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COLORIMETRIC AND FLUORIMETRIC DETERMINATION OF STEROIDS

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COLORIMETRIC AND FLUORIMETRIC DETERMINATION OF STEROIDS

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Abstract - This report is devoted to two classes of reactions. 1. Methods of colorimetric determination of ketosteroids which make use of the reaction of a carbonyl group with a hydrazine, a hydrazone or a hydrazide. Three of these methods use p-nitro- or 2,4-dinitrophenylhydrazine, thus allowing the determination of steroids bearing a carbonyl group at position 3, 17 or 20. Isoniazid gives colored hydrazones only with Δ^4- or $\Delta^{1,4}$-3-ketosteroids, whereas salicyloyl-hydrazide allows the fluorimetric determination of 17-ketosteroids. Under suitable conditions, phenylhydrazine gives a colored species only with 17,21-dihydroxy-20-ketosteroids (Porter-Silber reaction). These compounds can also be determined with 2-hydrazinobenzothiazole or with 3-methylbenzothiazolin-2-one hydrazone in the presence of an oxidant. 2. Other methods of determination of 21-hydroxy-20-ketosteroids : The three reactions cited above give positive results only when the steroid bears a hydroxy group at position 17. More generally, 21-hydroxy-20-ketosteroids can be determined through their reducing properties, using sodium molybdate, a tetrazolium salt or p-nitroso-N,N-dimethylaniline as reagent. The 21-hydroxy-20-keto group can be oxidized either with cupric acetate, to give the corresponding glyoxal derivative which is developed as a quinoxaline absorbing in the UV range, or with periodic acid to give formaldehyde which can be determined either colorimetrically or fluorimetrically.

INTRODUCTION

Thousands of papers deal with the colorimetric and fluorimetric determination of steroids, and it is obviously impossible to present even a very rough practical review of all that has been done in this field. Taking into account the fact that the description of methods specific for particular steroids would interest only a limited number of rather highly specialized analysts, we thought that we should limit ourselves to the description of some methods of functional group analysis which were checked in our laboratory, and which we consider as reliable.

In the colorimetric determinations described, Beer's law is obeyed at least up to the absorbance value 0.7. For the fluorimetric determinations, the linear relationship is observed within the limits given.

In all cases, the compounds mentioned under Results, as well as the compounds mentioned as reacting only weakly or not at all are the only compounds we tested. Therefore, these lists are not limitative.

All the reagents used were of analytical grade quality, and the solvents of reagent grade quality. By water is meant distilled water. For all reagents in solution, and unless otherwise stated, the concentration is always expressed in w/v if the solute is a solid, and v/v if it is a liquid.

For the sake of brevity, the limits within which a given temperature or a given time should be observed were not specified for each of the methods.

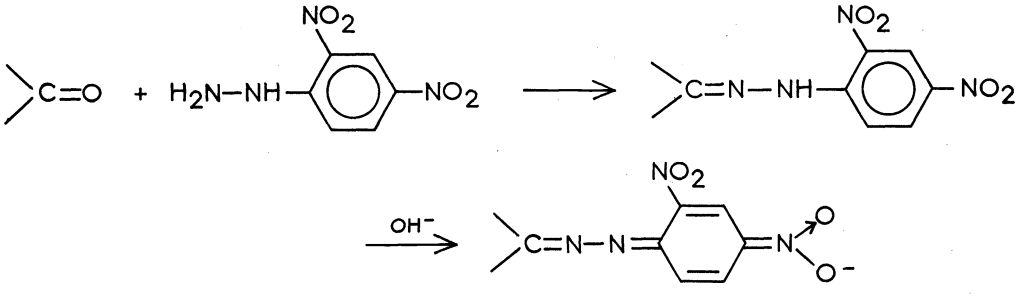
Temperature : When it is written : "Heat at t°C", it means that the temperature should be maintained at t ± 1°C. "Room temperature" means that the temperature is within the range 18 - 24°C.

Time : When it is written : "Let stand for x min" or "Heat for x min", it means x min ± 5 %. When it is written : "Let stand for x min and read", it means : start with reading after x min ± 5 %.

The absorbance was always read against the blank of the reagents.

A. 3-, 17-, AND 20-KETOSTEROIDS (Colorimetry)

1. Reaction with 2,4-dinitrophenylhydrazine (1)
Formation of the 2,4-dinitrophenylhydrazone in methanol-hydrochloric acid medium and development of the color with sodium hydroxide : orange to red color.



Chemicals. Methanol, concentrated hydrochloric acid, 4.0 M sodium hydroxide, and 2,4-dinitrophenylhydrazine.

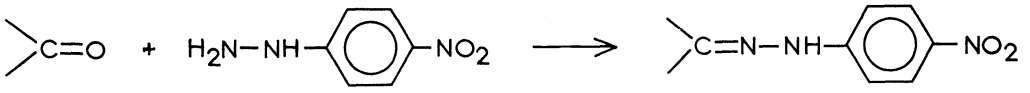
Reagent solution. An 0.10 % solution of 2,4-dinitrophenylhydrazine in a 1 : 3 (v/v) mixture of concentrated hydrochloric acid and methanol.

Method. To 0.50 ml of sample solution in methanol, add 0.50 ml of reagent, mix, and heat at 59°C for 90 min, the tubes being fitted with air-condensers and protected from direct light. Allow to cool for 2 min in a water bath, add 0.50 ml of 4.0 M sodium hydroxide with gentle shaking, and dilute with 5.0 ml of methanol. Let stand for 20 - 30 min at room temperature, and read.

Results

	λ max. (nm)	Sample size (µg) for A = 0.30 (1 cm - cell)
Dehydroepiandrosterone	430	80
Estrone	420	64
Hydrocortisone	470	16
Norethindrone	440	30
Norethynodrel	452	28
Prednisolone	480	14
Progesterone	460	26
Testosterone	450	25

2. Reaction with p-nitrophenylhydrazine (2)
Formation of the p-nitrophenylhydrazone, and development of the color with benzyltrimethylammonium hydroxide in dimethylformamide : red to violet color.



The exact mechanism of the development of the color upon the action of the base is unknown.

Chemicals. Dimethylformamide, ethanol, concentrated hydrochloric acid, a 40 % solution of benzyltrimethylammonium hydroxide in methanol, and p-nitrophenylhydrazine.

Reagent solutions. (a) An 0.040 % solution of p-nitrophenylhydrazine in ethanol containing 0.10 % (v/v) of concentrated hydrochloric acid. (b) Dilute 1.0 ml of 40 % solution of benzyltrimethylammonium hydroxide in methanol to 100.0 ml with dimethylformamide. Prepare fresh just before use.

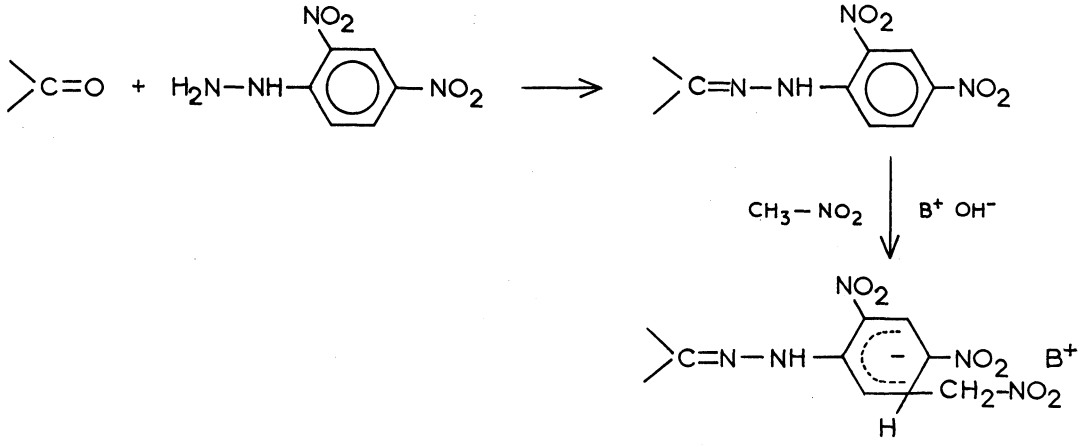
Method. To 0.50 ml of sample solution in ethanol, add 0.50 ml of reagent a, heat at 70°C for the given length of time, cool in a water bath, add 9.0 ml of reagent b, and read.

Results

	Heating time (min)	λ max. (nm)	Sample size (µg) for A = 0.30 (1 cm - cell)
Cortisone	20	550	21.5
Dehydrocholic acid	30	510	46.5
Dehydroepi-androsterone	50	530	215
Dexamethasone	30	570	34.0
Estrone	60	530	245
17β-Hydroxy-androstan-3-one	30	520	33.0
Progesterone	20	545	19.0

The reaction is much more sensitive with 3- than with 17- or 20-ketosteroids.

3. Reaction with 2,4-dinitrophenylhydrazine and nitromethane (3)
Formation of a 2,4-dinitrophenylhydrazone, and development with nitromethane in alkaline medium (Janovsky reaction). The excess of reagent does not interfere : violaceous-pink color.



Chemicals. Dimethylformamide, ethanol, nitromethane, concentrated hydrochloric acid, a 40 % solution of benzyltrimethylammonium hydroxide in methanol, and 2,4-dinitrophenylhydrazine.

Reagent solution. Dissolve 0.0250 g of 2,4-dinitrophenylhydrazine in a mixture of 10.0 ml of ethanol and 0.50 ml of concentrated hydrochloric acid, and dilute to 50.0 ml with ethanol.

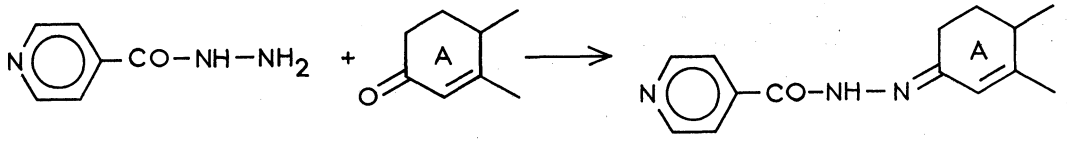
Method. Operate with protection against direct light. To 0.50 ml of sample solution in ethanol, add 0.50 ml of reagent and let stand at the given temperature for the given length of time. Cool to room temperature in a water bath if necessary, add 1.50 ml of nitromethane, 1.50 ml of dimethylformamide, and 0.30 ml of 40 % solution of benzyltrimethylammonium hydroxide in methanol. Shake for 30 seconds, and read at 565 nm. When operating the condensation at 100°C, the tubes should be fitted with air-condensers and the temperature of the water-bath should be rigidly homogeneous.

Results

	Temperature (°C)	Reaction time (min)	Sample size (µg) for A=0.30 (1 cm - cell)
Dehydroepiandrosterone	100	15	15.5
Estrone	100	15	15.0
17β-Hydroxyandrostan-3-one	18-24	30	23.5
Norethindrone	18-24	15	18.3
Norethynodrel	18-24	15	17.8
Prednisolone	100	15	10.0
Progesterone	100	15	8.4
Testosterone	18-24	10	21.6

B. Δ^4 - AND $\Delta^{1,4}$ -3-KETOSTEROIDS (Colorimetry)

Reaction with isoniazid (4)
Formation of a hydrazone in acidic medium : yellow color.



Chemicals. Methanol, concentrated hydrochloric acid, and isoniazid.

Reagent solutions. (a) A 0.80 % solution of isoniazid in methanol containing 1.0 % (v/v) of concentrated hydrochloric acid. (b) Dilute 12.50 ml of reagent a to 100.0 ml with methanol.

Methods. 1. To 2.0 ml of the solution of a Δ^4 -3-ketosteroid in methanol, add 2.0 ml of reagent b, let stand at room temperature for 1 h, and read at 380 nm.

2. To 2.0 ml of the solution of a $\Delta^{1,4}$-3-ketosteroid in methanol, add 2.0 ml of reagent a, let stand at room temperature for 3 h, and read at 405 nm.

Results

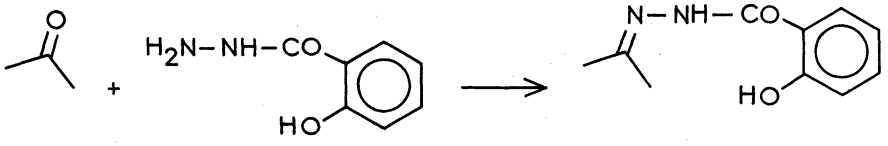
	Sample size (µg) for A = 0.30 (1 cm - cell)
Cortisone	42.0
17α-Ethynyl-17β-hydroxy-4,9,11-estratrien-3-one*	12.0
17α-Methyltestosterone	30.5
Norethandrolone	31.5
19-Norprogesterone	35.5
19-Nortestosterone	30.0
Progesterone	31.5
Testosterone	29.0
Dexamethasone	32.5
Prednisolone	34.0
Prednisone	29.0

* Let stand for 3 min only, and read at 420 nm.

Norethynodrel reacts also, probably because of its isomerization to norethindrone in the acidic medium. After 3 h (with reagent b), A = 0.30 is given by 63 µg, at 380 nm.
3-Ketosteroids with saturated ring A, or with the keto group located at other position, do not react (5).

C. 17-KETOSTEROIDS (Fluorimetry)

Reaction with salicyloylhydrazide (6)
Condensation with salicyloylhydrazide to give a salicyloylhydrazone, elimination of the excess reagent as a complex with the pentacyanoamminoferrate ion, and development in alkaline medium : blue fluorescence.



Chemicals. Benzene, methylene chloride, glacial acetic acid, 1.0 M sodium hydroxide, salicyloylhydrazide, and trisodium pentacyanoamminoferrate.

Reagent solutions. (a) An 0.10 % solution of salicyloylhydrazide in glacial acetic acid. Prepare freshly before use. (b) An 0.40 % aqueous solution of trisodium pentacyanoamminoferrate. Prepare freshly before use.

Method. Introduce 1.0 ml of sample solution in methylene chloride into the tube and evaporate to dryness on a water-bath at 60°C. Add 0.10 ml of reagent a, and heat at 60°C for 1 h. Cool to 20°C in a water bath, add 0.50 ml of methylene chloride and 5.0 ml of benzene, mix, and transfer into a 25 ml separatory funnel. Add 5.0 ml of reagent b, shake for 20 seconds, allow the phases to separate, and discard the aqueous layer. Wash the organic phase with 5 ml of water, and discard the aqueous layer. Add 5.0 ml of 1.0 M sodium hydroxide, shake for 20 seconds, allow the phases to separate, and collect the aqueous layer. Read at exc : 340 nm ; em : 420 nm.

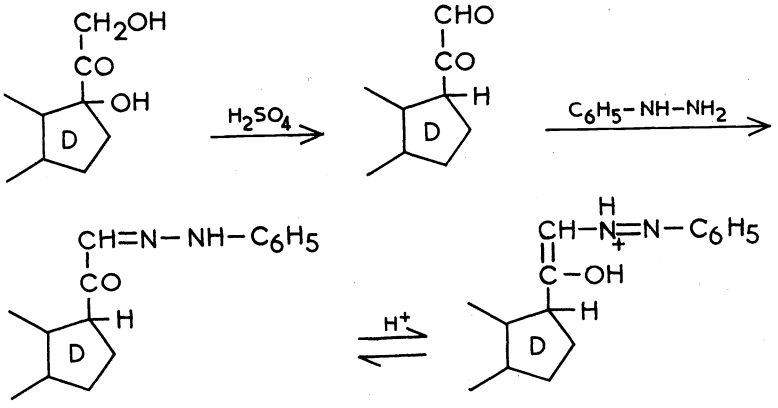
Results

	Determination limits (µg)
Androstane-3,17-dione	2.0-8.0
Androsterone	2.0-8.0
Dehydroepiandrosterone	1.6-8.0
Equilenine	1.5-7.5
Equiline	1.5-7.5
Estrone	2.0-8.0
11β-Hydroxyandrosterone	1.6-8.0

The reaction is weakly positive with 17β-hydroxy-3-oxo-androstane (50 µg gives the same fluorescence intensity as 4 µg of estrone). 3-Oxo-Δ^4-androstane and testosterone do not react.

D. 17,21-DIHYDROXY-20-KETOSTEROIDS (Colorimetry)

1. Reaction with phenylhydrazine (7)
Dehydration and rearrangement of the 17,21-dihydroxy-20 keto group in sulfuric acid medium to give a glyoxal side chain, and formation of the corresponding phenylhydrazone (8) : yellow color.



Chemicals. Methanol, concentrated sulfuric acid, and phenylhydrazine hydrochloride.

Reagent solution. Dissolve 0.065 g of phenylhydrazine hydrochloride in 100.0 ml of a cooled mixture of 310 ml of concentrated sulfuric acid and 190 ml of water.

Method. To 1.0 ml of sample solution in methanol, add 8.0 ml of reagent, heat at 60°C for 20 min, allow to cool, and read at 410 nm.

Results

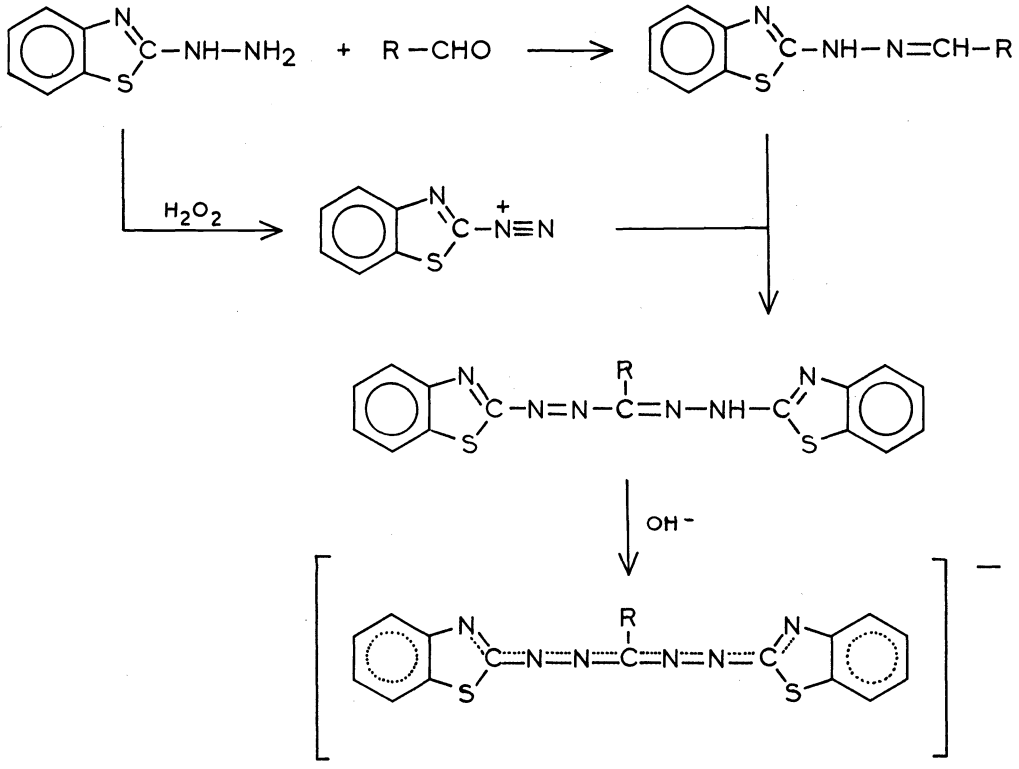
	Sample size (µg) for A = 0.30 (1 cm - cell)
Cortisone	40
Cortisone acetate	46
Cortisone hexahydrobenzoate	55
Dexamethasone	60
Dexamethasone acetate	70
Dexamethasone m-sulfobenzoate	184
Hydrocortisone	70
Hydrocortisone acetate	91

	Sample size (µg) for A = 0.30 (1 cm - cell)
Hydrocortisone hemisuccinate	108
Hydrocortisone hexahydrobenzoate	112
Prednisolone	60
Prednisolone hemisuccinate	98
Prednisolone hexahydrobenzoate	112
Prednisolone m-sulfobenzoate	236
Prednisone	40

The reaction is negative with 21-phosphates. Triamcinolone and 18-norcortisone do not react (9).

2. Reaction with 2-hydrazinobenzothiazole and hydrogen peroxide (10)
Condensation with 2-hydrazinobenzothiazole in alkaline medium and development by oxidation with hydrogen peroxide : blue-violet color.

The exact mechanism of the reaction in unknown. However, it may be postulated that a rearrangement occurs during the condensation, affording the hydrazone of an aldehyde, which then reacts with the diazonium salt generated in the reaction medium by oxidation of the excess reagent to give a formazan.



Chemicals. Chloroform, ethanol, 0.10 M hydrochloric acid, 0.10 M sodium hydroxide, an 0.75 % (w/v) solution of hydrogen peroxide (2.5 volumes oxygen), and 2-hydrazinobenzothiazole.

Reagent solution. Dissolve 0.040 g of 2-hydrazinobenzothiazole in 2.50 ml of 0.10 M hydrochloric acid, and dilute to 10.0 ml with water.

Method. Carefully evaporate to dryness on a steam bath 1 ml of sample solution in chloroform introduced into the tube. No trace amount of solvent should remain, since it inhibits the reaction. Introduce 1.0 ml of water, add 0.50 ml of reagent a and 0.50 ml of 0.10 M sodium hydroxide. Heat at 100°C for 10 min, cool for 5 min in a water-bath at 15°C, and add 2.0 ml of 0.75 % solution of hydrogen peroxide. Let stand for 10 min in the bath, add 2.0 ml of ethanol, mix, let stand for 5 min, and read at 580 nm.

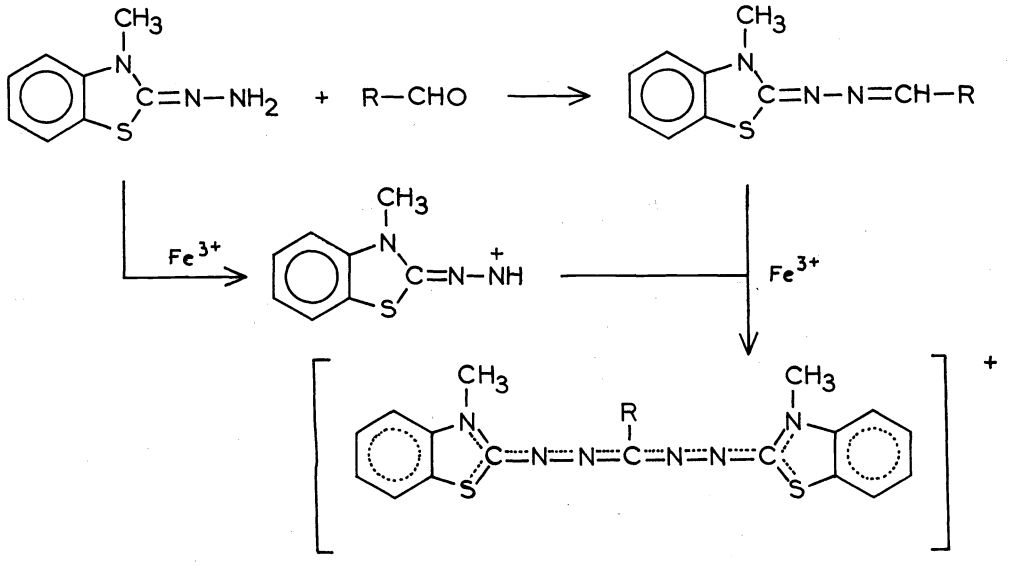
Results

	Sample size (µg) for A = 0.30 (1 cm - cell)
Cortisone	50
Dexamethasone	24
Hydrocortisone	39
Prednisolone	43
Prednisone	51
Triamcinolone	15

Readily saponifiable 21-esters can also be determined. Absorbance 0.30 is given by 36 µg of dexamethasone m-sulfobenzoate or 45 µg of cortisone acetate.

3. Reaction with 3-methylbenzothiazolin-2-one hydrazone and ferric chloride (10)
Condensation with 3-methylbenzothiazolin-2-one hydrazone and development by oxidation with ferric chloride : blue color.

The exact mechanism of the reaction is unknown. However, a mechanism similar to that postulated in the preceding method may be assumed.



Chemicals. Chloroform, 1.0 M hydrochloric acid, 0.10 M sodium hydroxide, ferric chloride hexahydrate, and 3-methylbenzothiazolin-2-one hydrazone hydrochloride.

Reagent solutions. (a) An 0.50 % aqueous solution of 3-methylbenzothiazolin-2-one hydrazone hydrochloride. (b) An 0.25 % aqueous solution of ferric chloride hexahydrate.

Method. Carefully evaporate to dryness on a steam-bath 1 ml of sample solution in chloroform introduced into the tube. No trace amount of solvent should remain, since it inhibits the reaction. Introduce 1.0 ml of water, add 0.50 ml of reagent a and 0.50 ml of 0.10 M sodium hydroxide. Heat at 100°C for 10 min, cool for 5 min in a water-bath at 15°C, add 0.50 ml of 1.0 M hydrochloric acid, and 2.0 ml of reagent b. Let stand for 1 h at room temperature, and read at 530 nm.

Results

	Sample size (µg) for A = 0.30 (1 cm - cell)
Cortisone	21
11-Desoxy-17-hydroxycorticosterone	21
Hydrocortisone	18
Prednisolone	19
Prednisone	17

Readily saponifiable 21-esters can also be determined.

E. 21-HYDROXY-20-KETOSTEROIDS (Colorimetry)

1. Reaction with sodium molybdate (11)
Reduction of the molybdate ion : blue color.

Chemicals. Glacial acetic acid and sodium molybdate.

Reagent solution. Dilute 0.50 ml of 25 % aqueous solution of sodium molybdate with 40.0 ml of glacial acetic acid.

Method. To 3.0 ml of sample solution in glacial acetic acid, add 4.0 ml of reagent, let stand for 2 h at room temperature, and read at 650 nm.

Results

	Sample size (µg) for A = 0.30 (1 cm - cell)
Cortisone	123
Hydrocortisone	187
Prednisolone	172
Prednisone	119

21-Esters do not react.

2. Reaction with Blue Tetrazolium (12)
Reduction of Blue Tetrazolium in alkaline medium to the corresponding formazan : pink color.

Chemicals. Chloroform, ethanol, a 10 % aqueous solution of tetramethylammonium hydroxide, and Blue Tetrazolium.

Reagent solutions. (a) Dilute 5.0 ml of 10 % aqueous solution of tetramethylammonium hydroxide to 50.0 ml with ethanol. (b) An 0.10 % solution of Blue Tetrazolium in ethanol.

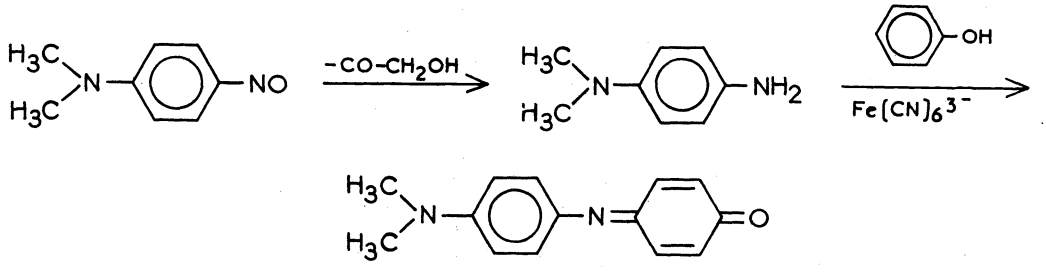
Method. To 1.0 ml of sample solution in ethanol, add 0.50 ml of reagent a, 0.50 ml of reagent b, and 2.0 ml of chloroform. Let stand at room temperature for the given length of time, exposed to subdued light, and read at 525 nm.

Results

	Reaction time (min)	Sample size (µg) for A = 0.30 (1 cm - cell)
Cortisone	5	17
Hydrocortisone	5	18
Hydrocortisone acetate	15	21
Prednisolone m-sulfobenzoate	10	37
Triamcinolone	20	10
Triamcinolone acetonide	15	24
Triamcinolone 16,21-diacetate	15	12

As may be seen from the above results, readily saponifiable 21-esters can also be determined. Prednisolone 21-phosphate does not react. The given conditions are critical. Under slightly more drastic conditions (heating at 60°C) various 3-ketosteroids with unsaturated ring A also react (13).

3. Reaction with p-nitroso-N,N-dimethylaniline (14)
The p-nitroso-N,N-dimethylaniline is reduced into dimethyl-p-phenylenediamine, which is then developed by an oxidative reaction with phenol in the presence of ferricyanide ion : green color.



Chemicals. Ethanol, 0.10 M sodium hydroxide, potassium ferricyanide, p-nitrosodimethylaniline, phenol, and Clark and Lubs buffer for pH 9.8 : Mix 50.0 ml of an 0.20 M aqueous solution of both boric acid and potassium chloride with 40.8 ml of 0.20 M potassium hydroxide, and dilute to 200 ml with water.

Reagent solutions. (a) An 0.10 % solution of p-nitroso-N,N-dimethylaniline in ethanol. (b) An 0.10 % solution of phenol in ethanol. (c) A 1.0 % aqueous solution of potassium ferricyanide. Prepare freshly before use.

Method. To 1.0 ml of sample solution in ethanol, add 0.50 ml of reagent a, immerse the tubes in ice water for 5 min, and add 0.50 ml of 0.10 M sodium hydroxide. Plug the tubes with cotton-wool, and let stand at 0°C for the given length of time, protected against light. Add 2.0 ml of buffer for pH 9.8, 5.0 ml of reagent b, 0.50 ml of reagent c, let stand in a water bath at 20 ± 2°C for 10 min, and read at 650 nm.

This method can be applied to free ketols and esters like acetates. Less readily saponifiable esters, such as hemisuccinates, cyclohexanecarboxylates, and m-sulfobenzoates can also be determined, provided that the first step of the reaction is modified as follows :

To 1.0 ml of the sample solution, add 1.0 ml of reagent a, let stand at room temperature for 5 min, and add 1.0 ml of 0.10 M sodium hydroxide. Let stand at room temperature for the given length of time, protected against light, then proceed as above.

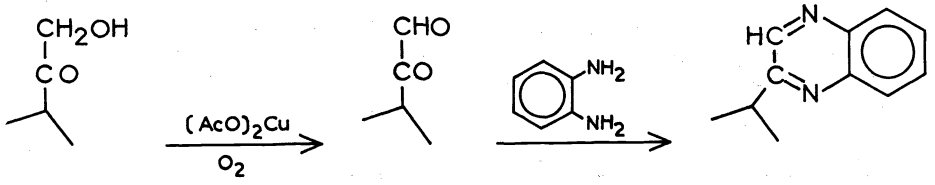
Results

	Reaction time (h)	Sample size (µg) for A = 0.30 (1 cm - cell)
Corticosterone	5	116
Cortisone	1	120
Cortisone acetate	1	134
Deoxycorticosterone acetate	5	139
Dexamethasone acetate	4	144
Hydrocortisone	2	120
Hydrocortisone acetate	2	134
Prednisolone	2	131
Dexamethasone hemisuccinate	2	173
Prednisolone cyclohexane-carboxylate	2	174
Prednisolone m-sulfobenzoate	2	210

21-Phosphates and sulfates do not react.

F. 21-HYDROXY- AND 17,21-DIHYDROXY-20-KETOSTEROIDS (UV spectrophotometry)

Oxidation with cupric acetate (15)
Oxidation of the ketol group to the corresponding glyoxal, which is developed as a quinoxaline derivative. The absorption maximum is located in the near-UV range.



Chemicals. Methanol, 1.0 M and 5.0 M hydrochloric acid, cupric acetate monohydrate, and o-phenylenediamine.

Reagent solutions. (a) Dilute 10.0 ml of a 3.0 % aqueous solution of cupric acetate monohydrate to 100.0 ml with methanol. (b) To 1.0 g of o-phenylenediamine, add 5.0 ml of 1.0 M hydrochloric acid and about 80 ml of water, and after dissolution dilute to 100.0 ml with water.

Method. To 1.0 ml of sample solution in methanol, add 0.10 ml of reagent a, heat at 50°C for 5 min, allow to cool in a water-bath, add 0.50 ml of reagent b, and heat again at 50°C for 5 min. Allow to cool in a water-bath, add 2.0 ml of 5.0 M hydrochloric acid, and read at 330 nm.

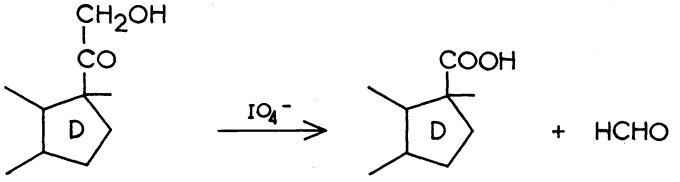
Results

	Sample size (μg) for A = 0.30 (1 cm - cell)
Cortisone	33.5
Deoxycorticosterone	39.5
Dexamethasone	45.5
Hydrocortisone	43.0
Prednisolone	40.0
Prednisone	40.0
Triamcinolone	53.0

21-Esters do not react.

G. 21-HYDROXY- AND 17,21-DIHYDROXY-20-KETOSTEROIDS (Colorimetry)

Periodate oxidation (16)
Oxidation of the ketol group with periodate ion, and development of the formaldehyde formed with phenylhydrazine and ferricyanide (Schryver reaction) : red color.



Chemicals. Ethanol, 1.50 M and concentrated hydrochloric acid, 0.10 M sodium hydroxide, 0.025 M aqueous solution of sodium metaperiodate, potassium ferricyanide, and phenylhydrazine hydrochloride.

Reagent solutions. (a) To 40.0 ml of 0.025 M aqueous solution of sodium metaperiodate, add 10.0 ml of 1.50 M hydrochloric acid. (b) A 1.0 % aqueous solution of phenylhydrazine hydrochloride. Prepare freshly. (c) A 2.0 % aqueous solution of potassium ferricyanide. Prepare freshly.

Method. To 1.0 ml of sample solution in ethanol, add 0.50 ml of reagent a, and let stand for 30 min at room temperature. Add 1.50 ml of 0.10 M sodium hydroxide, 2.0 ml of reagent b, and 1.0 ml of reagent c. Mix, chill in ice water for 5 min, add 5.0 ml of concentrated hydrochloric acid, mix, and dilute with 5.0 ml of ethanol. Let stand for 15 min at room temperature, and read at 520 nm.

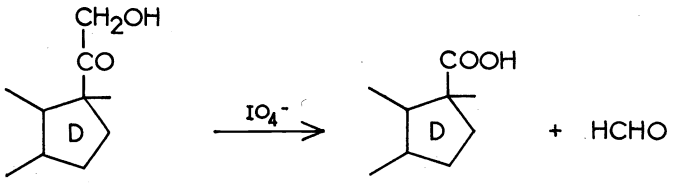
Results

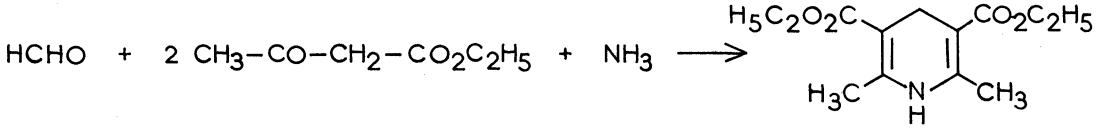
	Sample size (μg) for A = 0.30 (1 cm - cell)
Cortisone	170
Deoxycorticosterone	215
Prednisolone	255
Prednisone	170

21-Esters do not react.

H. 21-HYDROXY- AND 17,21-DIHYDROXY-20-KETOSTEROIDS (Fluorimetry)

Periodate oxidation (17)
Oxidation of the ketol group with periodate ion, and development of the formaldehyde formed with ethyl acetoacetate and ammonia to give 2,6-dimethyl-3,5-bis(ethoxycarbonyl)-1,4-dihydropyridine : blue fluorescence.





Chemicals. Ethanol, 1.0 M hydrochloric acid, 1.0 M sodium hydroxide, 0.01 M aqueous solution of sodium metaperiodate, ammonium acetate, stannous chloride dihydrate, and ethyl acetoacetate.

Reagent solutions. (a) A solution of 0.250 g of stannous chloride dihydrate in 100.0 ml of 1.0 M hydrochloric acid. Prepare freshly. (b) A 4.0 % solution of ethyl acetoacetate in 20 % aqueous solution of ammonium acetate.

Method. To 1.0 ml of sample solution in ethanol : water (1 : 49), add 0.20 ml of 0.01 M aqueous solution of sodium metaperiodate, and let stand at room temperature for 20 min. Add 0.80 ml of reagent a, 0.80 ml of 1.0 M sodium hydroxide, 1.20 ml of water, and 1.0 ml of reagent b. Heat at 60°C for 20 min, allow to cool, filter, and read at exc : 366 nm ; em : 470 nm.

Results

	Determination limits (µg)
Cortisone	6 - 30
Deoxycorticosterone	6 - 30
Prednisolone	8 - 40
Prednisone	6 - 30

21-Esters do not react.

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