Quantitative separation and estimation of steroids by thin-layer chromatography

I. Determination of progesterone and testosterone propionate in oil solutions

Introduction

Within the last few years there has been a great increase in the use of thin-layer chromatography for the separation of steroids including progesterone and testosterone propionate. However, all these reports have been concerned mainly with the procedure as a qualitative rather than a quantitative technique.

The present communication describes a simple and accurate method for the estimation, on a micro scale, of progesterone and testosterone propionate in mixtures of the two compounds. We feel that the proposed method will contribute to simplicity and accuracy obtainable for quantitative thin-layer chromatography in assaying mixtures of steroids.

The elaborated method has been adapted to the determination of steroid mixtures in commercially available vegetable oil solutions.

Experimental

Materials. Reagents: The INH reagent was prepared by dissolving 0.4 g of isonicotinic hydrazide in methanol; 0.5 ml hydrochloric acid (37 %) was added and filled up to 100 ml with methanol. The reagent is set aside for 24 h before use. The solvents used were of p.a. purity grade.

Adsorbent: Fluorescent Kieselgel HF_{254}, E. Merck, Darmstadt.

Reference standards: Progesterone, Testosterone propionate, Organon, Oss (The Netherlands).

Apparatus. Thin-layer chromatography outfit with regulation thickness spreader, Desaga, Heidelberg.

Agla micrometer syringe, Burroughs Welcome & Co, London.

Ultraviolet lamp (254 mμ), Hanau.

Preparation of plates

Chromatoplates were prepared following the technique described by STAHL[1]. Plates 20 × 20 cm were coated (layer 0.5 mm thick) with a slurry prepared by mixing 35 g Kieselgel HF_{254} with 85 ml of water in a mortar with the aid of a pestle. A batch suffices for six plates. The plates were air dried for 10 min at room temperature and thereafter activated by heating at 130° for 4 h and stored in a desiccator until use.

Determinations were performed in duplicate against standards run on the same plate.

Procedure

3.0 ml of the oil solution (30 mg progesterone and 45 mg testosterone propionate) were diluted with 6.0 ml chloroform. Twice 20 μl of this solution and 20 μl of each corresponding standard solution were applied with the aid of a microsyringe on to a Kieselgel HF_{254} coated plate along the starting line as 2 cm horizontal lines. The chromatogram was run by the ascending technique with 100 ml of the solvent cyclohexane–ether (8:2, v/v), which had been poured into the chromatographic chamber

previously. When the solvent had reached a point within 1 cm from the upper edge of the plate (about 50 min) the plate was removed from the chamber, air dried for about 5 min and the separated steroids were located by means of low-wavelength

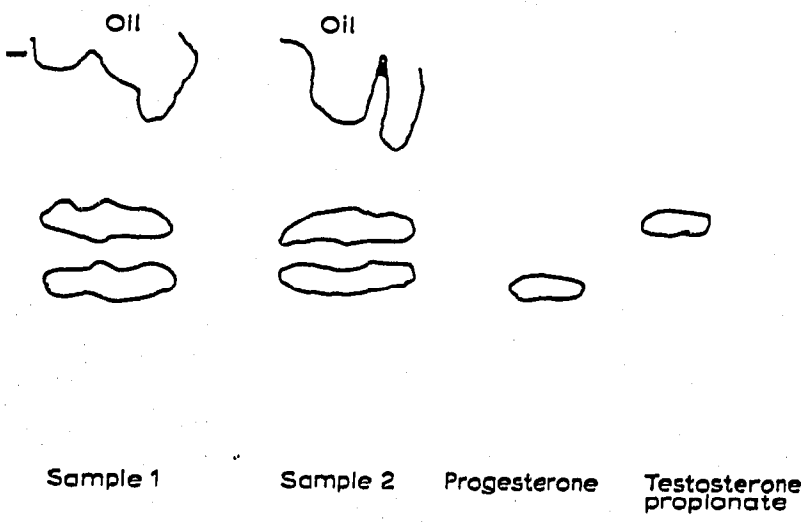

Fig. 1. Separation of steroids in oil solution.

ultraviolet light (Fig. 1). The oil migrated with the front and was quantitatively separated from the steroids. The U.V. absorbing zones were marked with an ample margin around the zone and quantitatively scraped off the plate each into a 50 ml glass stoppered flask. After the addition of 5 ml of methanol the flasks were well shaken for about 30 min to ensure complete extraction of the steroids and then centrifuged.

Progesterone

3.0 ml aliquots of each supernatant centrifuged progesterone solution of the samples and standard were pipetted into a 50 ml flask. After the addition of 4 ml INH reagent to each flask the solutions were allowed to stand for 1 h and the absorbancies determined at 380 mμ against a reagent blank.

$$\text{Per cent of progesterone in the sample} = \frac{E_{\text{sample solution}}}{E_{\text{standard solution}}}$$

where $E_{\text{sample solution}}$ = mean of extinction of sample 1 and sample 2.

Testosterone propionate

3.0 ml aliquots of each supernatant centrifuged testosterone solution of the samples and standard were pipetted into 50 ml glass stoppered flasks and the determination of testosterone propionate was carried out as described for progesterone.

Standard solution of progesterone

Dissolve 33.3 mg progesterone in chloroform and fill up to 10 ml with chloroform.

Standard solution of testosterone propionate

Dissolve 50.0 mg of testosterone propionate in chloroform and fill up to 10 ml with chloroform.

Results and discussion

The work reported here is the result of many trials with different adsorbents, solvent systems, and extraction, detection and estimation procedures.

For the sample application on the chromatoplate chloroform proved to be the most convenient solvent. The more polar solvents, such as methanol or ethanol, tended to leave the steroid as a ring rather than a zone. Warming up the chromatoplate while applying the solution in order to increase the rate of evaporation and thus speed up the solution application was found to be unnecessary.

The sensitivity of the method used for the quantification of steroids extracted from the adsorbent permits the determination of ketosteroids in quantities as small as 50 μg. This is important since steroids, because of their high physiological potency, are generally used in low concentrations (0.2–2 mg/ml). For this reason the very sensitive method suggested by UMBERGER[2] was the method of choice. This method is based on the rapid formation in acidified ethanol solution of isonicotinylhydrazones of Δ^4-3-ketosteroids having a double bond conjugated with a carbonyl group (ε = about 11.000).

To study the precision of the procedure six analyses were carried out with standard steroid vegetable oil solutions prepared in our laboratory and containing exactly known quantities of ketosteroids. As can be seen from Table I good reproducibility with a satisfactory standard deviation was obtained.

TABLE I

ANALYSIS OF STANDARD STEROID OIL SOLUTIONS

Analysis No.	*Progesterone 39.6 µg added*	*Testosterone propionate 60.0 µg added*
	µg found	*µg found*
1	41.65	60.0
2	38.72	58.08
3	38.21	60.47
4	39.48	59.77
5	37.39	57.51
6	39.05	58.93
Mean	39.08	59.12
Standard deviation P = 0.05	1.65	1.85

In Table II results of determinations with commercial samples are given. As can be seen the obtained results are in good agreement with the labelled amount of the respective steroid.

TABLE II

ANALYSIS OF COMMERCIAL STEROID OIL SOLUTIONS

Preparation No.	*Progesterone 10 mg labelled*	*Testosterone propionate 15 mg labelled*
	mg found	*mg found*
1	9.45	14.23
2	9.47	14.90
3	9.53	14.75

Work on the quantification of other steroid mixtures after thin-layer chromatographic separation is being continued in this laboratory.

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A sensitive non-specific localization test for thin-layer chromatography

During the chromatography of unknown mixtures considerable time and effort can be saved if the total number of components and their R_F values can be determined immediately.

This requires a spotting or localization agent capable of reacting with a large variety of compounds of different chemical structure.

Once the number and R_F values of all the constituents are determined, more specific localization and identification tests can be applied.

Among the spotting agents of wide applicability, the sulfuric acid test, the chlorine–*p*-toluidine test (ref. 1, Test No. 32) and the iodine vapor test (ref. 1, Test No. 72 and No. 73) are the most widely used.

The sulfuric acid test requires a highly corrosive spray and if used as a 50% aqueous solution as usually recommended, will often give a very non-uniform droplet size thus capable of damaging the thin layer of adsorbent.