absorption process was much faster than the acid-catalyzed isomerization of the drug.

The compound obtained by opening of the lactone ring, a metabolite found after administering spironolactone [3—5] was hard to detect in plasma extracts using the HPLC system described above because of its short retention time. A

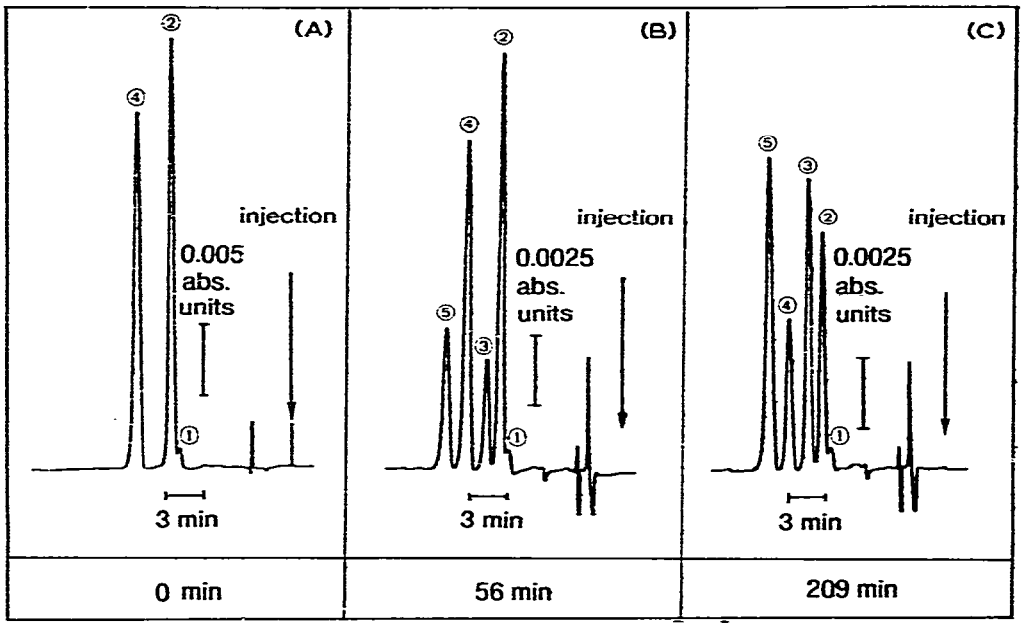

Fig. 3. HPLC chromatograms of incubates of spirorenone and its 1,2-dihydro derivative in 0.1 *N* HCl at $t = 0$ (A), $t = 56$ min (B), and $t = 209$ min (C). 1 = Impurity of spirorenone; 2 = spirorenone; 3 = 17(α-1') form of spirorenone; 4 = 1,2-dihydro-spirorenone; 5 = 17(α-1') form of 1,2-dihydro-spirorenone.

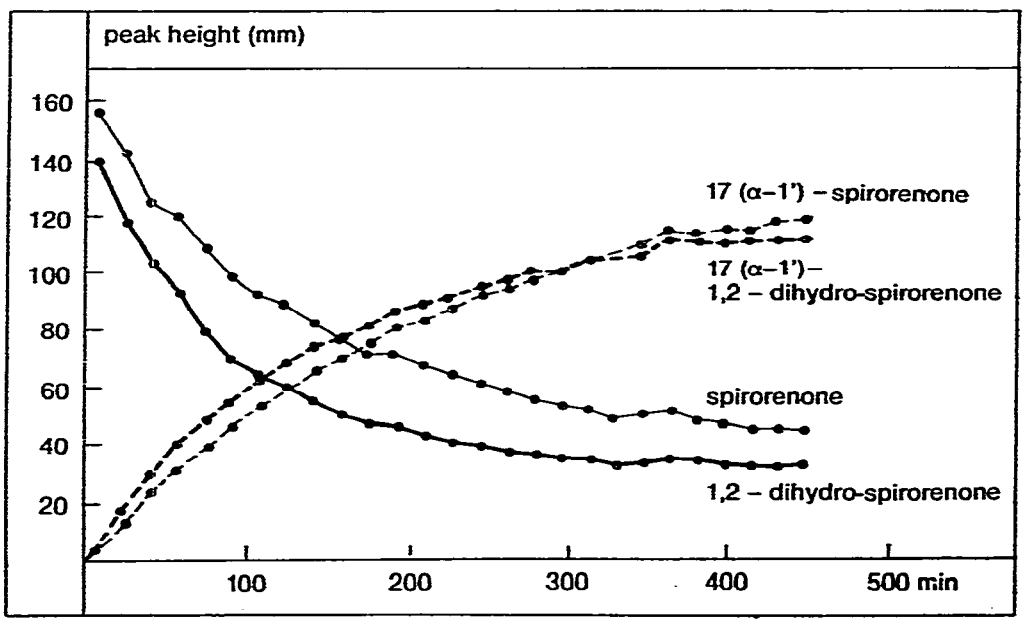

Fig. 4. Time course of acid-catalyzed rearrangement of spirorenone and 1,2-dihydro-spirorenone.

TABLE II

INDIVIDUAL PHARMACOKINETIC PARAMETERS OF TWO TEST SUBJECTS AFTER ORAL ADMINISTRATION OF 10 AND 40 mg OF SPIRORENONE, RESPECTIVELY

Dose (mg)	Test subject	Absorption $t_{1/2}$ (h)	Maximum (h)	Concentration (ng/ml)	Elimination $t_{1/2}$ (h)
10	1	0.4	2	49	5.3
	2	1.0	3	40	5.6
40	1	0.5	3	111	8.9
	2	0.5	3	98	9.0

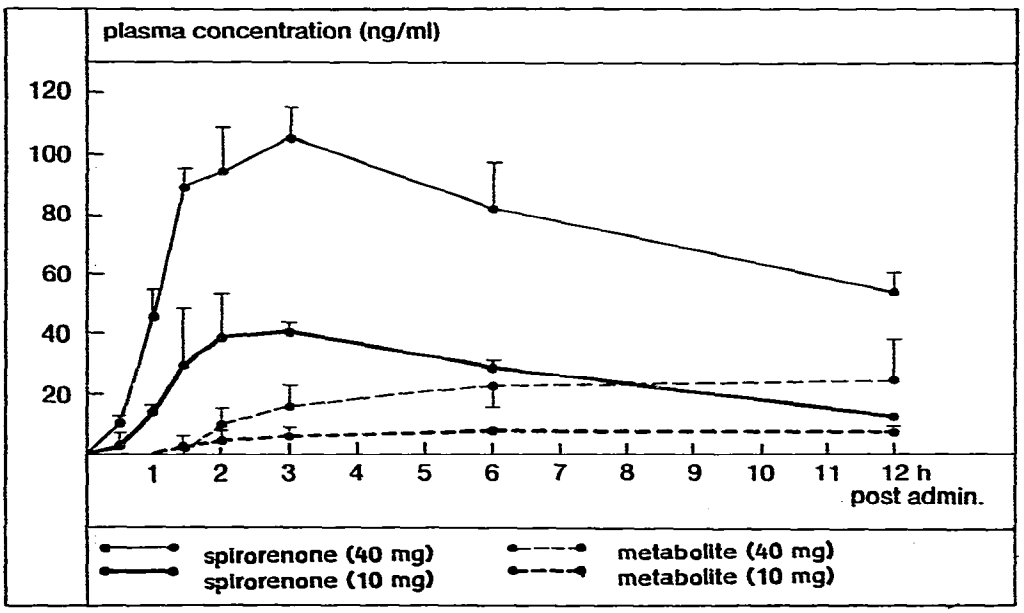

Fig. 5. Plasma levels of spirorenone and its metabolite (means) after oral administration of 10 and 40 mg of spirorenone to two male volunteers.

lot of matrix constituents were observed at this range of retention times so that this possible metabolite should not be regarded further.

A metabolite of spirorenone, however, chromatographically characterized as 1,2-dihydro-spirorenone (cf. below), could be measured in the plasma of the test subjects. It was detectable only after at least 1.5 h after drug administration, suggesting a relatively low rate of formation. Its concentration then constantly rose up to the end of the study period (Fig. 5).

Metabolite characterization

On HPLC analysis of plasma samples from test subjects having received oral doses of spirorenone, a metabolite was observed which had the same retention time as 1,2-dihydro-spirorenone (Fig. 2). On TLC analysis of the same plasma samples in the eluent system described above the metabolite again co-chroma-

tographed with the 1,2-dihydro derivative. And, moreover, the remission spectra on TLC plates of pure substance and of the metabolite after separation from plasma constituents were both identical with a UV maximum at 280 nm. Spirorenone, on the other hand, showed two maxima, at λ(1)= 250 nm and λ(2) = 290 nm (Fig. 6). Therefore it is quite probable that the metabolite observed in the HPLC chromatrogram corresponds to 1,2-dihydro-spirorenone.

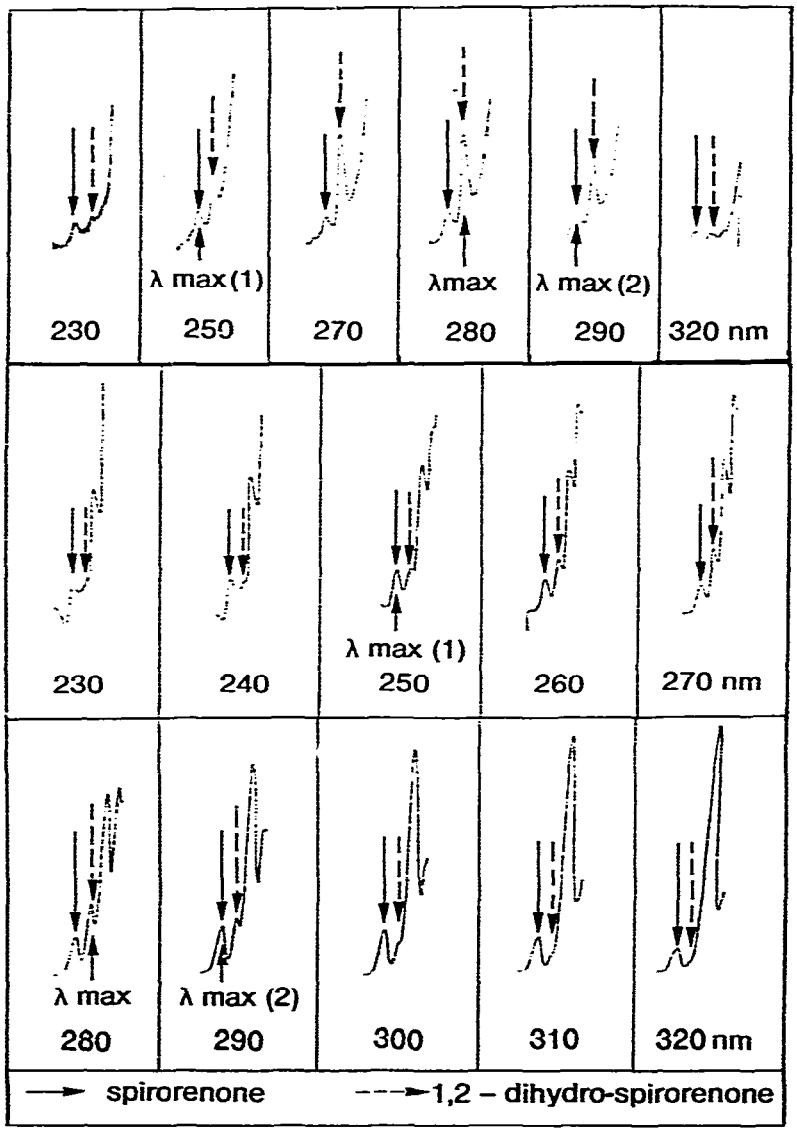

Fig. 6. UV absorption spectrum of spirorenone and its metabolite on a TLC plate after sepration from a plasma sample obtained from test subject No. 2, 11.5 h after oral administration of 40 mg of spirorenone (upper part) and as pure substances (lower part).

DISCUSSION

The present paper describes an assay procedure for the determination of spirorenone plasma levels to be expected after therapeutic drug administration. Simultaneously the concentration of a metabolite can be measured that was chromatographically characterized as 1,2-dihydro-spirorenone. Further studies including the isolation and final identification of this compound, however, still have to be performed. They are under progress at the present time.

1,2-Dihydro-spirorenone has been demonstrated to have an anti-aldosterone activity [1] about five times that of spironolactone. So if its identity could be verified in human plasma this would mean the appearance of an active metabolite probably prolonging the pharmacological activity of spirorenone itself.

The concentrations of the metabolite have been measured in this study by comparison with a standard curve of 1,2-dihydro-spirorenone although there was no explicit identification of the compound of interest. This was, however, possible since the UV spectrum of the metabolite and of 1,2-dihydro-spirorenone were identical. So if after ultimate isolation and identification of the metabolite it should — against all expectation — prove not to be the 1,2-derivative, the concentrations would nevertheless have been exactly measured.

Apart from HPLC and TLC with UV detection we had tried other methods for the determination of spirorenone in biological samples including fluorescence and electrochemical procedures and gas chromatography—mass spectrometry. However, the high temperature to volatilize the compound (300°C) or the unfavourable fluorescence and electrochemical data showed that UV detection combined with HPLC separation was the only way to measure those very low concentrations. In conclusion, the HPLC method described above seems to be a sensitive and selective assay suitable for further pre-clinical and clinical trials with spirorenone.

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