Thin-Layer Chromatographic Method for the Determination of Flurandrenolone Acetonide and Some Closely Related Foreign Steroids

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Abstract □ A method is presented for the separation and determination of flurandrenolone acetonide (6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione-16,17-acetonide) and some closely related steroids. This is achieved by TLC on silica gel using a one-dimensional multiple development technique. The related foreign steroids are determined semiquantitatively while flurandrenolone acetonide is determined quantitatively by extracting its spot from the plate, and comparing it to a standard treated similarly.

Keyphrases □ Flurandrenolone acetonide, related steroids—separation, determination □ TLC—separation, identification □ Colorimetric analysis—spectrophotometer

Interest in this laboratory for the determination of steroid purity using TLC has made necessary the development of TLC solvent systems for the separation and quantitation of closely related steroids. A system for the separation of some estrogens (1) and another for the separation of a mixture of closely related 6-fluoro-16α-hydroxycorticosteroids (2) have been reported recently. These solvent systems proved to be very useful for the separation of related foreign steroids, intermediates, or decomposition products from the therapeutically active steroid.

This paper describes a TLC method for the semiquantitation of the related foreign steroids that may possibly exist, or the steroids which may be formed under artificial decomposition of flurandrenolone acetonide. These related foreign steroids were arbitrarily chosen after examining the different methods of flurandenolone acetonide syntheses (3, 4) and it was concluded that their presence was a possibility. Also included are some of the products resulting from severe hydrolytic deacetonation (5) and chromic acid oxidation (6) of flurandrenolone acetonide. The closely related foreign steroids were determined semiquantitatively by visualizing the developed plate under short wavelength UV light and comparing their intensities to known amounts, while flurandrenolone acetonide was determined quantitatively by extracting the silica gel, color development with tetrazolium blue reagent, and measuring the absorbance at 520 mμ.

EXPERIMENTAL

Reagents—All chemicals, if not otherwise mentioned, were of the highest grade commercially available.

Solvent System—Benzene–ethylacetate (1:1) (2).

Flurandrenolone Acetonide Reference Standard Solution—A solution containing exactly 10 mg. of flurandrenolone acetonide per milliliter of chloroform–methanol (1:1).

Related Foreign Steroids Solution—A mixture containing 1 mg. of each of the following steroids[1]/5 ml. of chloroform–methanol (1:1): (*a*) 6α-fluoro-16α,17α,21-trihydroxy-pregn-4-ene,3,11,20-trione-16,17-acetonide; (*b*) 6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione-16,17-acetonide-21-acetate; (*c*) 6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione-16,17-acetonide-11-acetate; (*d*) 6α-fluoro-11β,16α,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione (flurandrenolone).

Specially treated ethanol, tetrazolium blue reagent, and tetramethylammonium hydroxide solution were prepared according to the method of Jakovljevic (7).

METHOD

The TLC plate[2] (20 × 20 cm.) was activated before use by heating at 110° for 10–15 min. The developing chamber was lined with blotting paper and allowed to stand with the solvent for 30 min. before use.

Ten milligrams of the sample was weighed accurately and dissolved in 1 ml. of chloroform–methanol (1:1). The plate was divided into seven equal sections and the following amounts were applied 2.5 cm. from the bottom edge of the plate using micropipets.[3]

Section 1, 5 μl. of the related foreign steroids solution; Section 2, 10 μl. of the ample solution; Section 3, 10 μl. of flurandrenolone acetonide standard solution; Section 4, was used as the plate blank; Section 5, 15 μl. of the related foreign steroids solution; Section 6, 10 μl. of the sample solution; Section 7, 10 μl. of flurandrenolone acetonide standard solution.

The plate was developed in the chamber allowing the solvent front to travel 15 cm. after passing through the point of application. It was then removed from the chamber and the solvent allowed to evaporate for about 3 min. Developing and evaporation of the solvent were done at room temperature (25°). The plate was redeveloped twice more in the same direction as the first time. After the third removal of the solvent the spots were detected using a short wavelength UV light,[4] and marked.

Evaluation of the Plate—In order to determine the percentage of related foreign steroids in flurandrenolone acetonide, each extra spot, other than the main compound, appearing in the sample sections was compared with the spot having the same mobility (R_f value) in the sections containing the related foreign steroids. Sections 1 and 5, equivalent to 1 and 3% of related foreign steroids, respectively, were used for the semiquantitation of possible impurities.

For the quantitative determination of flurandrenolone acetonide, the marked areas of the main spot in Sections 2 and 6 representing the sample, and Sections 3 and 7 representing the standard, as well as an equivalent area from the blank, Section 4, were removed and quantitatively transferred to separate glass-stoppered centrifuge tubes. Ten milliliters of absolute ethanol were added to each tube followed by vigorous shaking for 3 min. using a Vortex mixer.[5] The tubes were centrifuged for about 10 min. at 2000 r.pm. until the supernatant was clear. A 4-ml. aliquot from each tube (equivalent to 40 mcg. of flurandrenolone acetonide) was pipeted into separate 10-ml. volumetric flasks. The contents of all flasks were evaporated to dryness using mild heat and an air stream, then the color was developed with the tetrazolium blue reagent (7). The plate blank absorbance was subtracted from both the sample and the standard absorbances, and the corrected values were used for the calculation.

[1] The steroids used were obtained from Syntex Corp., Palo Alto, Calif.

[2] Precoated 250-μ thin-layer plates (Silica Gel F_{254}) supplied by Brinkmann Instruments, Inc., Westbury, N. Y.

[3] Micropipets, Microcaps, Drummond Scientific Co., Broomall, Pa.

[4] Chromato-Vue equipped with a short wavelength lamp (about 254 mμ), Ultraviolet Products, Inc., Calif.

[5] Vortex Jr. Mixer, Scientific Industries, Inc., Queens Village, N. Y.

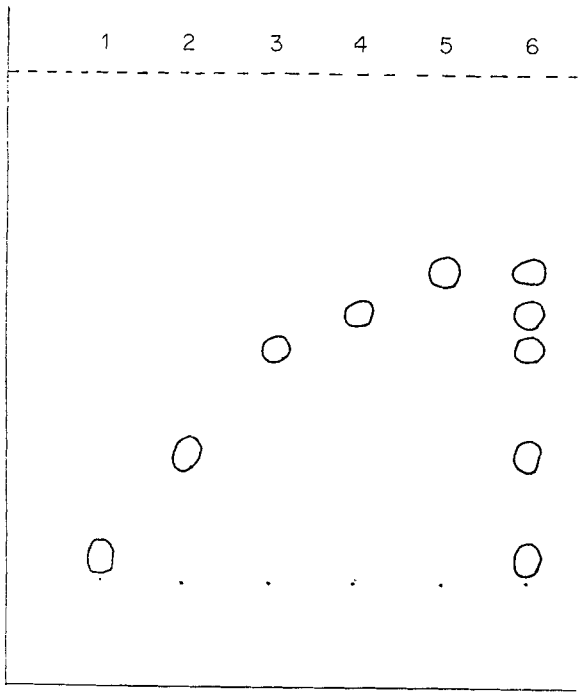

Figure 1—*1. Flurandrenolone; 2. flurandrenolone acetonide; 3. 6α - fluoro - 11β,16α,17α,21 - tetrahydroxy-pregn - 4 - ene - 3,20-dione - 16,17 - acetonide - 11 - acetate; 4. 6α - fluoro - 16α,17α,21-trihydroxy - pregn - 4 - ene - 3,11,20 - trione - 16,17 - acetonide; 5. 6α - fluoro - 11β,16α,17α,21 - tetrahydroxy - pregn - 4 - ene - 3,20-dione - 16,17 - acetonide - 21 - acetate; 6. mixture. Each spot represents 2 mcg.*

DISCUSSION

In recent years, TLC has become recognized as a valuable method for the separation and identification of closely related compounds. Many solvent systems for the TLC of steroids (1, 2, 8, 9) have been reported for the separation of closely related steroids and the resolution (2, 10) of α- and β- steroid pairs.

The related foreign steroids test (11–13) is now becoming one of the most important tests of steroidal drugs. This test uses TLC as a tool to detect any structurally related compounds that may exist in the therapeutically active steroid.

The method described here has been used satisfactorily in this laboratory for the semiquantitative determination of the related foreign steroids that may be present in flurandrenolone acetonide. It was also useful in stability studies where flurandrenolone was detected after severe hydrolytic deacetonation (5) of flurandrenolone acetonide. This reaction was found (7, 14) to occur in some formulations containing flurandrenolone acetonide that were aged by accelerated conditions.

The oxdiation product formed upon exposing flurandrenolone acetonide to chromic acid (6) was isolated and identified by IR, TLC, and melting point, and found to be 6α-fluoro- 6α,17α,21-trihydroxy-pregn-4-ene-3,11,20-trione-16,17-acetonide. This compound separated by the TLC solvent system used in this method, was incorporated in the related foreign steroids mixture.

Although several solvent systems have been reported for the TLC of corticosteroids, it was found that none of them could resolve the mixture used in this work. The development of a new TLC system (2) in this laboratory made it possible to have complete resolution of this particular mixture. This was achieved after developing the plate three times in the same direction. Figure 1 shows the thin-layer chromatogram of the separated steroids individually and as a mixture. Their R_f values were reported previously (2). To overcome any unevenly distributed plate layers, the sample and the standard were applied in duplicates. It was found that the results were reproducible.

Table I—Variability Data[a]

Average Absorbance of the Duplicate Samples	Plate Blank Absorbance	Corrected Absorbance
0.290	0.010	0.280
0.299	0.005	0.294
0.290	0.010	0.280
0.315	0.010	0.305
0.310	0.008	0.302
0.307	0.012	0.295
0.294	0.014	0.280
0.320	0.012	0.308

[a] Numbers represent spectrophotometric readings at 520 mμ. Average $\bar{x}$ = 0.293; range R = 0.028; variance S^2 = 0.00013742; SD = 0.01172262; RSD = ±4.000894.

The order of the plate sections makes it possible to have the sample close to two levels of the related foreign steroids; namely 1 and 3%, thus facilitating the visual semiquantitation under short wavelength UV light.

For the quantitative elution of flurandrenolone acetonide from the scraped silica gel, absolute ethanol was used instead of 95% ethanol since a clearer separation was obtained.

In order to evaluate the precision of this method, 100 mcg. of a flurandrenolone acetonide standard material was spotted in duplicate on eight TLC plates, which were then developed, eluted, and assayed as mentioned. It was found that the plate blank absorbances vary from 1.67–4.76% of the sample absorbances. The quantitative determination of flurandrenolone acetonide by this method showed (Table I) a relative SD of ±4.0% for eight independent assays.

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