Stability Method for Determination of Flurandrenolone in Pharmaceutical Formulations

By IVAN M. JAKOVLJEVIC, PAUL E. HARTSAW, and GEORGE E. DRUMMOND

A method is proposed which combines Florisil column chromatography, and tetrazolium blue reagent. The method describes a solvent system which eliminates the interference of more polar decomposition products such as flurandrenolone desacetonide and possible D-homo isomerization derivatives.

THE COMPLEXITY of today's formulations such as creams, lotions, and ointments makes the assay of flurandrenolone (6α-fluoro-16α-hydroxyhydrocortisone-16, 17α-acetonide) very difficult. If the formulation contains certain other ingredients, such as antibiotics, antifungal agents (iodochlorhydroxyquin), or coal tar, the assay is even more difficult since some of these components often exceed the flurandrenolone content by several hundredfold.

The isolated flurandrenolone from the formulations was assayed by the tetrazolium blue method using specially prepared reagents, the use of which results in a highly reproducible assay.

EXPERIMENTAL

Apparatus

Beckman model DU spectrophotometer with 1-cm. cells.

Reagents

Ethanol, Absolute.—To each liter of ethanol absolute were added 35 mg. of tetrazolium-blue reagent and 5 ml. of a saturated alcoholic potassium hydroxide solution. The mixture was allowed to stand several hours and was then distilled, rejecting the first and last 10% of the distillate.

Chloroform, A.R.—The ethanol contained as a preservative was washed out completely with water, then dried with anhydrous sodium sulfate, filtered, and an exact amount of ethanol absolute was added to make 0.5%. This was necessary because of variable amounts of ethanol found in commercially available chloroform. Close control of the ethanol content of chloroform was necessary, since flurandrenolone is partially eluted from the column when the ethanol content is increased to about 1% or more.

Use of the specially treated solvents described above is required whenever ethanol or chloroform are mentioned in the procedure.

Ethanol, 5%.—Five milliliters of ethanol absolute was pipeted into a 100-ml. volumetric flask and diluted to the mark with chloroform.

Acetic Acid, Glacial, A.R.

Tetramethylammonium Hydroxide (TMAH Solution).—Ten milliliters of a commercial 10% aqueous TMAH was pipeted into a 50-ml. volumetric flask and diluted to the mark with ethanol.

Received July 23, 1965, from the Analytical Development Laboratory, Eli Lilly and Co., Indianapolis, Ind.

Accepted for publication September 15, 1965.

The authors acknowledge the assistance of Mr. Norbert R. Kuzal in reviewing this paper.

Tetrazolium Blue Recrystallized, Fisher Certified Reagent.

Color Reagent.—In a 50-ml. volumetric flask, 75 mg. of tetrazolium blue recrystallized was dissolved in 90% ethanol (ethanol absolute–water, 9:1, v/v), and the volume made up to the mark with the same solvent.

Florisil, "Floridine," 60–100 Mesh.—The Florisil was washed with several portions of hot hydrochloric acid. The washing was continued with room-temperature acid until all yellow color was removed. The hydrochloric acid was removed by washing with water, then the washing was continued with ethanol and last with chloroform. The washed Florisil was spread in a thin layer and dried at 105–110° for 6 hr.

Glass Wool.—The glass wool was also washed with solvents in the following order: hydrochloric acid, water, ethanol, and chloroform.

Standard Solution.—Ten milligrams of a flurandrenolone standard was weighed on a micro-balance, transferred into a 100-ml. volumetric flask, and diluted to volume with chloroform.

It was found that the purity of solvents as well as specially cleaned glassware was very important since the final solution in which the formazan will be formed contains only 4 mcg./ml. or less of flurandrenolone.

Chromatographic Columns.—Chromatographic tubes 1.1–1.3 cm. i.d. and about 30 cm. long were used. The tubes were fitted with Teflon stopcocks. A slurry of 4.0 Gm. of Florisil in ethanol was added into the column. When the Florisil was settled, enough chloroform was added to wash out the ethanol completely.

METHOD

The following procedure has the advantage of being the same for creams, lotions, and ointments if the content of flurandrenolone is in the usual concentration of about 0.05%.

Sample.—A 2-Gm. sample, equivalent to 1 mg. of flurandrenolone, was weighed into a 50-ml. glass-stoppered conical flask; 30 ml. of chloroform was added by a graduated cylinder and stirred on a magnetic stirrer for 10 min. The mixture was filtered into a 50-ml. volumetric flask through a funnel 5 cm. in diameter fitted with Whatman No. 1 filter paper containing 15 Gm. of anhydrous sodium sulfate. The filter paper and sodium sulfate were washed with small portions of chloroform until the volume reached 50 ml.

Standard.—Ten milliliters of the standard solution, equivalent to 1 mg. of flurandrenolone was pipeted into a 50-ml. glass stoppered conical flask.

With a graduated cylinder, 20 ml. of chloroform was added and the solution was treated as described under *Sample*, beginning with: "The mixture was filtered . . ." Because a loss of about 1% was found in the filtration process, the standard was treated the same way as the sample.

Chromatography.—From the 50-ml. filtrates of both sample and standard, 10-ml. aliquots (equivalent to 200 mcg. of flurandrenolone) were pipeted onto prepared columns. The flow rate was adjusted to about 50 drops/min.

The flurandrenolone solution was allowed to drain into the column. The column was then washed with 35 ml. of chloroform. The elution of flurandrenolone was achieved with 5% ethanol in chloroform and the eluate was collected in a 25-ml. volumetric flask, filling the flask to the mark with the eluate.

Color Development.—The contents of both the standard and sample eluates were well-mixed, and a 5-ml. aliquot from each flask (equivalent to 40 mcg. of flurandrenolone) was pipeted into separate 10-ml. volumetric flasks.

For a reagent blank, 5 ml. of ethanol was pipeted into a third 10-ml. volumetric flask, and the contents of all three flasks were evaporated to dryness using mild heat and an air stream.

To each of the three flasks, 5 ml. of ethanol was pipeted to redissolve the residue. (The 5-ml. aliquot from the 25-ml. eluate was first evaporated and then redissolved in 5 ml. of ethanol, because the ethanol–chloroform ratio in the eluates would vary from column to column due to the chloroform prewash. It was found that tetrazolium blue has different rates of reaction in different ethanol–chloroform mixtures.)

One milliliter of the color reagent was pipeted into each flask, and the flasks were then placed in a 40 ± 1° water bath. After allowing 5 min. for the solutions to come to equilibrium temperature, 1 ml. of TMAH solution was pipeted into each flask at 1-min. intervals (flasks remaining in bath). The flasks were stoppered with glass stoppers and occasionally swirled. Exactly 10 min. after TMAH solution had been introduced, the flasks were removed from the bath in consecutive order. Each flask was allowed to stand exactly 5 min. at room temperature, at which time the contents were diluted to the mark with glacial acetic acid.

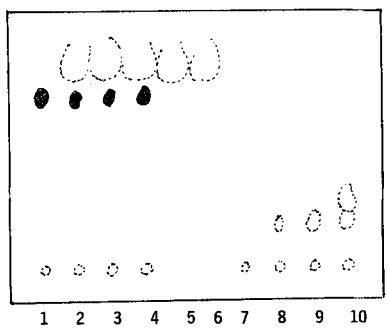


Fig. 1.—Key: *1*, flurandrenolone standard; *2*, cream, 100%; *3*, cream, 80%; *4*, cream, 70%; *5*, placebo cream, fresh; *6*, placebo cream, aged. (All 5% ethanol eluates.) Key: *7*, flurandrenolone standard; *8*, cream, 100%; *9*, cream, 80%; *10*, cream, 70%. (Ethanol absolute eluates.) The cross-hatched spots represent flurandrenolone.

Thin-Layer Chromatography.—Two flurandrenolone creams, after being deteriorated experimentally at prolonged elevated temperatures, were assayed by the described procedure. They were found to contain 80 and 70% of the labeled amount of flurandrenolone. These creams were chromatographed by the thin-layer chromatography technique using an undeteriorated cream assaying 100% as a control. The samples were treated as described under *sample*, then 15 ml. out of the 25-ml. eluates were evaporated to dryness. To be able to detect certain more polar components such as tetrahydroxy flurandrenolone (II) and its possible D-homo isomerization products (III), the columns were eluted, after the regular 5% ethanol also with absolute ethanol evaporating the eluates to dryness.

The residues from the 5% ethanol elution were dissolved in 0.1 ml. of chloroform. The residues from the absolute ethanol elution were dissolved in 0.1 ml. of a 1:1 chloroform–ethanol mixture. Both sets of residues were then spotted on Silica Gel G–F plates for thin-layer chromatography.

Multiple-stage development was applied. The chromatogram was first developed in ethyl ether, then in a chloroform–ethanol mixture (9:1, v/v). The plate was observed under a low wavelength ultraviolet light, and the areas of quenching marked. After that, the plate was sprayed with the tetrazolium-blue reagent. The results of the thin-layer chromatography are shown in Fig. 1.

The creams which assayed 80 and 70% had a spot of more polar compound that was detected by U.V. light and was also slightly reactive to the tetrazolium blue reagent. The 70% cream showed more of this less mobile spot than the 80% cream, while 100% showed none. The point of application exhibited a slight amount of material even in the case of the standard.

Ethanol eluates, as expected, showed small amounts of more polar material with the highest concentration being found in the 70% cream. This more polar material was also slightly reactive to the tetrazolium blue reagent.

The thin-layer chromatography of a fresh cream, as well as placebo cream, did not show any of the above-mentioned spots. An aged placebo cream appeared the same as the fresh placebo cream.

DISCUSSION

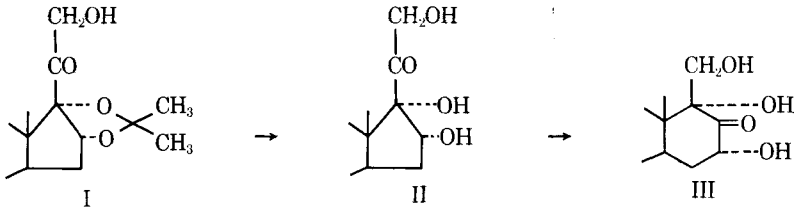
Formulations with a Lower Concentration of Flurandrenolone.—The creams and lotions with 0.02% or less of flurandrenolone contain too much water to be assayed by the proposed method. For these cases a pre-extraction step is necessary. An amount of such a formulation, equivalent to 1 mg. of flurandrenolone, but not more than 10 Gm., was weighed into a 125-ml. separator. With a graduate, 25 ml. of water and 25 ml. of chloroform were added to the sample and extracted. The chloroform extract was filtered through 5 Gm. of anhydrous sodium sulfate in a 50-ml. volumetric flask as in the regular procedure. The water layer was extracted with two additional 10-ml. portions of chloroform and the chloroform extracts filtered through the same filter. The extracts were diluted to 50 ml. and were chromatographed as in the procedure.

Ointments of lower concentration were first extracted with ethanol to remove the major part of

TABLE I.—VARIABILITY DATA[a]

Date	7/6/64		7/7/64		7/8/64		7/9/64		7/10/64		7/13/64	
	Std.	Sa.	Std.	Sa.	Std.	Sa.	Std.	Sa.	Std.	Sa.	Std.	Sa.
Analyst *A*	0.360	0.360	0.358	0.357	0.355	0.358	0.361	0.360	0.366	0.359	0.359	0.356
Analyst *B*	0.352	0.355	0.356	0.359	0.355	0.359	0.364	0.366	0.359	0.357	—	—
Analyst *C*	0.358	0.356	—	—	0.362	0.357	0.355	0.357	—	—	0.358	0.356

[a] Numbers represent photometer readings.



I II III

Scheme I

the vehicle. Chloroform could not be used since it dissolves all ingredients and a high blank results. The ethanol extract was diluted with water and re-extracted quantitatively with chloroform. The addition of 1 ml. of *0.5 N* hydrochloric acid in the chloroform extraction prevents emulsion formation.

Cream formulations containing coal tar were first extracted with a mixture of 85% methanol and *n*-heptane with the *n*-heptane removing most of the coal tar. The methanol phase was diluted with water and re-extracted quantitatively with chloroform. Iodochlorhydroxyquin, added to some formulations as an antifungal agent, does not interfere even if badly decomposed by aging: a part of it was removed by the column procedure as a wash, and the other part was retained by the Florisil.

Reuse of Columns.—The column for the standard solution was used each working day for 2 wk. It was necessary to wash the column immediately after each assay with 30 ml. of ethanol followed by 30 ml. of chloroform.

The columns used for the samples of creams and ointments were reusable for 6–8 days if washed as above but with 50 ml. of each solvent. The columns used for the formulations containing iodochlorhydroxyquin and coal tar were discarded after being used once.

Color Reagent.—Contrary to several literature reports, it was found that the color reagent can be stored for a period of 1 month if refrigerated when not in use.

Color Stability.—Although it is known that formazans are not stable in acid solutions (1), it was found that 3 ml. of glacial acetic acid when added as in the described procedure stabilizes the formed formazan for about 12 hr. During this time a plot of absorbances *versus* time exhibits a horizontal line.

Precision and Accuracy.—For this study a cream base has been used to which an exact amount of flurandrenolone standard was added. The study included three analysts and several days. The precision was found to be ±1.56% for two standard deviations, and the recovery 99% (Table I).

Stability Study.—This study included creams, lotions, and ointments aged by accelerated conditions. The first decomposition product, the formation of more polar flurandrenolone desacetonide (II), could not be eluted even with a 5% ethanol in chloroform mixture. This fact is important since even traces of this compound would significantly increase the results of pure flurandrenolone (I), because flurandrenolone desacetonide has a much higher response to the tetrazolium blue reagent. (Scheme I.) The products caused by further changes, the eventual isomerization of desacetonide flurandrenolone, and the possible formation of D-homo derivatives (III) similar to triamcinolone (2), which are also more polar than flurandrenolone, being tetrahydroxy compounds, could not be eluted from the column by the proposed method.

Comer and Hartsaw (3) have shown by radioisotope thin-layer chromatography that an extraction procedure in assaying cream flurandrenolone after storage at elevated temperature gives results that are somewhat higher compared to radioisotope thin-layer chromatography.

Flurandrenolone was found to be stable in all mentioned formulations, and decomposed only after prolonged intervals at elevated temperatures.

REFERENCES

(1) Lau, P. T. S., *Org. Chem. Bull.*, **36** (3), 1(1964).
(2) Smith, L. L., *et al.*, *J. Am. Chem. Soc.*, **82**, 4616(1960).
(3) Comer, J. P., and Hartsaw, P. E., *J. Pharm. Sci.*, **54**, 524(1965).