2,4-Dinitrophenylhydrazine: A Sensitive Quantitative Reagent for Determining Microgram Quantities of Prednisone

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Abstract □ 2,4-Dinitrophenylhydrazine is suggested as a quantitative colorimetric reagent for the determination of microgram quantities of prednisone. The sensitivity of this reagent is compared with that of other steroid colorimetric reagents such as blue tetrazolium and isonicotinic acid hydrazide. Extraction procedures are presented which isolate prednisone from formulations containing salicylamide, acetaminophen, aspirin, and ascorbic acid, where the ratio of analgesics to steroid is 300 to 1. 2,4-Dinitrophenylhydrazine quantitatively determines prednisone in concentrations as low as 1.26×10^{-6} *M*.

Keyphrases □ 2,4-Dinitrophenylhydrazine—colorimetric reagent, analysis of prednisone □ Colorimetry—analysis, prednisone with 2,4-dinitrophenylhydrazine □ Prednisone—separation from analgesic mixtures, colorimetric analysis

Pharmaceuticals containing steroids in combination with aspirin, sodium salicylate, salicylamide, acetaminophen, ascorbic acid, and aluminum hydroxide gel are currently being marketed by pharmaceutical manufacturers. These steroids are usually the adrenocortical steroids (cortisone or derivatives of cortisone), of which prednisone is the most prevalent in these combinations. The ratio of the steroid to the analgesic component is usually in the order of 1 to 300.

The USP XVIII assay for prednisone tablets (1) cannot be used to assay these combinations without some modification of the extraction technique. The USP XVIII extraction procedure will not separate prednisone from these components, and the blue tetrazolium reagent (2) will not develop the characteristic steroid color unless the prednisone is completely separated. It was observed in this laboratory that salicylamide inhibits the development of this color (3). Graham *et al.* (4) found that a salicylamide–hydrocortisone ratio of 60:1 reduces the absorbance of the blue tetrazolium color by 2%.

Other commonly used reactions for steroid analysis are the Porter–Silber reaction (5, 6) and the Umberger reaction (7). The Porter–Silber reaction uses phenylhydrazine–sulfuric acid as a colorimetric reagent which is specific for the 17,21-dihydroxy-20-keto side chain of corticosteroids. The Umberger reaction uses isonicotinic acid hydrazide, which reacts with Δ^4-3-ketosteroids, as a colorimetric reagent.

2,4-Dinitrophenylhydrazine is a common reagent used extensively in the determination of aldehydes and ketones. Mason *et al.* (8) found that cortisone forms a C-3 monohydrazone with 2,4-dinitrophenylhydrazine in the cold. The quantitative isolation and determination of progesterone as the 2,4-dinitrophenylhydrazone are given in the NF XIII monograph for progesterone injections (9). Testosterone forms a 2,4-dinitrophenylhydrazone, but a quantitative isolation and gravimetric determination are not possible (10).

Lappin and Clark (11) used 2,4-dinitrophenylhydrazine as a colorimetric reagent in the determination of trace amounts of carbonyl compounds. They enhanced the sensitivity of the reagent by adding a solution of sodium or potassium hydroxide to an alcoholic solution of the 2,4-dinitrophenylhydrazone, producing a very intense wine-red color.

Gornall and MacDonald (12) quantitatively determined corticosteroids in extracts of urine, blood plasma, and tissues, using 2,4-dinitrophenylhydrazine. The concentration of these ketosteroids in the final extracts of these substances was in the milligram or microgram range. This low concentration prevented precipitation of the steroid hydrazone; however, the addition of alkali to the alcoholic hydrazone solution produced a wine-red color having a maximum absorbance between 425 and 495 nm., depending upon the specific ketosteroid being determined. The lowest concentration of steroid quantitatively determined by Gornall and MacDonald (12) was 6.38 μmoles/l. (cortisone) or 6.38×10^{-6} *M*.

The work of Gornall and MacDonald (12) suggested that their techniques could be applied to the assay of prednisone in tablets containing 1 mg. or less of prednisone and 350 mg. or more of salicylamide, acetaminophen, or aspirin. To eliminate possible interference by these analgesic components, the prednisone should be isolated before the formation of the 2,4-dinitrophenylhydrazone. Aspirin may be separated by extraction with 1 *N* sodium bicarbonate, and salicylamide and acetaminophen may be separated by extraction with aqueous alkaline solutions (pH 10 or more) or with acidic ferric chloride. Alkaline solutions of pH 10 or more should not be used because of the possibility of the degradation of the steroid.

In the present study, prednisone was extracted from the tablet excipients with methanol. The methanol extract was concentrated to a residue, which was dissolved in either sodium bicarbonate or acidic ferric chloride solutions and then extracted with chloroform.

Table I—Composition of Samples

Ratio	Synthetic Mixture	Commercial Tablet A (Colored, Uncoated)	Commercial Tablet B (White, Uncoated)	Commercial Tablet C (Colored, Enteric Coated)
	Prednisone	Prednisone	Prednisone	Prednisone
	Salicylamide	Salicylamide	—	Salicylamide
	Aspirin	Acetaminophen	Aspirin	—
	Ascorbic acid	Ascorbic acid	Ascorbic acid	Ascorbic acid
	Starch	—	Aluminum hydroxide gel	—
	Magnesium stearate	—	—	—
	Sunset yellow dye	—	—	—
Salicylamide–prednisone	465:1	250:1	—	300:0.75
Aspirin–prednisone	462:1	250:1	325:0.75	—
Ascorbic acid–prednisone	35:1	25:1	20:0.75	50:0.75

To compare the sensitivities of three colorimetric reagents that could be used for the determinative step, aliquots of the same chloroform solution of the isolated prednisone were treated with the following: (*a*) blue tetrazolium, the USP XVIII steroid reagent (2); (*b*) isonicotinic acid hydrazide, Umberger's reagent (7); and (*c*) 2,4-dinitrophenylhydrazine, as used by Gornall and MacDonald (12).

The samples used in this study are shown in Table I.

EXPERIMENTAL

Apparatus—A recording UV visible spectrophotometer with 1-cm. cells was used.

Reagents—*Methyl Alcohol*—Use analytical reagent grade, acetone free.

Acidic Ferric Chloride—Mix 12 ml. of 60% $FeCl_3 \cdot 6H_2O$ with 48 ml. of 0.05 *N* hydrochloric acid.

Sodium Bicarbonate—Use 1 *N* solution.

Chloroform—Use analytical reagent grade.

Acid–Methanol (1:3)—Mix 1 volume HCl (C.P. reagent, sp. gr. 1.18) with 3 volumes of methyl alcohol. Prepare once a week.

Acid–Methanol (1:7)—Mix 1 volume of hydrochloric acid with 7 volumes of methyl alcohol. Prepare once a week.

2,4-Dinitrophenylhydrazine—Dissolve 100 mg. of 2,4-dinitrophenylhydrazine in 100 ml. of acid–methanol (1:3) (may be kept 1 week if stored in the cold).

Sodium Hydroxide—Protect prepared solution (4.0 *N*) from carbon dioxide to prevent formation of sodium carbonate, which would precipitate during the final dilution with methanol.

Blue Tetrazolium—Dissolve 50 mg. of blue tetrazolium in 10 ml. of 95% ethyl alcohol. Heating on steam bath facilitates solution of the blue tetrazolium. Filter solution if not clear.

Tetramethylammonium Hydroxide—Dilute 1 ml. of 10% aqueous tetramethylammonium hydroxide to 10 ml. with 95% ethyl alcohol.

Isonicotinic Acid Hydrazide—Dissolve 100 mg. of isonicotinic acid hydrazide in about 100 ml. of methanol, add 1 ml. of acid–methanol (1:7), and dilute to 200 ml. with methanol.

Standard Solution—Use prednisone USP reference standard, 0.01 mg./ml.

Procedure—*Sample Preparation*—Transfer a weighed amount of the powdered sample, equivalent to about 0.75–1 mg. of prednisone, to a 50-ml. conical flask. Add 30 ml. of methanol, place a small funnel in the flask (to act as a small condenser), and keep near the boiling point while stirring for 30 min., preferably with a magnetic stirrer. Allow to cool to room temperature, and filter into a 100-ml. volumetric flask. Wash the conical flask with five 10-ml. portions of methanol, pouring each portion through the filter. Dilute the combined filtrate and washings to 100 ml.; mix well.

Extraction—Samples containing salicylamide and/or acetaminophen and ascorbic acid: Concentrate a 50-ml. aliquot of the sample preparation to a residue on the steam bath, using a small air current. In each of three 60-ml. separators, place 5 ml. of acidic ferric chloride solution. Add 15 ml. of water each to Separators 2 and 3. Add 15 ml. of water to the sample residue, mix well to dissolve all water-soluble material, and add to Separator 1. Wash the residue beaker with 20 ml. of chloroform, and add to Separator 1. Shake for 1 min. and allow layers to separate. Draw off the chloroform layer from Separator 1 into Separator 2, and shake for 1 min. Then add the separated chloroform layer in Separator 2 to Separator 3 and shake. Salicylamide and acetaminophen should be completely removed from the chloroform layer in Separator 3; if the aqueous layer is

Table II—Determination of Prednisone in Synthetic and Commercial Samples with Three Reagents

Sample	Declaration, mg./Tablet	DNPH[a] Found, mg./Tablet	DNPH[a] Percent of Declaration	Blue Tetrazolium Found, mg./Tablet	Blue Tetrazolium Percent of Declaration	INH[b] Found, mg./Tablet	INH[b] Percent of Declaration
Synthetic mixture	1.016[c]	1.016	100.0	1.064	104.7	0.998	98.2
	1.016	1.016	100.0	1.064	104.7	0.998	98.2
	1.016	0.982	96.6	1.038	102.2	0.954	93.9
Av.	1.016	1.005	98.9	1.055	103.9	0.983	96.8
Commercial Tablet A	1.000	0.91	91.0	0.89	89.0	0.88	88.0
	1.000	0.97	97.0	0.92	92.0	0.92	92.0
Av.	1.000	0.94	94.0	0.905	90.5	0.90	90.0
Commercial Tablet B	0.750	0.74	98.7	0.79	105.3	0.69	92.0
	0.750	0.75	100.0	0.74	98.7	0.68	90.7
Av.	0.750	0.745	99.4	0.765	102.0	0.685	91.4
Commercial Tablet C	0.750	0.70	93.3	0.71	94.7	0.62	82.7
	0.750	0.70	93.3	0.75	100.0	0.63	84.0
Av.	0.750	0.70	93.3	0.73	97.4	0.625	83.4

[a] 2,4-Dinitrophenylhydrazine reagent. [b] Isonicotinic acid hydrazide reagent. [c] Synthetic mixture, mg./1 g.

Table III—Recovery Determinations of Prednisone in Combinations

Sample	DNPH[a] Added, mg.	DNPH[a] Found, mg.	DNPH[a] Percent of Recovery	Blue Tetrazolium Added, mg.	Blue Tetrazolium Found, mg.	Blue Tetrazolium Percent of recovery	INH[b] Added, mg.	INH[b] Found, mg.	INH[b] Percent of Recovery
Standard prednisone	2.12	2.01	94.8	2.12	2.04	96.2	2.12	1.99	93.9
	0.530	0.539	101.7	0.530	0.537	101.3	0.530	0.501	94.5
	0.530	0.526	99.2	0.530	0.509	96.0	0.530	0.508	95.8
Av.			98.6			97.8			94.7
Standard prednisone + Tablet A	1.164	1.156	99.3	1.165	1.110	95.3	1.164	1.093	93.9
	1.250	1.292	103.4	1.250	1.236	98.9	1.250	1.220	97.6
Av.			101.4			97.1			95.8
Standard prednisone + Tablet B	1.034	1.012	97.9	1.034	1.058	102.3	1.034	0.982	95.0
	1.044	1.081	103.5	1.044	1.054	100.9	1.044	1.031	98.8
Av.			100.7			101.6			96.9
Standard prednisone + Tablet C	1.150	1.152	100.2	1.150	1.246	108.3	1.150	1.129	98.2
	1.048	1.063	101.4	1.048	1.181	112.7	1.048	1.030	98.3
Av.			100.8			110.5			98.2

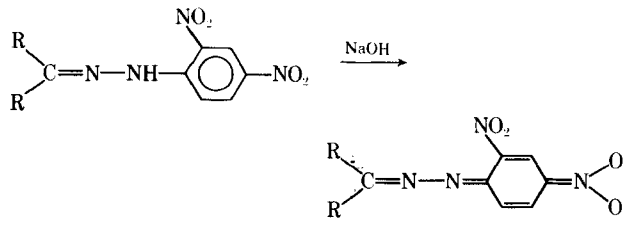
[a] 2,4-Dinitrophenylhydrazine reagent. [b] Isonicotinic acid hydrazide reagent.

purple (indicating the presence of salicylamide or acetaminophen), extract the chloroform layer from Separator 3 with another portion of water and acidic ferric chloride solution. Repeat the extraction procedure, using three 20-ml. portions of chloroform to wash out the residue beaker and passing each portion successively through Separators 2 and 3. Wash the combined chloroform extracts with 20 ml. of water. Concentrate the washed extracts to about 25 ml., transfer quantitatively to a 50-ml. volumetric flask, and dilute to volume with chloroform.

Samples containing aspirin and ascorbic acid: Concentrate a 50-ml. aliquot of the sample preparation to a residue on the steam bath. Add 20 ml. of 1 *N* sodium bicarbonate to the sample residue, mix well to dissolve all water-soluble material, and place in a separator. Wash the residue beaker with 20 ml. of chloroform, and add the wash to the separator. Shake for 1 min.; allow layers to separate. Draw off the chloroform layer into a second separator containing 20 ml. of 1 *N* sodium bicarbonate, and shake for 1 min. Repeat the extraction procedure, using three 20-ml. portions of chloroform to wash out the residue beaker and passing each portion successively through both separators. Wash the combined chloroform extracts with 20 ml. of water. Concentrate the washed extracts to about 25 ml., transfer quantitatively to a 50-ml. volumetric flask, and dilute to volume with chloroform.

Synthetic mixture: Use the combined extraction procedure for samples containing salicylamide and/or acetaminophen and samples containing only aspirin.

Color Development—*2,4-Dinitrophenylhydrazine*—Evaporate to dryness an aliquot of the chloroform solution of extracted prednisone equivalent to about 100 mcg. of prednisone. Avoid excessive heating of the dry residue. Dissolve the residue in 1 ml. of methanol, and transfer to a small (5-ml.) glass-stoppered tube. Quantitatively rinse the residue beaker with four 1-ml. portions of methanol, and add to the small tube. Carefully concentrate the methanol solution in the tube to about 1 ml. Wash down the sides of the tube with 1 ml. of methanol, and add 2.0 ml. (pipet) of 2,4-dinitrophenylhydrazine reagent. Loosely stopper the tube, and heat for 2 hr. at 60° (a constant-temperature heating block is extremely useful for this purpose). Cool to room temperature, and transfer quantitatively to a 25-ml. volumetric flask containing 2.0 ml. (pipet) of 4.0 *N* sodium hydroxide and 10 ml. of methanol.



Scheme I

Mix well, dilute to volume with methanol, and allow to stand for 30 min.

Prepare a reagent blank by carrying a mixture of 2.0 ml. of methanol and 2.0 ml. of 2,4-dinitrophenylhydrazine reagent through the color development.

Evaporate a 10-ml. aliquot of the standard prednisone solution to about 1 ml., transfer quantitatively to a small glass-stoppered tube, and treat like the sample.

Scan the sample and standard solutions between 750 and 400 nm., using the reagent blank as the reference. The maximum absorbance occurs at about 480 nm.

Blue Tetrazolium—Evaporate to dryness, in separate 25-ml. glass-stoppered conical flasks, an aliquot of the chloroform solution of extracted prednisone equivalent to about 100 mcg. of prednisone and a 10-ml. aliquot of the standard prednisone solution. Add 10 ml. of 95% ethyl alcohol to each of the residue flasks and to a blank flask. Add 2.0 ml. of blue tetrazolium reagent to each flask, mix well, and then add 2.0 ml. of the diluted tetramethylammonium hydroxide solution. Mix well; allow to stand 90 min. in the dark. Scan the sample and standard solutions between 750 and 400 nm., using the reagent blank as the reference. The maximum absorbance occurs at about 525 nm.

Isonicotinic Acid Hydrazide—Evaporate to dryness, in separate 25-ml. glass-stoppered conical flasks, an aliquot of the chloroform solution of extracted prednisone equivalent to about 100 mcg. of prednisone and a 10-ml. aliquot of the standard prednisone solution. Add 10 ml. of the isonicotinic acid hydrazide reagent to each of the residue flasks and to a blank flask. Swirl residue flasks to ensure complete solution of the residues, and allow to stand at room temperature for 2 hr. Scan the sample and standard solutions between 600 and 300 nm., using the reagent blank as a reference. The maximum absorbance occurs at about 410 nm.

RESULTS AND DISCUSSION

The results of the prednisone determinations in which the three colorimetric reagents were used are tabulated in Table II. The average prednisone values obtained with 2,4-dinitrophenylhydrazine ranged from 93.3 to 99.4% of the known or declared amount of prednisone; the range was 90.5–103.9% with blue tetrazolium and 83.4–96.8% with isonicotinic acid hydrazide. A comparison of these values indicates the accuracy and precision of the assays performed with 2,4-dinitrophenylhydrazine as the colorimetric reagent.

Recovery determinations for sample solutions spiked with known weights of prednisone and for solutions of standard prednisone (a procedural standard) carried through the entire assay procedure are tabulated in Table III. The average percentage recoveries from the procedural standard were 98.6% (2,4-dinitrophenylhydrazine), 97.8% (blue tetrazolium), and 94.7% (isonicotinic acid hydrazide); from the three spiked commercial samples, they were 100.9, 103.1, and 96.9%, respectively.

When the hydrazone reaction mixture is mixed with methanolic alkali, a black color immediately develops; on standing, this color changes to wine-red (sample and standard tubes) or to light yellow or tan (reagent blank). The wine-red color of the steroid hydrazone is stable, and its wavelength of maximum absorbance differs from that of the yellow or tan color produced by the excess reagent. Nevertheless, a reagent blank is advisable because it will reduce or eliminate background absorption over the range scanned.

For assay purposes, a standard curve is not essential. No error is encountered in the comparison of the absorbances of the sample and standard solutions, provided the concentrations of these solutions are approximately equal. However, to establish or demonstrate the linearity of absorbance and concentration at the microgram level, a standard curve was prepared. This curve shows linearity over a range of 0.45–5 mcg. of prednisone/ml., and it passes through the origin.

Interferences may be encountered from any compound containing carbonyl groups. These interferences may be eliminated by the proper extraction technique in the isolation of the prednisone. The samples used in this study contained ascorbic acid, which forms a hydrazone with the 2,4-dinitrophenylhydrazine reagent. However, in isolating prednisone from the other active ingredients, ascorbic acid is removed and is not present in the final chloroform solution of the extracted prednisone.

The behavior of the 2,4-dinitrophenylhydrazones of steroids in alkaline solution is not clearly established. However, Gornall and MacDonald (12) and others (11, 13, 14) suggested that the very intense wine-red color is due to the formation of the resonating quinoidal ion I (Scheme I).

Each carbonyl produces one quinoidal ion; therefore, the formation of more than one quinoidal ion per molecule of steroid should greatly increase the molar absorptivity of the steroid hydrazone. For cortisone, Gornall and MacDonald (12) reported a molar absorptivity of 43,600 at 475 nm. The average molar absorptivity of the prednisone hydrazone calculated from the standard absorbances obtained for the standard curve was 51,646 at 480 nm.

Gornall and MacDonald (12) found that the C-3 and C-20 carbonyls of cortisone are completely converted to hydrazones and its C-11 carbonyl is partially converted on heating 90 min. at 59°. These workers stated that the 2,4-dinitrophenylhydrazone of a carbonyl group that has reacted completely appeared to have a molar absorptivity in the vicinity of 20,000. By using the average calculated molar absorptivity of 51,646, and 20,000 as representative of a fully reacted carbonyl group, it appears that the C-3 and C-20 carbonyls of prednisone are completely converted, while its C-11 carbonyl, like the C-11 carbonyl of cortisone, is partially converted to the hydrazone.

The complete reaction of the C-3 and C-20 carbonyls of prednisone suggests that an accurate quantitative determination with 2,4-dinitrophenylhydrazine depends on the absence of any degradation products of prednisone in the sample being analyzed. The most common type of prednisone degradation is the oxidation of the C-17 side chain. This results in the loss of the C-20 carbonyl from the prednisone molecule and the formation of a complex mixture of organic acids together with neutral compounds such as aldehydes and ketones (4). This method of prednisone isolation will not separate prednisone and any neutral decomposition products (aldehydes or ketones); therefore, the 2,4-dinitrophenylhydrazone color formed with a degraded sample will not be truly representative of prednisone. TLC may be used to determine the condition of the extracted prednisone.

A portion of the synthetic mixture was heated on the steam bath in the presence of alkali and air. This procedure resulted in the degradation of prednisone at the C-17 side chain. Assay results of this decomposed prednisone extract are tabulated in Table IV. The 2,4-dinitrophenylhydrazine and blue tetrazolium results indicate average values of 78.5 and 60.9%, respectively, of the true prednisone value of the mixture. The isonicotinic acid hydrazide results agree with those in Table II and suggest an intact prednisone molecule. However, the low results with 2,4-dinitrophenylhydrazine and blue tetrazolium, which depend on the intact C-17 side chain, definitely indicate the degraded state of the prednisone molecule. Quantitative agreement between the assay results obtained with all three colorimetric reagents (Table II) indicates a completely intact (not decomposed or degraded) prednisone molecule.

Use of a sample weight containing 0.75–1 mg. of prednisone prevents the use of column partition chromatography to isolate the prednisone. The amount of salicylamide, acetaminophen, or aspirin in this sample weight would overload the partition chromatography column normally used. However, by reducing the sample size by a factor of 20, column partition chromatography can be used (instead of separator extraction) for the isolation of the steroid. This would provide from 37 to 50 mcg. of prednisone for the formation of the 2,4-dinitrophenylhydrazone. The concentration of the hydrazone solution used for absorbance measurement would be 37–50 mcg./25 ml. or 1.48–2 mcg./ml. This concentration is in the linear range of 1–5 mcg./ml. (Fig. 1).

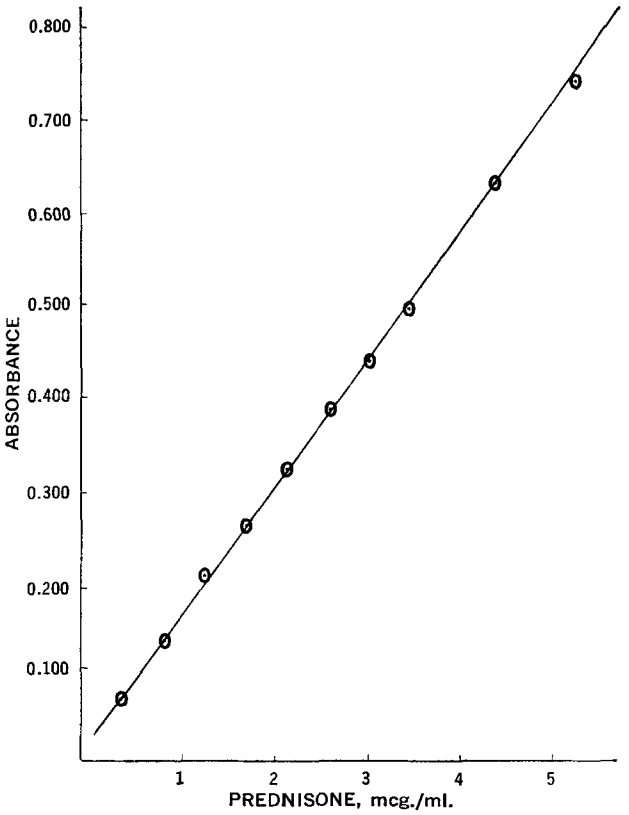


Figure 1—*Standard curve for prednisone with 2,4-dinitrophenylhydrazine reagent.*

CONCLUSIONS

2,4-Dinitrophenylhydrazine may be used as a colorimetric reagent for the quantitative determination of prednisone.

2,4-Dinitrophenylhydrazine is very sensitive for microgram quantities of prednisone. It is more sensitive than the commonly used steroid reagents, blue tetrazolium and isonicotinic acid hydrazide.

To compare the sensitivities of the three colorimetric reagents, prednisone was quantitatively determined in concentration of: (*a*) 1.17×10^{-5} *M*, 2,4-dinitrophenylhydrazine; (*b*) 2.03×10^{-5} *M*, blue tetrazolium; and (*c*) 2.89×10^{-5} *M*, isonicotinic acid hydrazide. The lowest concentration of prednisone determined with 2,4-dinitrophenylhydrazine and showing linearity with absorbance was 0.45 mcg./ml. or 1.26×10^{-6} *M* (1.26 μmoles).

Table IV—Prednisone Determinations Indicating Degradation of Prednisone in a Synthetic Mixture Containing 1.04 mg./g.

DNPH[a]		Blue Tetrazolium		INH[b]	
Found, mg./Tablet	Percent of Theory	Found, mg./Tablet	Percent of Theory	Found, mg./Tablet	Percent of Theory
0.83	79.8	0.60	57.7	0.96	92.3
0.77	74.0	0.55	52.9	0.97	93.3
0.85	81.7	0.75	72.1	1.01	97.1
Av. 0.817	78.5	0.63	60.9	0.98	94.2

[a] 2,4-Dinitrophenylhydrazine reagent. [b] Isonicotinic acid hydrazide reagent.

The experiments showed that microgram quantities of other ketosteroids might be similarly assayed.

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 11, 1971, from the *Food and Drug Administration, Chicago, IL 60607*

Accepted for publication June 9, 1971.

Specific Colorimetric Reaction for Quantitative Determination of 3-Hydroxy-$\Delta^{1,5}$-Diene Grouping in Steroids

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Abstract □ A quantitative colorimetric procedure, which appears to be specific for the 3-hydroxy-$\Delta^{1,5}$-diene grouping in steroids, was developed. The reaction results in the formation of a stable red color when the steroid reacts with aluminum chloride in a benzene–nitrobenzene system. The anabolic hormone, 17α-methyl-1,5-androstadiene-3β,17β-diol, was used to develop the procedure.

Keyphrases □ 3-Hydroxy-$\Delta^{1,5}$-diene steroids—reaction with aluminum chloride in benzene–nitrobenzene, colorimetric analysis □ Steroids—reaction with aluminum chloride in benzene–nitrobenzene, colorimetric analysis for 3-hydroxy-$\Delta^{1,5}$-diene group □ Colorimetry—analysis, 3-hydroxy-$\Delta^{1,5}$-diene steroids

An analytical procedure based on the color developed by heating an anabolic hormone with aluminum chloride, followed by condensation with anisaldehyde, was reported by Kato (1, 2). The functionality responsible for the reaction was found to be a double bond, which originally existed in the molecule or which was produced by the elimination of a hydroxyl group during the reaction. In all cases, a necessary condition was that these double bonds must not be conjugated with any carbonyl group present in the molecule. Cholestane, cholestanol, cholesterol, cyclohexene, and cyclohexanol were some of the many compounds reported by Kato that gave a positive reaction

The analysis of 17α-methyl-1,5-androstadiene-3β,-17β-diol (I) by Kato's procedure (1, 2) was tried but without success; the mixture turned an olive-green color and had a very poorly defined absorption maximum.

Tauber (3) and Lange *et al.* (4) reported color formation for many steroids in chloroform when reacted with 70% perchloric acid. Lange *et al.* (4) reported that colorless, well-forme dcrystals of sterol–perchloric acid were precipitated when sterols containing the 3-hydroxy-5-ene grouping were dissolved in chloroform and then treated with a stoichiometric amount of perchloric acid. When an excess of perchloric acid was used, the crystals redissolved and characteristic colors were produced. Alteration of either of the two essential groups in the sterol resulted in failure to give a precipitate, but color formation still took place.

Early work on the color formation of cholesterol when treated with sulfuric acid–acetic anhydride (Liebermann–Burchard reaction) (5, 6) and zinc chloride–acetyl chloride (Tschugaeff reaction) (7) has formed the basis for more recent work. The latter papers deal with colors formed with unsaturated steroids when treated with an acid–iron reagent (8–10).

When the steroid (I) was tested in these systems, it gave a positive color reaction with all of them. Since such a large variety of steroids give positive color reactions with these systems, it was desirable to try to obtain a reaction that would be specific for the reactive grouping of I.

In the course of the Kato reaction, it was noted that I produced a satisfactory red color by merely allowing aluminum chloride in nitrobenzene to react with the steroid at room temperature. The utilization of this color as an analytical procedure for I is reported here.

EXPERIMENTAL

Apparatus—The absorption curves were obtained using a spectrophotometer[1] with 1-cm. cells.

Reagents and Solvents—The following were used: aluminum chloride, anhydrous[2]; benzene, analytical reagent, thiophene free[2]; and nitrobenzene[3], m.p. 5–6°. The aluminum chloride and benzene were used without further purification. Solutions of aluminum chloride in nitrobenzene became increasingly dark (in the absence of added steroid) as the nitrobenzene aged. To minimize this effect conveniently, the nitrobenzene was washed twice with 0.5 *N* HCl and then three times with 0.5 *N* NaOH, dried with anhydrous

[1] Carey model 15.
[2] Mallinckrodt.
[3] Matheson, Coleman, and Bell.