colorant. The decomposition of FD&C Blue No. 2 appears to proceed by reduction to a semiquinone followed by oxidation.

3. There appears to be some evidence to indicate molecular changes in the lactose upon storage at high temperature. These changes seem to interfere with the spectra analysis in the ultraviolet range for colorants which absorb between 228 and 284 mμ.

As a result of this investigation, further studies are underway to thoroughly evaluate the thermal stability of lactose and lactose-and-colorants, both in aqueous solutions and in solid dosage forms. In addition studies are continuing to elucidate the degradation reaction for FD&C Blue No. 2.

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Drug Standards

Assay of Progesterone in Oil Injectables

By JACOB WOLFF

A method is presented for the quantitative determination of progesterone in oil solutions. The ketosteroid is isolated by partition chromatography using nitromethane on purified siliceous earth as the immobile phase and *n*-heptane as the eluent. The assay is based on the ultraviolet absorbance in ethanol solution at the maximum near 241 mμ. The identity of the separated progesterone is confirmed by determining the infrared spectrum in carbon disulfide solution.

The U.S.P. XVI (1) assay procedure for progesterone injection is a welcome simplification of the method in the previous edition (2). However, the assay is still based on the reaction with 2,4-dinitrophenylhydrazine, a general reagent for aldehydes and ketones. The melting point of the resulting hydrazone is rather high, 267–275°, and no other purity criteria are specified. Monty (3) reports that some chromatographically inhomogeneous 2,4-dinitrophenylhydrazones exhibit melting points no lower than the accepted values for the pure compounds. Umberger (4) used the color reaction with isonicotinic acid hydrazide for the determination of progesterone and testosterone. This reaction is more specific since it depends on the presence of a conjugated carbonyl linkage as in Δ^4-3-ketosteroids. Touchstone and Murawec (5) used spectrofluorometric techniques for the determination of progesterone in biological fluids. It appeared that a procedure based on the physical separation of progesterone from oil solutions, with its subsequent assay and identification, would be a more desirable approach than any of these.

Column chromatographic techniques have been successfully applied to similar problems.

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Carol (6) separated progesterone and testosterone using alcohol on purified siliceous earth as the immobile phase and iso-octane as the mobile solvent. Wotherspoon and Bedoukian (7) determined oxygenated compounds in essential oils by stripping from silica gel with ether after eluting the oils with pentane. Pesticides may be separated from fats and oils by the acetonitrile-hydrocarbon partition of Jones and Riddick (8) followed by chromatography on Florisil,[1] magnesium oxide, or purified siliceous earth. Umberger (4) obtained separation of progesterone or testosterone from oil, adequate for his colorimetric assay procedure, using Florisil and eluting with 2:1 iso-octane-chloroform. Tappi and co-workers (9) separated a number of steroidal hormones from each other and from olive oil using Florisil with successively more polar solvent mixtures. Snair and Schwinghamer (10) obtained a partial separation of estradiol dipropionate from oil using a column of siliceous earth and iso-octane thickened with polyethylene glycol 600, hexane being used as the mobile solvent. Monty (3) separated the 2,4-dinitrophenylhydrazones of aliphatic aldehydes and ketones on a nitromethane-Kieselguhr column with petroleum ether as the mobile solvent.

The proposed method was chosen after rather extensive experimentation with immiscible solvent systems and adsorbants. While many other techniques separated the main portion of the oil in the control samples, there was a steroid-like fraction which tended to remain with the progesterone throughout the various manipulations. Only the proposed procedure reduced the background absorbance to negligible proportions.

PROCEDURE

Materials.—Infrared spectrophotometer, a double beam Perkin-Elmer model 21; adsorbant, purified siliceous earth,[2] immobile solvent, nitromethane C.P.;[3] eluent, redistilled *n*-heptane; chromatographic column.

Insert a plug of purified cotton at the constriction of a chromatographic tube about 200 mm. long and about 25 mm. in internal diameter. Mix 8.0 ml. of nitromethane with 7.0 Gm. of adsorbant in a 150-ml. beaker until uniformly moistened. Transfer to the chromatographic tube in three approximately equal portions, tamping down with a packing rod after each addition. Arrange a long-stemmed, glass-stoppered, 250-ml. separator to serve as a reservoir for maintaining a constant level of liquid in the tube.

Standard Preparation.—Dissolve in alcohol 20.0 mg. of U.S.P. Progesterone Reference Standard, previously dried in vacuum over silica gel for 4 hours, dilute with ethanol to exactly 100 ml., and mix. Dilute 5.0 ml. of the solution with ethanol to make exactly 100 ml. and mix.

Assay Preparation.—Transfer, by means of accurately calibrated hypodermic syringe, 1.00 ml. of oil solution to a suitable volumetric flask so that the final solution will contain about 1 mg. of progesterone per ml. Dissolve in redistilled *n*-heptane, make to volume, and mix.

Assay.—Transfer 4.0 ml. of the assay preparation to the prepared chromatographic column. Pass 135 ml. of redistilled *n*-heptane through the column and discard the eluate.[4] Place a 200-ml. volumetric flask under the column as a receiver, add an additional 175 ml. of *n*-heptane to the column, and collect the entire eluate. Fill to the mark with *n*-heptane, and mix.

Transfer a 50-ml. aliquot to a 150-ml. beaker and save the remainder for the Identification Test. Evaporate to dryness under a gentle current of air on a steam bath. Remove the last traces of heptane by adding 1 ml. of methanol and redrying. Dissolve the progesterone by warming a few minutes in about 10 ml. of ethanol. Cool and dilute to exactly 100 ml. with ethanol.

Determine the absorbances of the sample and standard solutions relative to an alcohol blank at the peak near 241 mμ and at 300 mμ in a suitable spectrophotometer using matched 1-cm. cells. Calculate the quantity of progesterone in mg. in the aliquot taken from the formula $4\ A_u/A_s$, where A_u is the absorbance of the sample at 241 mμ minus the absorbance of the sample at 300 mμ, and A_s is the corresponding value for the standard.

Identification Test.—Evaporate the remainder of the eluate to dryness as discussed, starting with a 400-ml. beaker and transferring to a 5- or 10-ml. glass-stopper Erlenmeyer flask for the final evaporation. Dissolve the residue in 1.0 ml. of carbon disulfide, and determine the infrared absorption spectrum between 2 and 15 μ in a 1-mm. sodium chloride cell, using carbon disulfide in a matched cell as the blank. The resulting spectrogram should show all the significant absorption bands—and no others—that are found on a spectrogram similarly obtained with 3 mg. of U.S.P. Progesterone Reference Standard in 1.0 ml. of carbon disulfide.

DISCUSSION

In most chromatographic procedures, 1 ml. or less of immobile solvent is used per gram of adsorbant.

TABLE I.—ANALYSIS OF SYNTHETIC SOLUTIONS

Progesterone, mg./ml.	Adjuncts	% Recovered	
10.00	None	98.6	100.8
25.11	None	100.0	101.0
99.96	8% Benzyl alcohol 2% Ethanol	98.2	98.2

[1] Marketed by Floridin Co., Tallahassee, Fla.
[2] Acid-washed Celite 545, Johns-Manville Corp.
[3] Certified reagent grade from Fisher Scientific Co. was found to be satisfactory without further purification.

[4] The optimal volume of fore-run may vary by about 10 ml. with different batches of *n*-heptane or nitromethane. When new bottles of these reagents are used, determine the proper fore-run by the following procedure: Collect the last 25 ml. of the fore-run separately, and collect a tail cut of 25 ml. by passing through more eluant after collecting the main fraction in the 200-ml. flask. Evaporate these fractions as in the assay and make up to 25 ml. with ethanol. Determine the absorbance at 220, 241, and 300 mμ. An absorbance at 241 mμ exceeding that at 220 mμ indicates the presence of progesterone in the fraction involved, and the volume of the fore-run should be adjusted accordingly.

TABLE II.—ANALYSIS OF COMMERCIAL PROGESTERONE INJECTABLES

Sample	Declared mg./ml.	Adjuncts	Suggested Procedure % of Declared	Suggested Procedure Average	U.S.P. Assay % of Declared
1	100	8% Benzyl alcohol	86.2		
		2% Ethanol	87.0		
			86.8		
			87.6	86.9	90.6[a]
2	25	0.5% Chlorobutanol	95.0		
			93.8	94.4	96.8
3	10	0.1% Propylparaben	85.8		
			86.6	86.2	81.1
4	25	0.1% Propylparaben, in vegetable	80.3		
		oil	80.9	80.6	83.5
5	50	0.1% Propylparaben	86.5		
		20% Benzylbenzoate	87.5	87.0	88.9
6	25	0.5% Chlorobutanol	94.2		
			92.9		
			92.0	93.0	91.3
7	25	1:4000 Benzethonium octylacetate,	75.8		
		in peanut oil	75.4	75.6	75.3

[a] Determined m.p. at 260–264° (267–275° in U.S.P. XVI).

Here it was found that a relative excess of immobile solvent gave more consistent results. Evaporation to dryness and redissolving in alcohol are necessary to remove not only the heptane, but also the nitromethane which absorbs strongly in the ultraviolet region.

The progesterone recovered from oil injectables is usually slightly contaminated with ultraviolet absorbing substances. Consequently, the absorption spectra of samples and standards of equal concentration do not coincide exactly. While this difference may be appreciable at 220 mμ, it was never greater than 2% at 241 mμ. Prewashing the column with heptane did not reduce the background. Equilibrating the mobile and immobile solvents with each other increased the background, possibly by increasing the amount of nitromethane in the eluate. The interference, in any case, does not seem to affect the assay results. The absorbance of progesterone at 300 mμ is very low. On the samples, however, it ranged from 1–5% of the absorbance at the maximum around 241 mμ. It was found empirically on the samples of known concentration that subtracting this absorbance from that at the peak gave better results.

RESULTS

The recovery of progesterone from oil solutions was determined on solutions of known concentration. Weighed samples of U.S.P. Progesterone Reference Standard were dissolved by warming in 1 ml. of ethanol. About 4 ml. of sesame oil was added and the ethanol removed by heating on a steam bath for several hours. The solution was cooled and diluted to the desired volume. To check the reliability of the procedure under adverse conditions, the sesame oil used was from an old, somewhat oxidized sample. Duplicate determinations on three such samples are given in Table I and indicate that the recoveries are accurate within a 2% range.

In Table II, the results of the analysis of seven commercial preparations by the proposed method are compared with the results by the U.S.P. XVI (1) procedure. Except for samples 1 and 3, the results of the two procedures are in fair agreement. In sample 1, the difference may be explained in part by the impurities present in the hydrazone obtained in the U.S.P. procedure as indicated by the low melting point. Only two of the seven samples, 2 and 6, meet the requirements of the U.S.P. by either method of analysis.

The infrared absorption spectra of the progesterone recovered in the samples examined were indistinguishable from those of the reference standard.

SUMMARY

A method has been proposed for the assay of progesterone in oil solution, based on the isolation of the active ingredient by partition chromatography. The method is more specific than the current U.S.P. method and yields comparable results.

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