A SPECIFIC METHOD FOR THE DETERMINATION OF Δ^4-3-KETOSTEROIDS WITH p-NITROPHENYLHYDRAZINE

(FUNDAMENTAL STUDIES ON CLINICAL CHEMISTRY VI.[1])

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ABSTRACT

A new method for colorimetric determination of Δ^4-3-ketosteroids with p-nitrophenylhydrazine was studied and the standard procedure for the concentration of 10 μg - 50 μg of steroid samples was established. The principle of this method is based on the observation that the hydrazone formed with p-nitrophenylhydrazine produces a stable reddish purple color which has an absorption maximum at 540 mμ in an alkaline solution of dimethylformamide.

The present method is specific for Δ^4-3-ketosteroids and gives a maximum absorption in longer wave-length regions with a markedly higher intensity than the other methods.

Most published methods for the determination of Δ^4-3-ketosteroids such as progesterone (pregn-4-ene-3,20-dione), testosterone (17β-hydroxyandrost-4-ene-3-one) and corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione) depend usually upon the ultraviolet absorptions of free steroids or their derivatives[2-8]. One of the main disadvantages of these methods is that in the case of crude biological extracts, non-steroidal impurities tend to interfere by showing absorptions in the same ultraviolet regions.

Nakamura et al.[9] suggested that various carbonyl compounds could condense with p-nitrophenylhydrazine to give hydrazones, which were soluble in alcohol and gave a red or blue color with dimethylformamide (DMF) and tetraethylammonium hydroxide.

In an earlier study[10] on the colorimetric determination of cholesterol

in serum, it was found that only Δ^4-3-ketosteroids such as progesterone and testosterone reacted with the p-nitrophenylhydrazine-hydrochloric acid reagent (PNPH) to form hydrazones, which produced reddish purple colors showing maximum absorptions at 540 mμ in an alkaline solution of DMF. On the contrary, steroids having a keto group in other positions, such as dehydroepiandrosterone (3β-hydroxyandrost-5-ene-17-one), pregnenolone (3β-hydroxypregn-5-ene-20-one) and estrone (3-hydroxy-estra-1:3:5(10)-triene-17-one), developed no colors under the same conditions. It has, furthermore, been shown that the concentration of hydrochloric acid affected the formation of hydrazone, and Δ^4-3-keto-steroids alone reacted with PNPH reagent at a higher concentration of hydrochloric acid.

The present report describes a method for the colorimetric determination of Δ^4-3-keto steroids, which is based on the observations mentioned above.

EXPERIMENTAL

Reagents and Apparatus

1. Methanol: Reflux 2 liters of methanol containing 0.2 g of 2,4-dinitrophenylhydrazine and 0.1 ml of sulfuric acid for 2 hours and distill. Store in brown bottle.
2. p-Nitrophenylhydrazine reagent (PNPH): Dissolve 20.0 mg p-nitrophenylhydrazine (m.p. 157 - 158°C) with carbonyl-free methanol. Add 2.0 ml of conc. hydrochloric acid and make to 50.0 ml with methanol. This should be prepared daily.
3. Sodium hydroxide solution 1.0 ± 0.05 N: Prepare as an aqueous solution from concentrated carbonate-free sodium hydroxide solution, removing precipitate by filtration through a glass filter.
4. Dimethylformamide (DMF): Purify by distillation under reduced pressure in an all-glass apparatus.

All other reagents and solvents were of reagent-grade and used without purification.

5. Reaction vessels: Test tubes approximately 15×150 mm, fitted with 15/25 standard-taper ground-glass stoppers were used. These could be readily attached to a reduced pressure still head fitted with 15/25 standard taper joint and a capillary tube for distillation of aliquots of the samples to dryness.

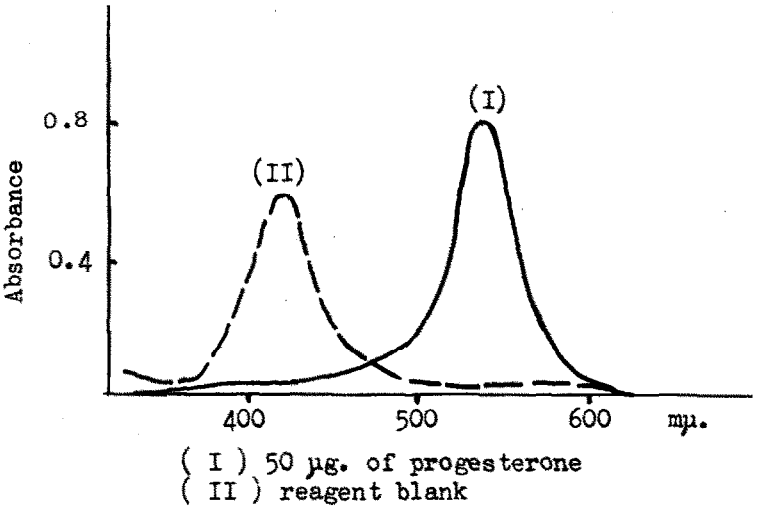


Fig. 1. Comparison of the absorption curves of solutions of the hydrazone derivative of progesterone and the reagent blank prepared by the standard procedure.

Development of Method

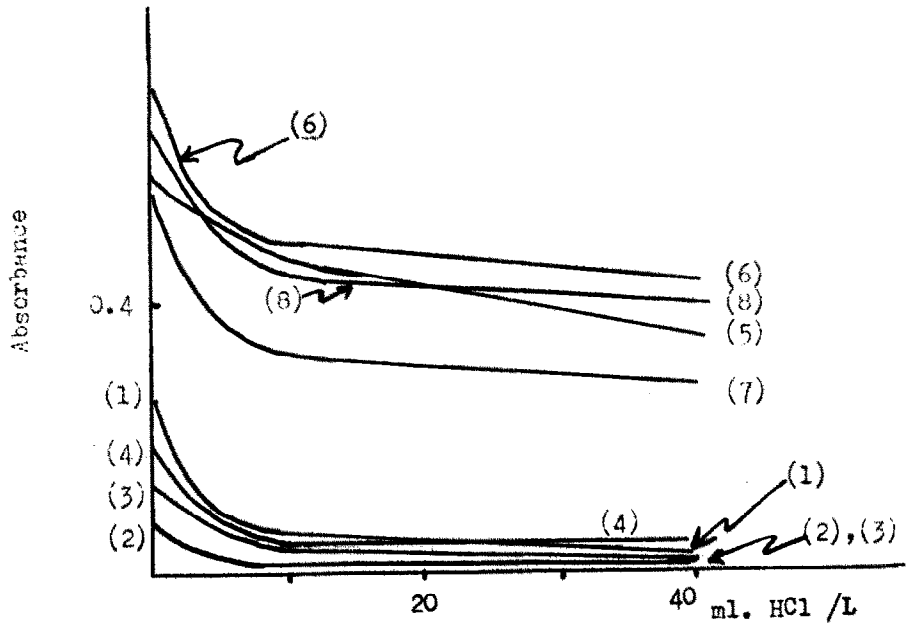
Progesterone was used as the reference standard during most of this investigation. Sufficient experiments were also made with other Δ^4-3-ketosteroids to show that the same procedure can be applied equally well to them. A variety of factors influencing these reactions were examined here, in an attempt to raise the sensitivity and specificity as highly as possible for Δ^4-3-ketosteroids.

1. Concentration of p-nitrophenylhydrazine: Various concentrations of p-nitrophenylhydrazine were tested and maximum absorbance was obtained when the concentration was between 0.4 and 0.8 mg per ml, although the color of the reagent blank increased. A concentration of 0.4 mg per ml in acidic methanol was selected as most convenient and satisfactory.

2. Kind and concentration of acid: By varying the concentration of hydrochloric acid at the PNPH concentration of 0.4 mg per ml, the curves shown in Fig. 2 were obtained. When the concentration of conc. hydrochloric acid was 40 ml per liter (the molar ratio of acid to hydrazine was 160 to 1), only Δ^4-3-ketosteroids such as progesterone, prednisolone (11β,17α,21-trihydroxypregn-1,4-diene-3,20-dione), cortisone (17α,21-dihydroxypregn-4-ene-3,11,20-trione)-acetate and hydrocortisone (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) reacted, and other steroids having a keto group in other positions developed no colors. On the other hand, when the concentration of hydrochloric acid was 0.4 ml per liter (the molar ratio of acid to hydrazine was 1.6 to 1.0) the keto group in other positions as well as Δ^4-3-keto group reacted and developed the color, which showed an absorbance at 520 mμ in alkaline solution of DMF. Furthermore, absorbance of the reagent

blank against DMF increased roughly in an inverse proportion to the concentration of hydrochloric acid. Several acids other than hydrochloric acid were tested and found to be unsatisfactory and inconvenient. These acids were acetic acid, p-toluensulfonic acid, salicylsulfonic acid, perchloric acid and phosphoric acid. When organic acids such as acetic acid and p-toluensulfonic acid were used, the reagent blank developed a similar color to that of the sample. On the other hand, when phosphoric acid was used, addition of alkali precipitated a salt and determination was impossible. From these observation, it has been found that the concentration of 40 ml of hydrochloric acid per liter was most convenient and satisfactory for the specific determination of Δ^4-3-ketosteroids.

3. Effect of temperature: The procedure was carried out at various temperatures between room temperature to refluxing temperature of the methanolic solution. The development of the reaction at 40, 50 and 60°C are shown in Fig. 3. At 60°C the intensity reached its maximum value after 20 minutes, but began to diminish slowly after 30 minutes and decreased to 90% of maximum value after 2 hours. At 50°C, the intensity taken at any time after 40 minutes would be satisfactory, whereas at 40°C it took about 2 hours to attain the maximum value. For practical purposes, 60 minutes at 50°c was selected as the most convenient reaction period.



(1) pregnenolone (2) epiandrosterone acetate
(3) estrone (4) 17α,21-dihydroxypregnane-3,11,20-trione-21-acetate (5) prednisolone (6) progesterone
(7) cortisone acetate (8) hydrocortisone

Fig. 2. Effects of concentration of hydrochloric acid upon the reaction of p-nitrophenylhydrazine with 30 μg of ketosteroids.

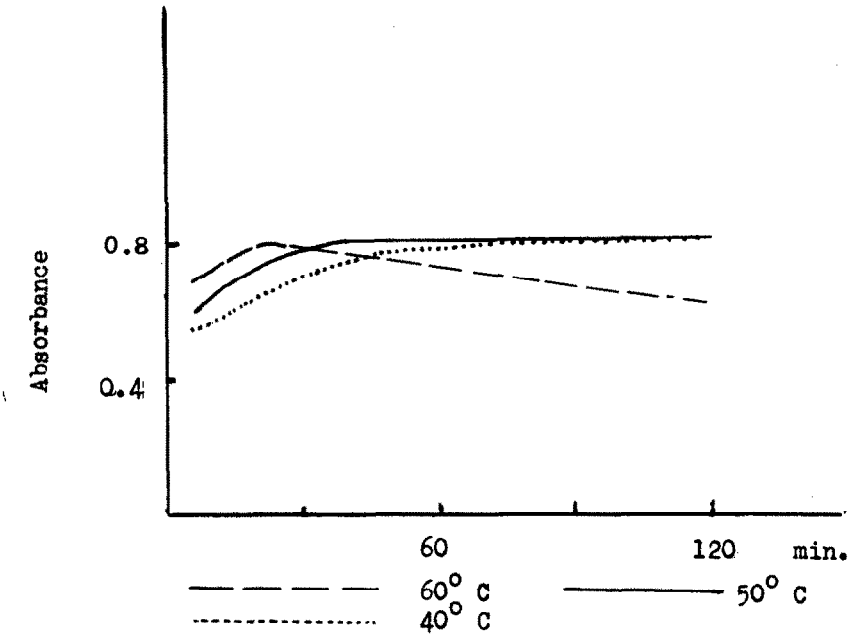


Fig. 3. The effect of temperature upon the time of reaction of p-nitrophenylhydrazine with 50 μg of progesterone and development of color as measured by absorption at 540 mμ.

4. Addition of alkali: While the reaction mixture was still yellow after the heating period, addition of a sufficient amount of alkali produced a purple color. The reagent blank gave an orange color that faded slowly to yellow on standing. The amount of alkali used proved to be critical. When 0.5 N sodium hydroxide was used, the color tended to fade more rapidly. A large quantity of 2 N sodium hydroxide proved to be critical due to precipitation of salt. Increasing the concentration of alkali to 4 N also caused precipitation on standing. It was found that 0.5 ml of 1 N sodium hydroxide was most convenient and satisfactory.

Since the absorption of CO_2 from air tanded to cause more rapid fading of color and precipitation, the reaction was generally carried out in a stoppered tube. The colored hydrazoneswere stable for hours under these condition.

5. Absorption spectra: The absorption spectrum of the colored solution produced from 50 μg of progesterone by the standard procedure gave a strong absorption maximum at 540 mμ which was not shifted with time, nor by varying the concentration of the other reagents. The absorption curve of the reagent blank was measured against DMF and was found to give a smaller blank reading at longer wave length regions (Fig. 1).

6. Formation of hydrazones: In order to develop the experiment on the mechanism of the color reaction, the preparation of 2,4-dinitro-

phenylhydrazones and p-nitrophenylhydrazones of progesterone and pregnenolone was attempted. The hydrazones of progesterone were found rather difficult to prepare and purify. Since this steroid has two carbonyl groups of different reactivity, the reaction products were likely to be a complicated mixture. However, the data presented in the previous paper[10] have demonstrated that the formation of hydrazones of cholest-4-ene-3,6-dione was affected by the concentration of acid. From these observations the derivatives were finally prepared by the following procedure:

Progesterone mono-p-nitrophenylhydrazone (I): To 5 ml of methanol, 100 mg of progesterone was added. A second solution was prepared by dissolving 54 mg of p-nitrophenylhydrazine in the mixture of 5.0 ml of methanol, 5.0 ml of conc. hydrochloric acid and 15.0 ml of water, and was poured into the progesterone solution. The mixture was heated at 60°C on a water bath, when the yellow derivative promptly began to separate. After digestion on a water bath for 10 minutes, the solution was cooled and a granular yellow product was then collected. This was recrystallized from acetone and water to give yellow needles, m.p. 224 - 226°C (decomp.).

Anal. Calcd. for $C_{27}H_{35}O_3N_3$: C,72.13; H,7.85; N,9.35. Found: C,72.34; H,8.11; N,9.50.

Progesterone di-p-nitrophenylhydrazone (II): To a solution of 100 mg of progesterone and 300 mg of p-nitrophenylhydrazine in 30 ml of methanol, 0.1 ml of conc. hydrochloric acid was added. The mixture was refluxed on a water bath for 75 minutes and was added to 20 to 30 ml of water. The precipitate was washed with 10 ml of hot benzene. Recrystallization from acetone with a small amount of water gave yellow orange microcrystals, m.p. 266 - 267°C (decomp.).

Anal. Calcd. for $C_{33}H_{40}O_4N_6$: C,67.78; H,6.90; N,14.37. Found: C,67.98; H,7.08; N,14.50.

Pregnenolone p-nitrophenylhydrazone (III): To a solution of 125 mg of pregnenolone and 68 mg of p-nitrophenylhydrazine in 35 ml of ethanol, 0.2 ml of methanolic hydrochloric acid (prepared by diluting 5.0 ml of conc. hydrochloric acid to 50 ml with methanol) was added. The mixture was refluxed on a water bath for 30 minutes and was added 20 to 30 ml of water. The precipitate was recrystallized from acetone and water to give micro yellow crystals, m.p. 262 - 263°C (decomp.).

Anal. Calcd. for $C_{27}H_{37}O_3N_3$: C,71.81; H,8.26; N,9.31. Found: C,71.29; H,8.04; N,9.21.

Progesterone di-2,4-dinitrophenylhydrazone (IV): This hydrazone was prepared by the procedure of Klein[11] The elemental analysis of this derivative was in agreement with theoretical values.

Progesterone mono-2,4-dinitrophenylhydrazone (V): By the same procedure as for preparation of (I), 100 mg of progesterone was treated with 300 mg of 2,4-dinitrophenylhydrazine. Recrystallization from benzene

and n-hexane gave orange microcrystals, m.p. 203 - 205°C (decomp.).
Anal. Calcd. for $C_{27}H_{34}O_5N_4$: C,65.57; H,6.93; N,11.33.

Found: C,65.26; H,7.05; N,11.33.

The absorption spectra of solutions of appropriate concentrations (1.0 to 2.0 mg/dl) were taken. DMF was the customary solvent. Spectra of alkaline solutions which were prepared by diluting a stock solution of the derivatives with sodium hydroxide were also taken. The results are given in Table I.

Absorption maximum of 2,4-dinitrophenylhydrazone was in the shorter wave length regions and its molecular extinction coefficient was smaller than those of the corresponding p-nitrophenylhydrazones, both in neutral and alkaline medium (Table I).

7. Calibration curve: The linear relationship between the optical density and the concentration of progesterone was obtained in a range of 10 to 50 μg.

8. Specificity: In order to study the specificity and sensitivity of this method for ketosteroids, apparent molecular extinction coefficients (ε')[12] and absorption maxima (λmax) were determined. The compounds to be tested were dissolved in absolute methanol at the concentration of 60 μg per ml for Δ^4-3-ketosteroids and 100 μg per ml for other ketosteroids. According to the procedure described below, 0.5 ml of these solutions were measured. The absorption curve of the reaction product in an alkaline solution of DMF was determined at room temperature. Apparent molecular extinction coefficients were calculated as an average of several determinations that showed generally good agreement with each other at the wave length of maximum absorption for each hydrazone. The results are given in Table II. Furthermore, non-steroidal ketones such as acetone, cyclohexanone, methyl ethyl ketone, methyl isobutyl ketone, santonine and ascorbic acid were tried. The results are given in Table III.

From the data presented, it may be concluded that the reaction is highly specific for Δ^4-3-ketosteroids.

Procedure

Into a test tube, pipette 0.5 ml of methanol. This is treated throughout exactly the same as the other samples and serves as a regent blank. Transfer to similar test tubes suitable aliquots of the samples to be analyzed. Evaporate to dryness under reduced pressure with gentle warming and dissolve the residue in 0.5 ml of methanol. Alternatively, the residue can be taken up in such a volume of methanol that the 0.5 ml aliquot can be transfered to test tube and analyzed directly. To each test tube, add 0.5 ml of PNPH reagent and mix. Allow the reaction to proceed for 60 minutes in a constant level water bath maintained at 50 ± 1°C. After cooling for several minutes, add 0.5 ml of 1 N sodium hydroxide and 5.0 ml of DMF to each tube with gentle shaking. Transfer the content of each tube to a colorimeter cuvette and read the per cent transmission or absorbance against the reagent blank at a wave length centered on 540 mμ.

DISCUSSION

In the field of steroid chemistry, various hydrazines have been used

Table I. Absorption Spectra of Hydrazones

Compound [a]	λ_{max}^{DMF} mμ	($\varepsilon \times 10^{-4}$)	$\lambda_{max}^{DMF\text{-}NaOH}$ mμ	($\varepsilon \times 10^{-4}$)
Progesterone mono-DNPH (V)	395	(2.83)	483	(3.59)
Progesterone di-DNPH (IV) [b]	390	(4.98)	478	(5.81)
Progesterone mono-PNPH (I)	424	(3.46)	543	(5.16)
Progesterone di-PNPH (II)	417	(4.36)	535	(8.00)
Pregnenolone PNPH (III)	406	(2.41)	522	(3.46)

a) DNPH and PNPH denote 2,4-dinitrophenylhydrazone and p-nitrophenylhydrazone respectively.
b) The spectum of this compound in ethanol has already been discussed by Djerassi, Anal. Chem. 20, 880 (1948).

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to form derivatives and permit the separation, purification and identification of ketosteroids. 2,4-Dinitrophenylhydrazine[14] and isonicotinic acid hydrazide[3] have generally been used for the determination of Δ^4-3-ketosteroids. It should be pointed out that these methods serve only to give an estimate of the total number of carbonyl groups, whether they are in the steroid nucleus or not, and the hydrazine reagents also show maxima in the same ultraviolet regions.

It is evident from the data of Table II that conditions of the standard procedure are optimum for Δ^4-3-ketosteroids. The wave length of maximum absorption for each Δ^4-3-ketosteroid was 540 mμ. The reaction of C_3 keto groups without conjugation of double bond, such as etiocholanedione (5β-androstane-3,17-dione) (11), 5α-androstane-3,17-dione (12) and cholestane-3-one (25), was about one-tenth that of Δ^4-3-ketosteroids and gave a maximum absorption at 520 mμ.

While Δ^4-3-ketosteroids and the corresponding $\Delta^{1,4}$-diene-3-keto-steroids showed practically the same selective absorption at 240 mμ, the absorption maxima of their colored solutions under the standard procedure differed markedly (540 mμ for the former and 565 mμ for the latter) and the apprent molecular extinction coefficient of hydrazone of $\Delta^{1,4}$-diene-3-ketosteroid (prednisolone) was reduced to about half of the former (Table II). This should prove quite useful for the purpose of differentiation between these types of compounds. The differences of these spectra were similar to those given by Djerassi[13] et al. for 2,4-dinitrophenylhydrazone, whereas their absorption maxima were displaced to shorter wave length.

Pregnenolone, having no Δ^4-3-keto group but a 20-keto group, developed no color. If pregnenolone would form a hydrazone under the standard procedure, the absorption maximum might be expected at 522 mμ from Table I. The 20-keto groups of progesterone and pregnenolone, therefore, failed to react under the present procedure. Because cortisone (17α,21-dihydroxypregn-4-ene-3,11,20-trione) and hydrocortisone behaved like progesterone in parallel experiments it may be concluded that the 20-keto and 11-keto groups of these compounds fail to react and develop a color. Furthermore, 17-keto groups of etiocholanolone (3α-hydroxyetiocholane-17-one) (6), epiandrosterone (3β-hydroxyandrostan-17-one) (7) and estrone (10) also did not react to develop a color under the standard procedure.

It is indicated from the data of Table III that santonine, which has also $\Delta^{1,4}$-diene-3-keto group like prednisolone, and ascorbic acid failed to react. The other nonsteroidal ketones tested reacted to develop a color at 515 mμ, whereas apparent molecular extinction coefficients were

Table II. Absorption Maxima and Apparent Molecular Extinction Coefficients determined by the Standard Procedure

Compound	λ_{max} mμ	ε'_{max} [12]	Position of double bond and/or keto group
1) Testosterone	540	32,000	Δ^4-3,
2) 19-nor-Testosterone	540	28,600	Δ^4-3,
3) 19-nor-Methyltestosterone	540	27,200	Δ^4-3,
4) 19-nor-Testosterone phenylpropionate	540	28,100	Δ^4-3,
5) Etiocholanolone	-	-	17-,
6) Dehydroisoandrosterone	-	-	17,
7) Epiandrosterone acetate	-	-	17,
8) Epiandrosterone	-	-	17,
9) Androsterone	-	-	17,
10) Estrone	-	-	17,
11) Etiocholanedione	520	3,960	3, 17
12) 5α-Androstane-3,17-dione	520	4,080	3, 17
13) Androst-4-ene-3,17-dione	540	30,000	Δ^4-3, 17
14) 16-Keto-estrone	-	-	16
15) Progesterone	540	31,600	Δ^4-3, 20
16) Hydrocortisone	540	28,300	Δ^4-3, 20
17) Hydrocortisone acetate	540	27,500	Δ^4-3, 20
18) Cortisone acetate	540	30,400	Δ^4-3, 20
19) Desoxycorticosterone acetate	540	32,100	Δ^4-3, 11, 20
20) 17α.21-Dihydroxypregnane-3,11,20-trione 21-acetate	520	3,500	3, 11, 20
21) Pregnenolone	-	-	20
22) Prednisolone	565	15,600	$\Delta^{1,4}$-3, 20
23) Corticosterone	540	34,000	Δ^4-3, 20
24) Cholest-4-ene-3-one	540	30,700	Δ^4-3
25) Cholestanone	520	3,820	3

Table III. Absorption Maxima and Apparent Molecular Extinction Coefficients of Nonsteroidal Ketones determined by the Standard Procedure

	λ_{max} mμ	ε'_{max}[12]
Acetone	515	2,260
Methyl ethyl ketone	515	1,100
Methyl isobutyl ketone	515	1,690
Cyclohexanone	515	2,250
Santonine	-	-
Ascorbic acid	-	-

about one-twentieth of those of Δ^4-3-ketosteroids. However, these ketones could be removed by prolonged vacuum evaporation before the formation of hydrazones.

Table II shows that the apparent molecular extinction coefficients for Δ^4-3-ketosteroids were about 27,000 - 35,000 under the present procedure. On the other hand, the coefficients were about 11,000 - 12,000 by the colorimetric method with isonicotinic acid hydrazine[3] and about 20,000 - 25,000 by the colorimetric method with 2,4-dinitrophenylhydrazine[14] and thiosemicarbazide[4] and about 17,000 by the ultraviolet absorption method at 240 mμ.

The present method is highly specific for Δ^4-3-ketosteroids and gives a maximum absorption in longer wave length regions with a markedly higher intensity than the other methods. A complete assay can be carried out in approximately 3 hours.

Application of this method to the analysis of Δ^4-3-ketosteroids from biological origins is now under investigation and will be the subject of a future report.

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