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The Use of 2,4-Dinitrophenylhydrazine Derivatives of Ovarian Ketosteroids in Steroid Analysis

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This paper reports the development of a technically simple analytical technique for the characterization and quantitative determination of ovarian ketosteroids employing derivative formation with 2,4-dinitrophenylhydrazine (2,4-DNP), separation by thin-layer chromatography (TLC), and quantitative determination by spectrophotometry. The chemical identities of the derivatives were established by nitrogen analysis. The melting point, chromatographic mobilities of the free steroids and their derivatives on silica gel G in three systems, wavelength of maximum absorption in chloroform and ethanol, and molar absorption coefficient are reported for the 2,4-DNP derivatives of ovarian ketosteroids. The relationship between the absorption maximum in chloroform and the chemical structure of the derivatives has been analyzed.
INDEX DESCRIPTORS: ovarian Ketosteroids, 2,4-Dinitrophenylhydrazine derivatives, steroid analysis.

Steroid analysis is of fundamental importance for the field of steroid biochemistry. Steroids have been analyzed by a variety of means, including gas-liquid, paper, and column chromatography, spectrophotometry, radioimmunoassay, etc. We have employed a technique for the analysis of the ovarian ketosteroids based on the separation of their 2,4-dinitrophenylhydrazine (2,4-DNP) derivatives by thin-layer chromatography (TLC). The reagent 2,4-dinitrophenylhydrazine has been used for the characterization and determination of carbonyl groups for many years (1, 2). It has been used in the characterization of cortisone (3), progesterone (4, 5, 6), pregnenolone (7), 17-ketosteroids (8), and other ketosteroids (7, 9). The combination of the derivatization process with TLC has produced a technically simple analytical technique which has considerable range and power (8, 10, 11, 12, 13). The purpose of this paper is both to report the development of a technique for the derivatization, separation (by TLC), and quantitative determination of the ovarian ketosteroids, and to record the physical properties of the 2,4-DNP derivatives of these ketosteroids.

MATERIALS AND METHODS

Chemicals and Reagents

All solvents used were of reagent grade and were obtained from Fisher Chemical Company (St. Louis, Missouri). They were redistilled before use. Steroids were of the highest quality available, and were obtained either from Sigma Chemical Company (St. Louis, Missouri) or Mann Research Laboratories (New York, New York). They were analyzed by TLC, and recrystallized before use where necessary. The silica gel G was produced by E. Merck (Darmstadt, Germany, article no. 7731) and was obtained from Brinkmann Instruments, Inc. (Westbury, New York).

Preparation of Thin-Layer Chromatographic Plates

Thin-layer chromatographic plates were prepared according to Stahl (14), using a spreader and mounting board obtained from Brinkmann Instruments, Inc. The plate size was 20 × 20 cm. The silica gel G adsorbent was applied in a layer 0.25 mm thick. Plates were activated by heating at 90 C for 4 hours, and were then stored in a dessicator until use. Development was accomplished in developing tanks obtained from Brinkman Instruments, Inc.

Derivatization Technique

The steroids (100-200 μg) were dissolved in 200 μliters of absolute ethyl alcohol and allowed to react overnight with 400 μliters of a solution containing 125 mg of 2, 4-DNP dissolved in 15 ml ethyl alcohol and 400 μliters of concentrated hydrochloric acid. The reaction mixture was then evaporated under pre-purified nitrogen at 37 C on a temperature-controlled heating block. The reaction mixture was then redissolved in a minimum quantity (usually about 0.5 ml) of chloroform-ethanol (1:1/v:v) and chromatographed on a Sephadex LH-20 column (250 × 9 mm) to separate the derivatives from the unreacted 2,4/DNP. Only the colored compounds eluted before the orange-red 2,4-DNP were collected, since control experiments had established that this procedure resulted in a quantitative recovery of the steroid derivatives. The derivatives obtained from the LH-20 column were dried under a stream of pre-purified nitrogen on a temperature block and spotted on a silica gel G plate. The chromatogram was developed in benzene-ethyl alcohol (9:1/v:v) or benzene-ethyl acetate (3:1/v:v). The appropriate area of the plate was removed with a vacuum suction filter (Kontes Glassware Co., Vineland, New Jersey), the steroid derivative eluted with three successive 0.5 ml aliquots of chloroform, the resulting solution made up to a known volume with chloroform, and the absorbance of the resulting solution determined at the wavelength of maximum absorption in a Beckman DU-2 spectrophotometer.

Bulk amounts of the steroid derivatives were prepared for analytical purposes by reacting in ethyl alcohol 0.3 mmole of the steroid with 0.350 mmole of 2,4-DNP per carbonyl group. The steroid derivatives were isolated by filtration on a sintered glass funnel and recrystallized two times from ethyl alcohol-water. The derivatives of pregnenolone and epiandrosterone were recrystallized two additional times from benzene-hexane in order to remove impurities not removed by the original recrystallizations. Because of their insolubility in ethyl alcohol, the derivatives of androstenedione and progesterone were recrystallized two times from chloroform-ethyl alcohol. The purity of the compounds was checked by TLC on silica gel G, using benzene-ethyl acetate (3:1/v:v) and benzene-ethyl alcohol (9:1/v:v) as solvents. The pure derivatives were dried several days in an Aberhalden vacuum drying apparatus over phosphorus pentoxide at 64 C (boiling methyl alcohol) under a vacuum of 6 to 10 μm of mercury. The melting points of the pure, dried derivatives were determined with a Thomas-Hoover melting point apparatus (Arthur H. Thomas Co., *Philadelphia, Pennsylvania*). All melting points were uncorrected. Nitrogen analyses were performed by Dr. F. B. Strauss Microanalytical Laboratory (10 Carlton Road, Oxford OX2 7SA, England).

Table 1. *Spectral and Chromatographic Properties of the 2,4-DNP Derivatives of Ovarian Ketosteroids*

No.	Color	λmax, $CHCl_3$	ε, $CHCL_3$ $M^{-1}cm^{-1}$	Chromatographic Properties — Rf values[a]				
				Free Steroid		2, 4-DNP Derivative		
				I	II	I	II	III
1.	Red	390	23,900	0.28	0.56	0.61	0.61	0.11
2.	Red	390	32,800	0.22	0.41	0.55	0.69	0.08
3.	Yellow	364	22,600	0.45	0.67	0.60	0.68	0.07
4.	Yellow	366	27,300	0.36	0.53	0.49	0.50	0.06
5.	Yellow	364	23,400	0.39	0.56	0.53	0.55	0.05
6.	Yellow	364	23,500	0.73	0.59	0.88	0.93	0.20
7.	Yellow	364	24,900	0.43	0.51	0.60	0.66	0.08
8.	Yellow	366	22,600	0.22	0.47	0.30	0.64	0.08
9.	Orange	380	52,700	0.53	0.75	0.95	0.95	0.43
10.	Orange	374	45,600	0.27	0.57	0.86	0.93	0.08
11.	Orange	374	41,400	0.44	0.77	0.94	0.95	0.20

[a]System I = benzene-ethyl acetate (3:1/v:v); II = benzene-ethanol (9:1/v:v); III = benzene-hexane-ethyl acetate (7:7:1/v:v:v). Rf's for the two components of the free 2,4-DNP itself: System I, major component 0.43; minor component 0.91; System III, major component 0.06; minor component 0.46.

RESULTS

The following compounds were derivatized, being listed by the name of the parent steroid. A description of the chemical properties of these derivatives follows. The spectral and chromatographic properties are listed in Table I. The numbers in the list correspond to those in the Table.

1. *20a-Hydroxy-4-pregn-3-one.* 3-*a*,*β*-unsaturated-mono-2,4-DNP. Molecular weight (MW) 496.61 (calculated). Nitrogen determination (%N): Theoretical (Th): 11.28. Experimentally found (F): 11.24. Wavelength of maximum absorption in ethanol (λmax, EtOH) 380 nm. Melting Range (MR) 222-224 C. (All melting points were taken upon purified samples dried in an Abderhalden drying pistol for several days, just before the samples were submitted for nitrogen analysis. They are uncorrected. All the derivatives decompose upon melting).

2. *17β-Hydroxy-4-androsten-3-one* (testosterone). 3-*a*,*β*-unsaturated-mono-2;4-DNP. MW 468.56. %N: Th: 11.96. F: 12.00. λmax, EtOH 380 nm. MR 199.5-200 C.

3. *17β-Hydroxy-5a-androstan-3-one* (5*a*-Dihydrotestosterone). 3-mono-2,4-DNP. MW 470.57. %N: Th: 11.91. F: 11.75. λmax, EtOH 363-4 nm. MR 228.5-230 C.

4. *3β-Hydroxy-5a-androstan-17-one* (epiandrosterone). 17-mono-2,4-DNP. MW 470.57. %N: Th: 11.91. F: 11.80. λmax, EtOH 360 nm. MR 222-223 C.

5. *3β-Hydroxy-5-androsten-17-one* (dehydroepiandrosterone). 17-mono-2,4-DNP. MW 468.56 %N: Th: 11.96. F: 11.88. λmax, EtOH 355 nm. MR 232.5-234.5 C.

6. *3-Hydroxy-1,3,5(10)-estratrien-17-one* (estrone). 17-mono-2,4-DNP. MW 450.50. %N: Th: 12.44. F: 12.23. λmax, EtOH 360 nm. MR 283-284 C.

7. *3β-Hydroxy-5-pregnen-20-one* (pregnenolone). 20-mono-2,4-DNP. MW 496.61. %N: Th: 11.28. F: 11.30. λmax, EtOH 360 nm. MR 251-252 C. (Turns bright red just before melting).

8. *3β,17a-Dihydroxy-5-pregnen-20-one* (17*a*-hydroxypregnenolone). 20-mono-2,4-DNP. MW 512.61. %N: Th: 10.93. F: 10.80. λmax, EtOH 355 nm. MR 237.5-238.5 C.

9. *4-Pregnene-3,20-dione* (progesterone). 3*a*,*β*-unsaturated, 20-bis-2,4-DNP. MW 674.72. %N: Th: 16.61. F: 16.66. λmax, EtOH 365 nm. MR 284.5-285 C.

10. *17a-Hydroxy-4-pregnen-3,20-dione* (17*a*-hydroxyprogesterone). 3-*a*,*β*-unsaturated, 20-bis-2,4-DNP. MW 690.72. %N: Th: 16.22. F: 16.20. λmax, EtOH 365 nm. MR 276-277 C.

11. *4-Androsten-3,17-dione* (androstenedione). 3-*a*,*β*-unsaturated, 17-bis-2,4-DNP. MW 646.67. %N: Th: 17.33 F: 17.56. λmax, EtOH 370 nm. MR 292-293 C.

DISCUSSION

The 2,4-DNP Derivatization Procedures

Because free 2,4-DNP absorbs light in the same region of the spectrum as its ketosteroid derivatives, it is essential to remove all the unreacted 2,4-DNP from the reaction product. Column chromatography on Sephadex LH-20 yields a mixture of all the 2,4-DNP steriod derivatives purified from the free 2,4-DNP and therefore suitable for further separation of the derivatives by TLC. Earlier workers (15) removed the unreacted 2,4-DNP from the reaction product by oxidizing it to m-dinitrobenzene with Benedict's reagent. This procedure did not damage the steriods because Benedict's reagent will not oxidize the hydroxyl groups of steroids unless the reaction is continued for 15 minutes or longer. However, because the use of Benedict's reagent would complicate the chromatographic separation of the derivatives by producing relatively large amounts of m-dinitrobenzene, the Sephadex

LH-20 procedure was developed.

Because 2,4-DNP is a mild oxidizing agent, the possibility of oxidation of hydroxyl groups to carbonyl groups exists, with subsequent derivative formation from the oxidation product. However, Kuroda (16) has shown that the 2,4-DNP will not oxidize terpene and aromatic alcohols even when the solution is heated to 60 to 70 C for 1 to 2 hours. The possibility of artifact production from the oxidation of hydroxyl groups is therefore negligible.

Chloroform is the solvent of choice for determination of the spectra of these derivatives. Ethyl alcohol was used in much of the early work on 2,4-DNP derivatives (17) because the effects of alkali on the spectra could also be determined. Many of the 2,4-DNP derivatives of ketosteroids are virtually insoluable in ethyl alcohol, however, while readily dissolving in chloroform. Erratic shifts are observed in the absorption maxima when changing from one solvent to the other, so that there is no reliable way to relate the spectrum obtained in one solvent to that obtained in the other (18). Although the absorption maxima for these compounds were determined in both solvents, the use of chloroform is recommended.

Recoveries of the steroid derivatives were determined by taking a known amount of the steroid, derivatizing it, and purifying it through the TLC systems. Recoveries averaged 96%, which is similar to recoveries obtained by other investigators using a somewhat similar technique (8). These high recoveries demonstrate that the loss of steroid occasioned by derivatization and analysis is quite low.

Correlations between Structure and Spectral Properties

There are three general types of 2,4-DNP derivatives formed from ovarian ketosteroids. Compounds with α,β-unsaturated carbonyl groups form red derivatives which have absorption maxima in chloroform at 390 nm. Compounds with "saturated" carbonyl groups form yellow 2,4-DNP derivatives which have absorption maxima in chloroform from 364 to 366 nm, regardless of whether the carbonyl group is located in the 3 –, 17 –, or 20-position. Compounds which have both groups form orange bis-2,4-DNP derivatives which have absorption maxima in chloroform in the range from 374 to 380 nm.

Further examples of these classes may be found in the literature. The wavelength of maximum absorption in chloroform for the α,β-saturated compounds cyclopentanone (1), cyclohexanone (1), androstenedione (8), and pregnane-3,20-dione (7) absorb light in the same region as the α,β-saturated compounds reported in this paper.

The 3-mono-2,4-DNP of progesterone, and the derivatives of 4-cholesten-3-one, 4-pregnene-11β,17α,20β,21-tetrol-3-one, 20,21-diacetate, and testosterone acetate (7) all have absorption maxima similar to the compounds prepared in this study.

The mixed compounds found in the literature show the same intermediate wavelength of maximum absorption in chloroform found in the compounds in this series. An interesting variant is the bis-2,4-DNP of 4,16-pregnadiene-3,20-dione (7). This compound has one normal α,β-unsaturated carbonyl group. The other α,β-unsaturated carbonyl group has the double bond attached to the carbon atom at the junction of the D ring and the C20,21-side chain. Presumably this arrangement hinders the full development of resonance with the 2,4-DNP molecule and thus accounts for the lowered absorption maximum.

The type of carbonyl group is the most important factor in determining the absorption maximum, and not the position of the group on the molecule. Thus, testosterone has an absorption maximum of 390 nm, while 5α-dihydrotestosterone has an absorption maximum at 364 nm — the absorption maximum of an α,β-saturated carbonyl compound.

Advantages of Derivatization with 2,4-DNP in Steroid Analysis

The paramount advantage of derivatization with 2,4-DNP in steroid analysis is that the equipment and reagents are inexpensive and relatively uncomplicated. The derivatives can be used both for identification and quantitative determination of the parent steroid. Since more than one steroid in a mixture can be determined by this technique, the "steroid profile" of tissues, venous effluents, or *in vitro* incubations can easily be determined. Moreover, "unexpected" steroids can be easily detected.

The use of the 2,4-DNP derivatization procedure also allows the determination of two Rf's — one for the free steroid and one for the derivative. Preparation and identification of two derivatives will usually suffice to identify a steroid by comparison with known standards. The 2,4-DNP derivatization process is well-suited to this purpose.

The absorption maximum of the 2,4-DNP derivative provides information about the position of the carbonyl group present on the steroid. Moreover, the color of the derivative can be a decided asset in handling these compounds. The other customary methods for visualizing steroids (iodine adsorption, ultra-violet light detection, sulfuric acid charring) are not as convenient and reliable, although the first two methods do not damage the compounds (22). Also, the sensitivity of the ultra-violet light detection method is marginal at steroid levels frequently encountered.

Since the derivatization procedure is essentially quantitative, and since the molecular weight of the 2,4-DNP derivative approximates that of the free steroid molecule, preparation of the 2,4-DNP derivative at least doubles the amount of material which is available for analysis. Another useful aspect of the use of 2,4-DNP derivatives involves the "blank problem." Thin-layer chromatographic techniques require the elution of steroids from the thin-layer plate, allowing the introduction of any impurities present on the plate. None of the interfering substances absorb light in the wavelength ranges involved in the 2,4-DNP derivatives of ketosteroids (360 to 390 nm).

The free steroid can be regenerated from the 2,4-DNP derivative (22), providing a sample of the free steroid for further study.

Limitations of Derivatization with 2,4-DNP in Steroid Analysis

2,4-Dinitrophenylhydrazine will form derivatives only with ketosteroids. However, the only non-ketonic steroids usually found in ovarian tissues are 5-androstene-3β, 17β-diol (androstenediol), and 1, 3, 5 (10)-estratriene-3, 16α, 17β-triol (estriol), and 1, 3, 5 (10)-estratriene-3, 17β-diol (estradiol). Very little androstenediol is formed under any circumstances in ovarian tissue (24), while other methods must be used for estrogen analysis because of the extremely low concentration of these compounds in biological tissues and fluids.

The derivatization procedure is somewhat time-consuming. One full day is required for the use of this technique if one begins the derivatization the night before. However, efficient planning of the experiment will keep this factor from becoming a problem.

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