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Thin-Layer Chromatographic Stability Assay for C^{14}-Labeled Steroid in a Cream

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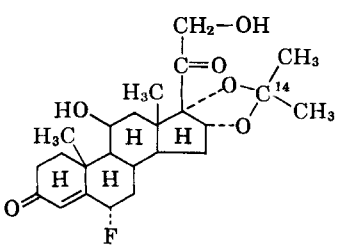
The acetonide of 6α-fluoro-16α-hydroxyhydrocortisone, prepared from C^{14}-labeled acetone, was formulated as a cream. Initial assays were made by a conventional tetrazolium method and also by a thin-layer chromatographic separation and subsequent scintillation measurement of the C^{14}-labeled steroid. The cream samples were aged under accelerated conditions, and subsequent assays by the two methods were compared to the initial results. Small assay interferences caused by the aging of the formulation were demonstrated on the tetrazolium method. The techniques used illustrated advantages of the thin-layer chromatographic scintillation method for the early evaluation of a proposed stability method for a cream formulation.

THIN-LAYER chromatographic techniques have been useful for the quantitative steroid assay as drug substances (1) and in stability assay of aqueous solutions (2). Common measurements of the eluted steroids are by ultraviolet spectrophotometric or tetrazolium colorimetric procedures. Formulation and stability studies of cream preparations showed that placebo preparations, on accelerated aging, sometimes gave positive interference by conventional techniques. It was noted also that in some preparations aging increased binding of the steroids, with a resultant loss of steroid during extraction.

The method reported here was designed to evaluate a proposed conventional stability method to determine whether the errors mentioned would be significant on aged samples of a cream formulation of the acetonide of 6α-fluoro-16α-hydroxyhydrocortisone (I). The cream containing the C^{14}-labeled steroid was prepared by normal procedures and placed in accelerated aging conditions. The stability method under study involved the red tetrazolium colorimetric assay of a chloroform extract of the steroid cream along with the cream placebo.



6α-Fluoro-16α-hydroxyhydrocortisone-16,17-acetonide

I

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EXPERIMENTAL

Four types of assays were made on each sample—namely, the red tetrazolium (RTZ) method under evaluation, the C^{14} assay of the RTZ chloroform extract, the C^{14} assay of the RTZ aqueous phase, and the C^{14} assay of the thin-layer chromatography (TLC) eluates.

The C^{14}-labeled steroid was prepared by the reaction of C^{14}-labeled acetone with 6-α-fluoro-16-α-hydroxyhydrocortisone. The labeled acetonide had an activity of 2.81 μc./mg. The cream of the labeled steroid was prepared by a conventional method. A placebo was prepared at the same time and the samples placed under accelerated aging conditions.

Sample Preparation.—The contents of one tube of a cream sample and of a placebo sample were emptied into 250-ml. volumetric flasks and the weights determined by difference. The cream was dissolved in methanol–chloroform solution (1:1).

Reagents.—(*A*) A solution of 250 mg. of 2,3,5-triphenyl tetrazolium chloride in 25 ml. of absolute ethanol was prepared fresh daily and protected from light.

(*B*) Five milliliters of commercial 10% solution of tetramethylammonium hydroxide solution was diluted to 50 ml. with absolute alcohol and filtered if necessary.

(*C*) A standard solution of steroid was prepared by dissolving 25 mg. of steroid in 100 ml. of chloroform.

(*D*) Toluene counting fluid was prepared by dissolving 380 mg. of 1,4-bis-2-(4-methyl-5-phenyl oxazolyl)-benzene (scintillation grade) and 19 Gm. of 2,5-diphenyloxazole (scintillation grade) in 3.79 L. of toluene.

(*E*) Diotol liquid scintillation solvent was prepared by dissolving 104 Gm. of naphthalene, 6.5 Gm. of 2,5-diphenyloxazole, 130 mg. of 1,4-bis-(5-phenyl-2-oxazolyl) benzene in 500 ml. of toluene, 500 ml. of dioxane, and 300 ml. of methanol.

(*F*) Tetrazolium blue spraying reagent was prepared daily by mixing 20 ml. of a 2 mg./ml. aqueous solution with 10 ml. of 10% sodium hydroxide solution.

RTZ Procedure.—An aliquot of the sample solution equivalent to approximately 1 Gm. of cream was transferred into each of three separators. An aliquot of the placebo solution equivalent to 1 Gm. of cream was transferred into each of four separators. The solutions were evaporated to dryness with the aid of steam heat and a stream of air. Water (15 ml.) was added to each separator, and 2 ml. of standard solution (*C*) was added to the three separators containing the placebo. The material in each separator was extracted with four 10-ml. portions of chloroform, and the chloroform extracts were filtered through anhydrous sodium sulfate and adjusted to 50 ml. in a volumetric flask.

Ten-milliliter aliquots were transferred from each flask into separate 25-ml. volumetric flasks and evaporated with steam heat and air. Five milliliters of absolute ethanol was pipeted into each flask and into an eighth 25-ml. volumetric flask as a reagent blank. One milliliter of tetrazolium solution (*A*) and 2 ml. of tetramethylammonium hydroxide solution (*B*) were added to each flask which then was allowed to stand in the dark for exactly 30 minutes. One milliliter of glacial acetic acid was added to each flask and the solutions mixed by swirling. The absorbances of the solutions were measured with a Beckman DU spectrophotometer in 1-cm. cells at 485 mμ *versus* the reagent blank.

$$\frac{\text{mg. steroid}}{\text{Gm.}} = \frac{\text{sample abs.} - \text{placebo abs.}}{(\text{placebo} + \text{std.})\ \text{abs.} - \text{placebo abs.}} \times \frac{0.5}{\text{ml. aliquot}} \times \frac{250}{\text{sa. wt.}}$$

C^{14} Assay of RTZ Chloroform Extract.—Aliquots of 10 ml. from each sample and each placebo plus standard extract from the RTZ method were transferred into separate counting vials and evaporated to dryness. Counting fluid (*D*, 10 ml.) was added to each vial and counted in a Packard Tri-Carb model 314EX liquid scintillation spectrometer for 10 min. (3). An internal C^{14} standard of NEC-103-toluene-1-C^{14} (2.54 mc./mmole) (New England Nuclear) was added to each vial and counted for 1 min.

$$\mu\text{c. } C^{14}/100\ \text{Gm. cream} = \frac{\text{disintegration/min. } C^{14}\ \text{std.}}{(\text{c.p.m. sa.} + C^{14}\ \text{std.}) - \text{c.p.m. sa.}} \times \frac{\text{c.p.m. sa.} - \text{c.p.m. (placebo)}}{10} \times \frac{50}{\text{ml. aliquot}} \times \frac{250}{\text{sa. wt.}} \times \frac{100}{2.2 \times 10^6}$$

C^{14} Assay of the RTZ Aqueous Phase.—The aqueous phase of each sample and placebo plus the standard from the extraction from RTZ method were drained into 250-ml. beakers and the separators rinsed with 20-ml. portions of ethanol. The solutions were evaporated to dryness, and the residues were transferred with ethanol into counting vials with ethanol and again evaporated to dryness. Ten milliliters of Diotol (*E*) was added to each vial and the solution counted for 10 min., then for 1 min. after the addition of an internal C^{14} standard.

$$\mu\text{c. } C^{14}/\text{aqueous phase}/100\ \text{Gm. cream} = \frac{\text{disintegrations/min. } C^{14}\ \text{std.}}{\text{c.p.m. (sa.} + C^{14}\ \text{std.)} - \text{c.p.m. sa.}} \times \frac{\text{c.p.m. sa.} - \text{c.p.m. placebo}}{\text{ml. aliquot}} \times \frac{250}{\text{sa. wt.}} \times \frac{100}{2.2 \times 10^6}$$

C^{14} Assay of Thin-Layer Chromatography (TLC) Eluates.—Aliquots of the original sample solution equivalent to approximately 1 Gm. of cream were transferred into each of three 125-ml. separators. An aliquot of the original placebo solution equivalent to approximately 1 Gm. of cream was transferred into a fourth separator. The aliquots were evaporated to dryness with the aid of steam heat and a stream of air. Heptane aliquots (40 ml.) were added to the separator, and the solution was extracted four times with 20-ml. aliquots of 85% methanol. The extracts were collected in a 250-ml. separator. After the addition of 80 ml. of distilled water to the 250-ml. separator, four more extractions were made with 20 ml. of chloroform.

TABLE I.—STABILITY ASSAY RESULTS BY RTZ METHOD AND TLC METHOD USING C^{14}-LABELED STEROID

Time, Mo.	RTZ, % of Initial	TLC, % of Initial	Difference, % of Initial
25°C.			
1	97.1	100	+2.9
3	106	101	−5.0
6	95.1	98.6	+3.5
9	97.7	90.8	−6.9
12	106	100	−6.0
		Av.	−2.3
37°C.			
1	96.7	96.8	+0.1
3	95.5	90.2	−5.3
6	88.3	86.3	−2.0
9	92.3	75.7	−16.6
12	86.7	78.6	−8.1
		Av.	−6.38
50°C.			
1	79.3	73.5	−5.8
3	80.9	59.4	−21.5
6	74.7	57.3	−17.4
		Av.	−14.9

Each extract was filtered through a layer of anhydrous sodium sulfate into a 150-ml. beaker. The chloroform was evaporated to dryness and the residue transferred to a 2-ml. volumetric flask with small portions of chloroform and adjusted to volume with chloroform.

The thin-layer plates [200 × 200 mm. Silica Gel G (Stahl)] were marked into six lanes 1.25 in. wide with a spatula. One lane was spotted with a placebo to be used for background count, and three lanes were spotted with the samples to be counted for C^{14}. The fifth lane was spotted with a sample, and the sixth lane was spotted with 0.05 mg. of standard steroid. The placebo and the samples were spotted by using a 50-µl. syringe to apply a total of 200 µl. in a line 1.25 in. from the end of the plate. The plate was placed into a chamber containing anhydrous ether and allowed to develop to 1 in. from the top of the plate. The plate was dried and the development in ether repeated. The plate was dried and placed in a developing chamber containing 30:70 ether–ethyl acetate and developed in the same manner.

The placebo lane and the three sample lanes were divided into segments 0.5 in. wide. A spatula was used to scrape each segment into separate counting vials. Methanol (1 ml.) was added into each vial to dissolve the steroid, and 10 ml. of counting fluid (*D*) was added into each vial. The solution was counted by liquid scintillation for 10 min., then counted for 1 min. after the addition of an internal C^{14} standard. The remaining sample lane and standard lane were sprayed with the tetrazolium reagent (*F*). The plate was heated gently on a steam bath to darken the color of the spots.

$$\frac{\text{disintegrations/min.}}{\text{segment}} = \frac{\text{disintegrations/min. } C^{14} \text{ std.}}{\text{c.p.m. (sa. + } C^{14} \text{ std.) − c.p.m. sa.}} \times \frac{\text{c.p.m. sa. − c.p.m. corresponding placebo}}{1}$$

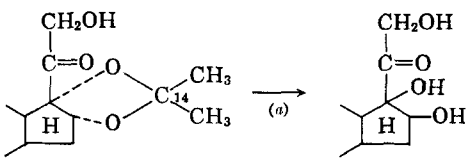
The segment of peak count corresponded to the R_f of the standard and sample when sprayed by the TZ reagent (*F*). The disintegrations of this segment plus the two adjoining ones were used as the total pure steroid spot.

$$\frac{\mu\text{c. } C^{14} \text{ steroid}}{\text{100 Gm. cream}} = \frac{\text{disintegrations/min. steroid segments}}{0.2} \times \frac{2}{\text{ml. aliquot}} \times \frac{250}{\text{sa. wt.}} \times \frac{100}{2.2 \times 10^6}$$

RESULTS AND DISCUSSION

The primary concern in the evaluation of the proposed method (RTZ) was the ability of the method to measure deterioration of the steroid in the cream preparation. Preliminary precision study of the RTZ method using 12 replicate assays on a cream sample gave a relative standard deviation of 2.8%. To obtain a valid comparison of the two methods, triplicate assays were performed by each method for each sample. The results reported in Table I are averages of triplicates. Apparent from the data was the favorable correlation of the two methods for the 25° stability samples.

On the 37° samples, the cream maintained its apparent physical properties, but the TLC method indicated a greater decreasing potency on aging. This effect is more predominant in the 50° aged samples, which were liquified on storage. Possible loss of assay could have proceeded by several routes.



I II + Acetone

The steroid acetonide (I) could have lost acetone to form the desacetonide (II). The acetone would be lost in the evaporation of the aqueous phase. The desacetonide (II) was found to remain at the point of application of the chromatogram. De-

TABLE II.—RADIOACTIVITY OF PHASES OF TETRAZOLIUM METHOD

Time, Mo.	Sample Aliquot	Chloroform Extract	Aqueous Phase
	µc./100 Gm. Cream		
25°C.			
0	1.026	1.001	0.00578
1	1.015	1.000	0.0101
3	1.084	1.092	0.00703
6	1.015	1.001	0.00935
9	1.012	0.998	0.0114
12	1.072	1.049	0.0119
37°C.			
1	1.000	0.977	0.0184
3	1.004	0.973	0.0217
6	1.028	0.990	0.0291
9	1.023	0.998	0.0354
12	1.004	1.049	0.0368
50°C.			
1	0.935	0.910	0.0570
3	0.899	0.889	0.0237
6	0.963	0.851	0.0530

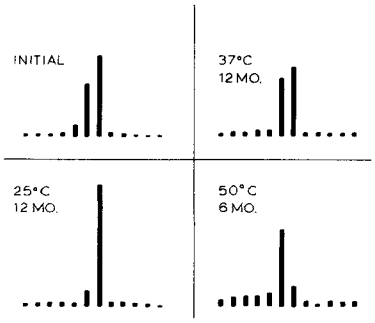


Fig. 1.—C^{14} counts per minute of segments of thin-layer chromatograms.

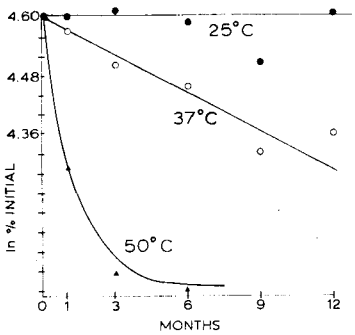
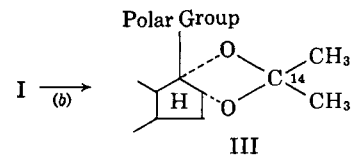
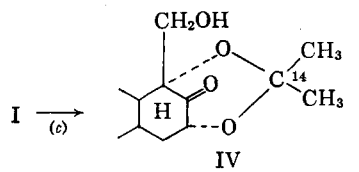


Fig. 2.—Ln plots of cream stability data.

terioration by reaction (*a*) would have been indicated by some loss of potency by the RTZ procedure, a complete loss by the TLC method, and the appearance of a BTZ positive color at point of application on the chromatogram. All of these phenomena were observed.



III



IV

Other possible courses of decomposition could have been through reaction (*b*) to form unknown III or reaction (*c*) to form the D-homoannulated type of derivative, IV (4). Both of these products, III and IV, would remain radioactive but be separated by the TLC method. There would result no loss in total radioactivity but loss of potency by the TLC method.

The activities of the sample aliquot, the chloroform extract, and the aqueous phase were measured to observe possible causes of assay errors and to observe properties of the decomposition products. (See Table II.)

Reaction sequence (*a*) would result in loss of acetone and activity in sample aliquot since it was evaporated before measurement. This was observed in the 50° samples. The ln plot of stability data (Fig. 2) illustrates that the decomposition at 50° did not follow first-order reaction kinetics; also the data indicate that decomposition followed more than one reaction course with the formation of unknown polar acetonide or acetonides. Gain of activity in the aqueous phase of the 37° and 50° aged samples suggested reaction (*b*), with a production of polar acetonides of type III. The lower results by the TLC method, compared with the activity in the chloroform extract, suggested reaction of type (*c*), with formation of compound of type IV with some difference in polarity from that of I.

Distributions of the C^{14} radioactivity in the various thin-layer chromatograms are shown in Fig. 1. The 25° 12-month sample appeared to have purity equal to the original. Only in the 37° and 50° samples were increased activities before and after steroid segments observed.

Since reactions of types (*a*) and (*b*) or (*c*) were observed, first-order reaction kinetics were not accurate theoretically. This inaccuracy was substantiated by large 95% confidence intervals found for the time required to reach 85% assay using pseudo first-order computer methods described (5) to calculate k at 25° from the activation energy. The RTZ method confidence interval was 0.34 to 302 years and the TLC method 0.48 to 26 years.

The failure of the 50° data to follow a first-order reaction caused this inaccuracy. Ln per cent initial *versus* months plots gave nearly straight lines for 25° data. Using these plots to determine k at 25°, a decomposition of 1.37% ± 0.126% per year for the RTZ method and 0.178% ± 0.039% per year for the TLC method were found. The accuracy of the RTZ method in terms of recovery by $CHCl_3$ extraction was demonstrated by the average loss of 1.5%, assuming no formation of water-soluble radioactive material. From pooled variance from triplicate assays, the relative standard deviations of the methods were calculated to be ±2.68 for the RTZ and ±2.64 for the TLC methods.

CONCLUSIONS

A thin-layer chromatographic procedure, utilizing C^{14}-labeled steroid, was developed to prove the accuracy of a chloroform extraction and red tetrazolium spectrophotometric stability assay of a cream preparation. The relative standard deviations for both methods were less than ±3%. The spectrophotometric method was found to be inaccurate on extreme accelerated aged samples. Rate of decomposition estimated by pseudo first-order reaction kinetics based on 25° data was found to be 1.34% per year by the spectrophotometric method and 0.178% per year by the thin-layer method.

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