Qualitative Color Test for Identification and Differentiation of Some Fluorinated Steroids

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A qualitative colorimetric test has been developed for the identification and differentiation of fludrocortisone acetate, triamcinolone, and triamcinolone acetonide in pharmaceutical formulations. The test is based on a newly discovered reaction for fluorinated steroids and depends upon the formation of an amber color from fludrocortisone acetate, a yellow chromogen from hydrolyzed triamcinolone, and a pink color from triamcinolone acetonide by reaction of phenol, hydroquinone, and the phosphoric-sulfuric acid mixture.

MANY color reactions of steroids are used in qualitative tests to demonstrate the presence of these various steroids in pharmaceutical preparations. For example, *m*-dinitrobenzene-potassium hydroxide (1) and 2,6-di-*tert*-butyl-*p*-cresol (2) are useful identity tests for various fluorinated steroids.

This investigation is a study of the newly discovered color reaction for fluorinated steroids with phenol, hydroquinone, and the phosphoric-sulfuric acid mixture as a specific method of identification and differentiation of fludrocortisone acetate, triamcinolone, and triamcinolone acetonide in pharmaceutical formulations.

EXPERIMENTAL

Method

Reagents and Equipment.—Sulfuric-phosphoric acid solution. To 850 ml. of concentrated sulfuric acid, 150 ml. of 85% phosphoric acid is added and mixed.

Phenol liquid, 85%, reagent grade.

Tetramethylammonium hydroxide solution, 10 ml. of 10% aqueous reagent is diluted to 100 ml. with ethanol.

Methylene chloride (dichloromethane), spectrophotometric grade.

Hydroquinone, purified.

Florisil, 60/100 mesh.

Purification column. The column consists of a glass tube of 10 mm. inside diameter and 150 mm. in length with a constricted end to retain the adsorbent. The column is packed with Florisil to the height of 40 mm. and is washed with 10 ml. of methylene chloride prior to use.

Test tubes, screw cap, 25 × 150 mm.

Unless specified, all chemicals are reagent grade.

Procedure

Sample Preparations.—*Creams, Lotions, and Ointments.*—A sample equivalent to 2 mg. of steroid is weighed into a test tube. Methylene chloride 20 ml., is added and the tube is covered with a plastic cap. The steroid is extracted by shaking for 2 minutes. Lotion samples are centrifuged at 1500 r.p.m. for 3 minutes. The upper, aqueous layer is discarded by means of an aspirator. Cream and ointment samples are filtered through No. 3 filter paper.

The methylene chloride solution is poured into a purification column. When the flow rate is slower than 75 drops per minute, it can be increased by the air pressure bulb fitted at the top of the column. The column is washed with 3 × 10 ml. of methylene chloride. The solvent is forced out by means of the rubber pressure bulb and the steroid is eluted with 10 ml. of methanol.

Suspensions and Sprays.—A sample is diluted with methanol to give the concentration of about 200 mcg. of steroid/ml.

Tablets.—A powdered sample equivalent to 4 mg. of triamcinolone or 1 mg. of 9α-fluorohydrocortisone is weighed into a test tube. Ethyl acetate, 10 ml. is added and the tube is covered with the plastic cap. Steroids are extracted by shaking for 3 minutes. The sample is filtered through No. 3 filter paper.

Color Development.—Aliquots of the sample extracts are placed in three test tubes designated A, B, and C. A 2-ml. portion of the methanolic sample or 1 ml. of the ethyl acetate tablet extract are the sample aliquots used. Glacial acetic acid (1 ml.) is added to tube A and the mixture is heated in a boiling water bath for 1 minute. Alcoholic tetramethylammonium hydroxide (1 ml.) is added to tube B and the tube is incubated at 50° for 15 minutes.

To all three tubes, 0.5 ml. of phenol, about 50 mg. of hydroquinone, and 5 ml. of sulfuric-phosphoric acid are added. The test tubes are mixed and heated in a boiling water bath for about 1 minute to speed up the color development.

A pink color in tubes A and C and yellow chromogens in tube B prove the presence of triamcinolone.

A pink color in all tubes indicates the presence of triamcinolone acetonide.

An amber color in tube A and orange-red color in tubes B and C indicate the presence of fludrocortisone acetate in a sample.

RESULTS AND DISCUSSION

The results summarized in Table I indicate that this method is useful for the identification and differentiation of fludrocortisone acetate, triamcinolone, and triamcinolone acetonide in a variety of formulated products.

For good color development, the steroid tested has to be free from interfering substances. This is accomplished by centrifugation or filtration to remove insoluble matter and, if necessary, absorption on a Florisil column, followed by elution of the steroid fraction.

Methylene chloride was the solvent selected for the extraction of creams, lotions, and ointments because it appeared to be better than other solvents tested, both from the standpoint of extraction efficiency and the removal of impurities from the Florisil column. The steroids were eluted from the

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TABLE I.—QUALITATIVE COLOR TEST OF STEROIDS

Name of Product	Type of Formulation	Developed Color[a] (Samples) A	B	C
Fludrocortisone acetate	Lotion	A	OR	OR
Fludrocortisone acetate, neomycin, gramicidin	Lotion	A	OR	OR
Triamcinolone acetonide	Lotion	P	P	P
Triamcinolone acetonide, neomycin, gramicidin	Lotion	P	P	P
Fludrocortisone acetate	Ointment	A	OR	OR
Fludrocortisone acetate, neomycin, gramicidin	Ointment	A	OR	OR
Fludrocortisone hemisuccinate, neomycin, gramicidin	Ointment	A	OR	OR
Triamcinolone acetonide	Ointment	P	P	P
Triamcinolone acetonide, neomycin, gramicidin	Ointment	P	P	P
Fludrocortisone acetate, neomycin, gramicidin	Cream	A	OR	OR
Triamcinolone acetonide	Cream	P	P	P
Triamcinolone acetonide, neomycin, gramicidin	Cream	P	P	P
Fludrocortisone hemisuccinate, neomycin, gramicidin	Suspension	A	OR	OR
Triamcinolone acetonide	Suspension	P	P	P
Triamcinolone acetonide	Spray	P	P	P
Fludrocortisone acetate	Tablet	A	OR	OR
Triamcinolone	Tablet	P	Y	P

[a] A, Amber; OR, orange-red; P, pink; Y, yellow.

column with methanol, but acetone or ether could also be used for this purpose. Dimethylformamide, an excellent solvent for the elution of steroids from Florisil columns, could not be used because it gives a yellow color with the steroids in the presence of tetramethylammonium hydroxide and sulfuric acid. Ethyl acetate was used for tablet extractions because no further purification of these extracts were required.

Either Florisil or aluminum oxide may be used as an adsorbent for fluorinated steroids. Florisil has been chosen in this method because of the easy removal of interfering substances from creams, lotions, and ointment formulations.

Triamcinolone and triamcinolone acetonide produce the same pink color in the presence of phenol, hydroquinone, and sulfuric-phosphoric acid. However, if a triamcinolone sample is heated in the presence of the alkaline solution at 50° for 15 minutes prior to the addition of reagents, yellow chromogens are obtained because triamcinolone is unstable in alkaline solutions at elevated temperatures. Experiments have shown that extensive degradation occurs at the temperature of 60° and above. Triamcinolone acetonide is slightly affected at 60° in the presence of tetramethylammonium hydroxide. Tetramethylammonium hydroxide has been chosen for the degradation purpose because it produces no heat after the addition of a sulfuric-phosphoric acid mixture. Heating fludrocortisone acetate at 100° in the presence of glacial acetic acid for 1 minute converts it to the free alcohol. Triamcinolone and triamcinolone acetonide are not affected when heated in the acid solution.

The color test was applied to various other steroids which are also used in pharmaceutical preparations. The results of these tests are given in Table II.

TABLE II.—COLOR PRODUCED WITH PHENOL, HYDROQUINONE, AND SULFURIC-PHOSPHORIC ACID

Name of Steroid	Developed Color
Cortisone acetate	Amber
Fluoxymesterone	Orange to red
Hydrocortisone	Orange to red
Prednisolone	Red to orange

The presence of both phenol and hydroquinone in the reaction mixture is very important. Either phenol or hydroquinone produces the same color, but a combination of both chemicals gives about twice the color intensity. About 50 mg. of hydroquinone and 0.5 ml. of phenol proved to be the most suitable for the color development. Sulfuric acid of at least 40% strength is needed for the reaction, but the 85% mixture gives the best results. An increase in the concentration of sulfuric acid speeds up the reaction, but concentrations above 85% give lower color intensities. Phosphoric acid is used for dilution purposes to keep the water content in the reaction mixture to a minimum.

The temperature is an important factor in the color development. An increase in temperature accelerates the degree of reaction, but considerable quantities of chromogens are destroyed when the reaction mixture is heated at high temperatures. Since the color development is more rapid at 100° this temperature has been chosen for completing the test in a minimum of time.

Although relatively large samples of steroids (2 to 4 mg.) have been used for analysis, it has been possible to detect as little as 10 γ/ml. of steroid in the final solution by this method.

REFERENCES

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