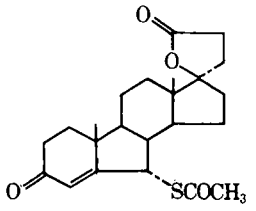
Fluorometric Microassay for Spironolactone and Its Metabolites in Biological Fluids

W. SADEE▲, M. DAGCIOGLU, and S. RIEGELMAN

Abstract □ Spironolactone (I), canrenone (II), and potassium canrenoate (III) can be analyzed in the low nanogram range utilizing a fluorescence reaction in sulfuric acid (62% v/v). The fluorescent species, a trienone, was used as an external standard. The methods were highly sensitive (~10 ng./ml. of I–III in plasma) and specific for 17-alkyl-17-hydroxy-4,6-dien-3-one steroids. The 7α-thioacetyl-4-en-3-one (I) was dethioacetylated under mild alkaline or acid conditions to the 4,6-dienone moiety prior to extraction. The ester glucuronide of III was quantitatively hydrolyzed at pH 13 for 10 min. at 100°. An estimate of acid-labile conjugates of I–III was achieved by reaction in methanolic 3.6% sulfuric acid for 30 min. at 100°. Spiro-γ-lactone congeners (*e.g.*, I and II) were separated from γ-hydroxycarboxylic acids (*e.g.*, III) by extraction into methylene chloride at pH 7–8. The methods were applicable to biological samples such as plasma, bile, urine, and gastric fluids.

Keyphrases □ Spironolactone—fluorometric analysis, biological fluids □ Canrenone and potassium canrenoate—fluorometric analysis, biological fluids □ Spectrophotofluorometry—analysis, spironolactone, canrenone, potassium canrenoate, and metabolites in biological fluids

In prior investigations (1–3), a major metabolite of the antimineralocorticoid spironolactone[1] (I) was identified to be canrenone[2] (II). Potassium canrenoate[2] (III) was also metabolized to II (1–4). A fluorometric micromethod, which only accounts for II, was developed by Gochman and Gantt (1). The purpose of the present study was to extend the applicability of the fluorescence analysis to Compounds I–III and to other metabolites such as the recently described ester glucuronide of canrenoate (IV) (4).



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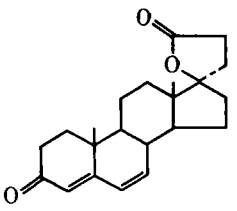

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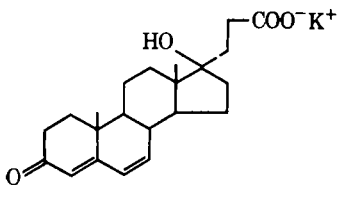


III

The mechanism of the fluorescence reaction was elucidated previously to exclude errors in measuring metabolites (5). The fluorescent species was isolated as yellowish crystals, to which the structure of the trienone V was assigned (5). This compound was used as the fluorescent standard. It became evident that any reduced metabolites would not yield the fluorescent trienones under the conditions used. The procedures were designed for estimating I and its metabolites in biological samples such as bile, plasma, gastric fluid, and urine in the rat, dog, and man.

EXPERIMENTAL

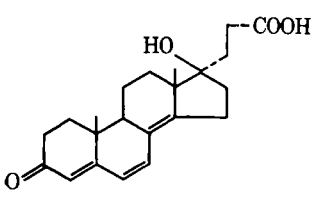
Reagents and Apparatus[3]—All of the solvents and chemicals used were of analytical reagent grade. Triple-distilled water and freshly distilled methylene chloride were used.

Assay Procedures—*External Standard Solution*—3-(17β-Hydroxy-3-oxoandrosta-4,6,8(14)-trien-17-yl)propionic acid (V) (5) was used as an external standard which represented the fluorescent species of I–III. One milliliter of a stock solution of 10 mg. V in 100 ml. methanol was diluted to 50 or 100 ml. each time with water. Appropriate quantities of these solutions up to 0.2 ml. were diluted to 5 ml. with 62% sulfuric acid (62 ml. of 96% sulfuric acid and 38 ml. of water) to yield standard solutions containing 40–160 ng./ml. The fluorescence of 2 ml. of these solutions was read at an activation maximum of 483 nm. and a fluorescence maximum of 525 nm. The fluorescence yield was linear over a broad concentration range of V (4 ng. to >1 mcg. V/sample). To compensate for the loss of fluorescence over time (about 3% within 10 min.), the standard should be prepared each time at the end of the extraction procedures.

The amounts of I–III, which were carried through the procedures as described below, were calculated from the fluorescence readings of the standard and the samples using a factor F, which resulted from the fluorescence yield of I–III and the recovery by the extraction procedures. For a better comparison, all amounts of I–III were calculated as III considering a relative molecular weight of I/II/III = 100/82/95. Since fluorescence readings of III and the standard V were compared having a ratio of relative molecular weights 95/82, a factor F = 1.17 would indicate a complete extraction and complete conversion to V.

For the detection of relatively high concentrations of I–III (>1 mcg./ml.), the absorption maximum at 483 nm. in 62% sulfuric acid can be utilized for colorimetric analysis.

Due to the high sensitivity and specificity of the method, plasma, urine, bile, and gastric fluid samples were directly extracted without prior purification. Plasma samples were deproteinated by diluting



V

[1] Aldactone.

[2] Compound II was formerly referred to as aldadiene, and Compound III was formerly referred to as aldadienic acid (1–3).

[3] Absorption spectra were recorded on a DBG Beckman spectrophotometer, and fluorescence was read on an Aminco-Bowman spectrophotofluorometer and on a Zeiss spectrophotofluorometer.

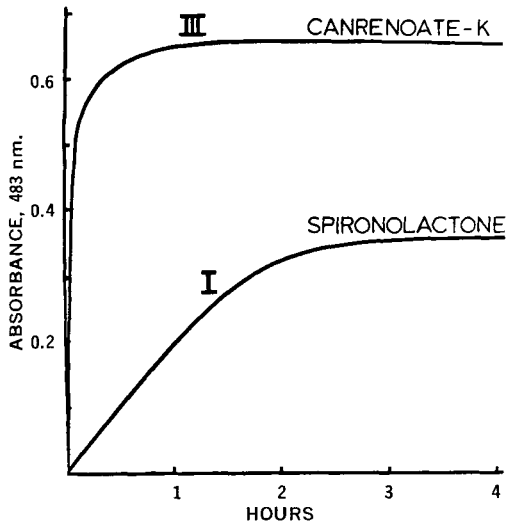


Figure 1—*Development of the absorption maximum at 483 nm. of spironolactone (I) (6.7 mcg./ml.) and potassium canrenoate (canrenoate-K) (III) (6.3 mcg./ml.) in 62% sulfuric acid (v/v).*

with a twofold volume of methanol and were centrifuged. The supernate contained practically 100% of I–III and other metabolites, as shown by control studies using fluorescence and tritium label. Spironolactone-20,21-^{3}H (I), with a specific activity of 590 μc./mg. in benzene solution, and potassium canrenoate-20,21-^{3}H (III), with a specific activity of 860 μc./mg., were received from a commercial source[4]. Samples were counted after adding a dioxane cocktail for aqueous samples, according to Bray (6), in a liquid scintillation counter[5] with ^{3}H-toluene as the internal standard. The samples should contain between 6 and 200 ng. I–III. Blank readings of plasma samples were equivalent to 2 ng. V. The major part of this blank reading is due to the sulfuric acid reagent (equivalent to as much as 1.5 ng.).

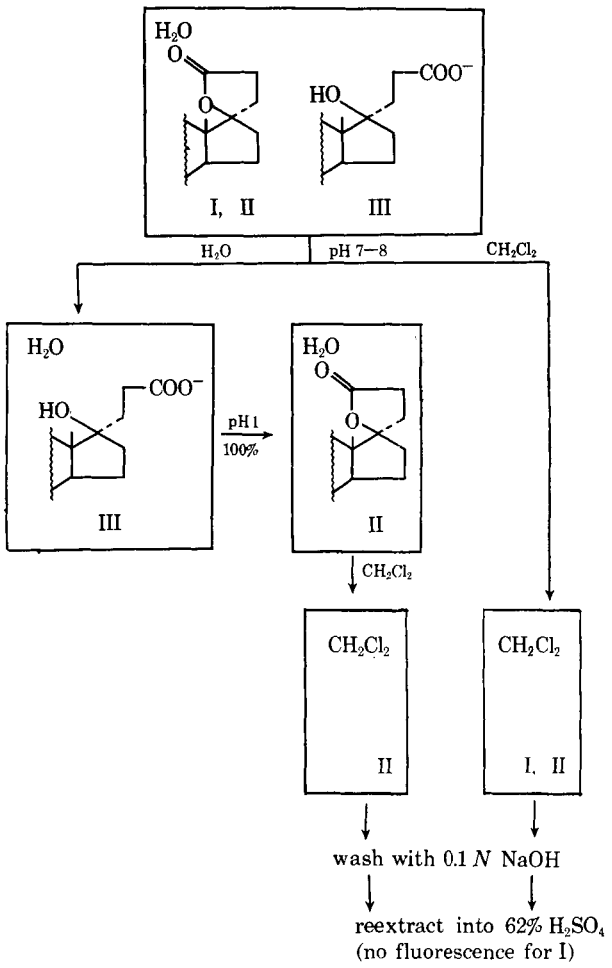
Procedure 1: Determination of II and III—To 0.3 ml. of an aqueous or methanolic solution of II and III, 2 ml. of 1% sodium bicarbonate solution was added; then the solution was shaken for 5 min. with 4.0 ml. of methylene chloride. After centrifuging, the organic layer containing the neutral γ-lactone II was quantitatively transferred to another test tube. To the aqueous phase containing the γ-hydroxycarboxylic acid III, 0.3 ml. 2 *N* HCl was added to yield a pH <1, at which III lactonizes to II. After 15 min. at room temperature, the solution was shaken for 5 min. with 4.0 ml. of methylene chloride and centrifuged. The organic layer was transferred to another test tube. To reduce the fluorescence blank, 2.0 ml. of 0.1 *N* NaOH was added to the methylene chloride layer which was shaken for 1 min. After centrifuging, 3.0 ml. of the organic layer was pipeted into glass-stoppered test tubes, 2.0 ml. of 62% sulfuric acid was added, and the tubes were shaken for 20 min. The organic layer was then removed by pipeting. At this point, the standard solutions, containing 40–160 ng. V, were prepared. The fluorescence of the sulfuric acid layers was read at 483–525 nm. against the appropriate standard solution. The factor for calculating amounts of II (expressed as III) and III per sample was $F = 1.74$.

Procedure 2: Fluorescence following Mild Alkaline Hydrolysis (Applicable to I–IV)—To 0.3 ml. of an aqueous or methanolic solution, 0.15 ml. of 0.3 *N* NaOH was added (pH 13), and the solution was heated in stoppered test tubes in a boiling water bath for 10 min. By using this procedure, I, II, and IV were hydrolyzed to III. After cooling, 0.2 ml. of 1 *N* HCl was added. The solution was kept for 15 min. at room temperature to lactonize III to II, which is more readily extractable. Then, 2 ml. of 1% sodium bicarbonate solution was added; the solution was extracted with 4.0 ml. methylene chloride by shaking for 5 min. and then centrifuged. The organic layer was transferred to another test tube and washed with 0.1 *N* NaOH, and the fluorescence was read after reextraction with 62% sulfuric acid as described in Procedure 1. The factors for converting fluorescence readings of I and III to amounts of III were $F_I = 1.93$ and $F_{III} = 1.84$, respectively.

Procedure 3: Fluorescence following Mild Alkaline and Acid Hydrolysis (Applicable to I–IV and Acid-Labile Conjugates, "Total Fluorescence Assay")—To hydrolyze the alkaline labile conjugates, *e.g.*, IV, 0.3 ml. of an aqueous or methanolic solution and 0.15 ml. of 0.3 *N* NaOH (pH 13) were combined and the solution was heated in stoppered test tubes in a boiling water bath for 10 min. as in Procedure 2. After cooling, 0.11 ml. of 18% sulfuric acid was added, yielding methanolic 3.6% sulfuric acid. The solution was again heated in stoppered test tubes in a boiling water bath for 30 min. By this procedure, I, III, IV, and acid-labile conjugates of I–III are converted to II. After cooling, 2 ml. of 1% sodium bicarbonate solution was added, and the procedure was carried on as described under Procedure 2. The factors for converting the fluorescence readings of I and III to amounts of III were $F_I = 2.02$ and $F_{III} = 1.90$, respectively.

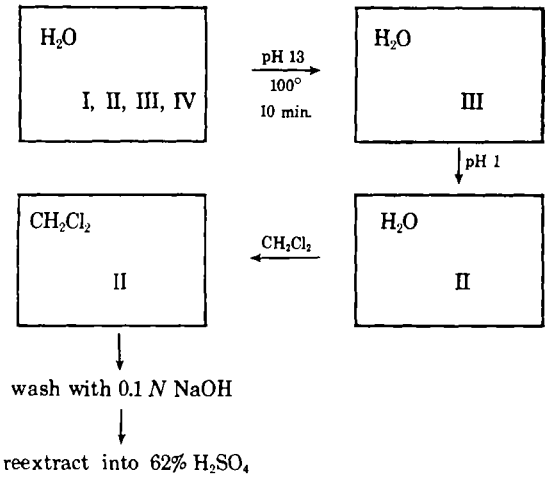
RESULTS AND DISCUSSION

The fluorescence reaction of a broad variety of steroids in sulfuric acid is widely used for quantitative analysis. Hydrocortisone and corticosterone yield an intense fluorescence in 80% sulfuric acid. However, a high yield of the greenish fluorescence obtained from II and III in comparatively low concentrated sulfuric acid (62%) requires rather unique features of the steroid moiety, *i.e.*, the 4,6-dien-3-one structure and a tertiary 17-hydroxyl function (5). Thus, hydrocortisone and corticosterone do not fluoresce in 62% sulfuric acid, whereas II and III yield 83.2% of the trienone V as judged by fluorescence. The formation of the trienone V in 62% sulfuric acid is shown in Fig. 1 (upper curve). The development of the absorption maximum at 483 nm. is correlated with the fluorescence of the trienone V (5).



Scheme I—Determination of II and III (Procedure 1)

[4] G. D. Searle Co., Chicago, Ill.
[5] Packard LSS 3380 AAA.



Scheme II—Fluorescence following mild alkaline hydrolysis (applicable for I–IV, Procedure 2)

This unique reaction in connection with the high yield of V resulted in extremely high sensitivity, specificity, and reproducibility of the fluorescence assay for II and III. The limiting factor of the assay procedures was the blank reading of the sulfuric acid and of the biological samples.

Recently, a GC procedure was described for II, which was proposed to be more specific than the fluorescence method (7). However, the specificity of this procedure is doubted, since I is dethioacetylated to II under the GC conditions, resulting in only one peak with the retention time of II. Furthermore, this thermolytic product was identified as II by mass spectroscopic evidence.

Acid-labile substituents such as hydroxy and thioacetyl ($SCOCH_3$) in positions 6 and 7 of 17-hydroxy-17-alkyl-4-en-3-ones should be expected also to yield the trienone system of V in concentrated solutions of sulfuric acid. However, very little fluorescence was generated by these steroids (8, 9). The development of the absorption of the 7α-thioacetyl derivative I in 62% sulfuric acid at 483 nm. is shown in Fig. 1 (lower curve). Based on the different rates of the formation of V from I or III, a differential assay for the dienones II and III and for the 7α-thioacetyl-enone I was developed. The following factors were considered in selecting a relatively low concentrated sulfuric acid (62%), which extended the extraction time to 20 min.

1. Naturally occurring steroids, which yield fluorescence by mechanisms other than the formation of the trienone system present in V, do not interfere in the assay.

2. Fewer side reactions occur, and the stability of the trienone V increases with decreasing concentrations of sulfuric acid.

3. The relative fluorescence yield of I, when compared to III, decreases with decreasing concentrations of sulfuric acid.

4. The enone I is a much weaker base than the dienones II and III and is, therefore, extracted to a smaller extent from methylene chloride into lower concentrated sulfuric acid. The extraction time of 20 min. using 62% sulfuric acid additionally allows a nearly complete conversion of the dienones II and III to the more basic trienone V during the extraction procedure, which increases the yield of extraction to over 95% for II and III. Therefore, the fluorescence yield generated from I (Procedure 1) was less than 5% when compared to the fluorescence yield of II and III, allowing accurate measurements of II and III in the presence of I.

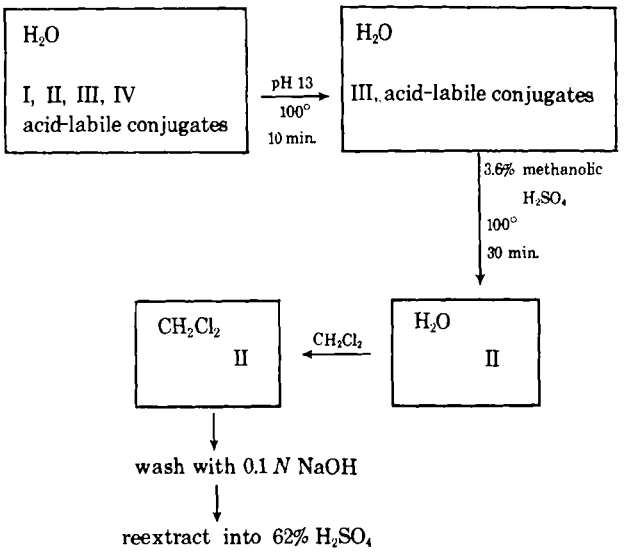
For the estimation of I, dethioacetylation to the dienone moiety was effected at pH 13 for 10 min. at 100° prior to extraction (~94% yield, Procedure 2). Also, acid hydrolysis in methanolic 3.6% sulfuric acid for 30 min. at 100° (Procedure 3) results in dethioacetylation of I and dehydration of possible 7α-hydroxy metabolites (10, 11), as well as in the conversion of possible 7α-thiol metabolites to the dienone moiety. Under the conditions of Procedures 2 and 3, the spirolactone moiety remained stable. More vigorous treatment was found to decompose these steroids.

The chemical nature of the neutral spiro-γ-lactone ring in contrast to the γ-hydroxycarboxylic acid was utilized in the analytical separations. While both forms were relatively stable at pH 5–8, the γ-lactones were rapidly hydrolyzed at pH 13, and the γ-hydroxycarboxylic acids were rapidly lactonized at pH 1 to 100%. Both forms were separated at pH 7–8 by extracting the neutral γ-lactones into methylene chloride. The γ-hydroxycarboxylic acid congeners were then converted at pH 1 to γ-lactones, which are more lipophilic and easier to extract. The extraction of biological acid contaminations from methylene chloride into 0.1 *N* NaOH did not result in hydrolysis of the γ-lactone congeners due to their high distribution coefficient (methylene chloride–water). The overall extraction yield (Procedure 1) was 91%.

Since Compound I can only be calculated by subtracting values for II and III obtained in Procedure 1 from the values obtained by Procedure 2 (sum of I, II, and III), I cannot be accurately measured in the presence of large amounts of II and III. Also, the polar glucuronic acid conjugate (IV) (4) interferes with the estimation of I and has to be separated if present. Compound IV was not found in measurable quantities in plasma of dogs and rats. Karim *et al.* (4) reported that in human studies, administration of ^{3}H-III resulted in recovery from the urine of the ester glucuronide (IV) as a major metabolite. We found that IV in human urine can be quantitatively hydrolyzed to III using mild alkaline hydrolysis (Procedure 2). Studies undertaken on the dog, rat, and man, to be reported subsequently, also resulted in evidence that still other polar conjugates are present in their plasma, bile, and urine samples. These others can be estimated by the "total fluorescence assay" (Procedure 3).

The assay procedures are described in Schemes I–III.

The methods, in addition to the use of tritium-labeled I and III, proved to be valuable for studying the fate of the spirolactones in the rat, dog, and man, which will be reported elsewhere.



Scheme III—"Total Fluorescence Assay" (Procedure 3)

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Plasma Levels of Spirolactones in the Dog

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Abstract □ A fluorometric micromethod for the determination of spironolactone (I) and some of its possible metabolites was utilized to follow plasma concentrations after intravenous administration of I, canrenone (II), and potassium canrenoate (III). Spironolactone (I) was eliminated from plasma of female dogs, with a half-life of less than 10 min., and was partially converted to II and III. The γ-lactone ring of II equilibrated with the γ-hydroxycarboxylic acid of III, resulting in similar plasma levels of II 2 hr. following equimolar intravenous doses of II and III. Canrenone (II) represented the predominant component in plasma. Plasma concentrations of II, following an equimolar intravenous dose of I, were significantly smaller (~40%). Thus, I was only partially dethioacetylated to II.

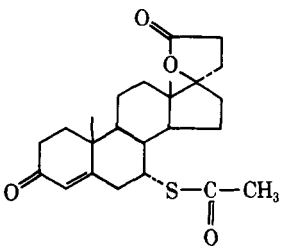
Keyphrases □ Spirolactones—plasma levels after intravenous administration, fluorometric analysis, dogs □ Spironolactone—plasma levels after intravenous administration, fluorometric analysis, dogs □ Canrenone and potassium canrenoate—plasma levels after intravenous administration, fluorometric analysis, dogs □ Plasma levels—spironolactone, canrenone, and potassium canrenoate after intravenous administration, dogs □ Spectrophotofluorometry—analysis, spirolactones

The class of spirolactones, synthesized by Cella and Tweit (1), were found to be potential competitive antagonists of mineralocorticoids (2, 3). One of these, spironolactone (I), is used as a potassium-sparing diuretic due to its high peroral activity. In spite of many studies on the pharmacological activity and metabolic and pharmacokinetic behavior of this compound (4–12), the major metabolic fate and the active principle of I remain unclear. Canrenone (II) was identified as a major metabolite (4, 5), which also possesses pharmacological properties similar to those of its parent compound (1, 6). Since a sensitive fluorometric assay was available for II but not for I (7), most pharmacokinetic studies were based on this method.

The plasma levels of II, obtained after high oral doses of I (100–1000 mg.), were in the low nanogram range in humans (4–12). This may be explained partially by a poor absorption of I due to its low solubility in water (20 mcg./ml.). Pharmaceutical formulations have been devised which apparently lead to a 10-fold enhanced GI absorption, using polysorbate 80 (8, 9, 13) or micronized powder (10, 14). The greater absorption could be correlated with increased dissolution rates in water (13).

The plasma levels of I in man were estimated to be in the range of one-fifth of the levels of II (7); however, in another study, I could not be detected at all (15). Similarly, contradictory results were reported for the appearance of I in urine (15, 16). No conclusive evidence could be presented as to the pharmacologically active principle (17).

The time course of drug action also has to be considered. Compound I shows a slow cumulative action on sodium excretion in the urine (12) and a maximum effect between 4 and 14 hr. following a single dose. Other reports indicated that it may take longer than 24 hr. for maximum effect. It was reported that II disappears from the plasma with a half-life of 4–5 hr. in man (18, 19); another report indicated 8 hr. (20). Neither of these values may represent the terminal log-linear phase of the biological half-life. Half-life studies of II have been undertaken with potassium canrenoate (III) in man (18–20). Compound III was reported to convert rapidly to II (18–20) and to the ester glucuronide of III as a major metabolite in man (20).



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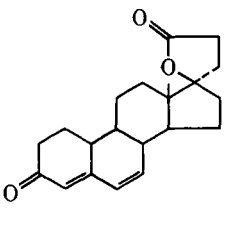

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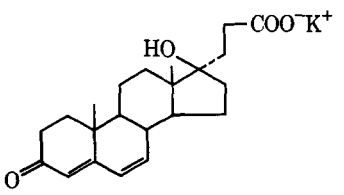

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