obtained from the standards. Distinct differences can be noted between the standards at the 90% and 110% concentrations.

RESULTS

The method has been applied to six commercial dosage forms containing reserpine and other active ingredients. The results are shown in Table I. (The active ingredients, rauwolfia serpentina and protoveratrines A and B,[3] were also separated on a thin-layer plate. Reserpine and the protoveratrines were not quantitated against standard reserpine and protoveratrine mixtures.) The commercial tablets were obtained on the open market in bottles of 50 or 100, and single tablets of each were used for assay. None of the active ingredients or excipients present in the tablets interfered in the determination of reserpine.

Table II shows a comparison of results obtained from the determination of reserpine and protoveratrine A in tablets containing reserpine, protoveratrine A, and hydroflumethiazide[1] by the proposed single tablet method *versus* the U.S.P. method (1) for reserpine and the protoveratrine A procedure of Craig and Jacobs (2), using multiple-tablet extracts.

DISCUSSION

Sensitivity.—From 2 to 6 μl. of the working standard solution was spotted on a plate. The plate was developed and sprayed with the reagent. The lower limits of reserpine and protoveratrine A that can be detected are 0.9 and 1.44 mcg., respectively.

Interferences.—Reserpine was hydrolyzed in an alkaline medium according to the method of Neuss (8). The products, reserpic acid and trimethoxybenzoic acid, were dissolved in chloroform at a concentration equivalent to that of the working standard solution and chromatographed in the usual fashion. Reserpic acid and trimethoxybenzoic acid do not react with the reagent. When the plate is exposed to ultraviolet light, faint fluorescent spots are observed at the origin.

When the standard stock solution of reserpine and protoveratrine A is exposed to bright light for approximately 4 hr., diluted, and chromatographed, fluorescent spots are observed at the origin and at an R_f of 0.5 to 0.58. These spots are not found on a developed plate when freshly prepared, light-protected standard solutions are chromatographed. Excipients and active components found in the seven formulations reported above do not interfere.

[3] Marketed as Rauprote tablets by The Vale Chemical Co., Inc., Allentown, Pa.

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Determination of Hydrocortisone and Hydrocortisone Acetate in Antibiotic Mastitis Preparation

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A modified column partition chromatographic method has been developed for the determination of hydrocortisone and hydrocortisone acetate in antibiotic mastitis preparations. A sodium bicarbonate trap under a methyl alcohol–water stationary phase in Celite is used with two mobile phases. First, the interfering oil is eluted with methylene chloride–isooctane, then the steroid to be assayed by the blue tetrazolium method is eluted with methylene chloride. This procedure shows a marked improvement over the U.S.P. XVII hydrocortisone ointment method in the removal of interferences and results in satisfactory assays for the two steroids in mastitis formulations. Recoveries of the steroids added to blank mastitis preparations ranged from 90.3 to 100.9 per cent. The average percentage of recovery for hydrocortisone and hydrocortisone acetate was 96.9 and 98.2, respectively.

CORTICOSTEROIDS have been incorporated in a large number of drug preparations, primarily for their anti-inflammatory activity. Two of the most common, hydrocortisone and hydrocortisone acetate, are often used in ointments and oils for the treatment of mastitis in dairy animals. These mastitis preparations frequently contain procaine penicillin G and a vegetable oil, *e.g.*, peanut oil, and may contain, in addition, one or more of the following: dihydrostreptomycin sulfate, neomycin sulfate, polymyxin B sulfate, sulfamerazine, sulfamethazine, sulfathiazole, sulfanilamide, methylpara-

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ben, propylparaben, papain, chlorobutanol, cobalt sulfate, and polyvinylpyrrolidone. Aluminum monostearate or colloidal silica is frequently used as a gelling agent.

Of the several chemical methods widely used for the assay of steroids (1–4), the blue tetrazolium method, official in U.S.P. XVII and N.F. XII, with minor modifications was chosen for this study.

The U.S.P. XVII method for hydrocortisone ointment proved unsatisfactory for mastitis preparations. Assays for hydrocortisone revealed positive interferences (ranging from less than 1 to 39% and varying with the manufacturer) when blanks were used which contained, in all cases, procaine penicillin in an oil base together with some of the above-mentioned adjuncts. The main interfering substances are procaine penicillin G and the oil.

A Celite chromatographic column with methyl alcohol–water as the stationary phase and methylene chloride–isooctane as the mobile phase is useful for removing fatty alcohols while retaining the steroid; the steroid can then be eluted with methylene chloride (5). This method is applicable to ointments containing steroids and is especially effective for removing the interfering substances contained in lanolin.

A modification of this method is presented for the determination of hydrocortisone and hydrocortisone acetate in antibiotic mastitis preparations. It consists of the following: (*a*) inserting a sodium bicarbonate trap under the methyl alcohol–water stationary phase to prevent procaine penicillin from interfering; (*b*) changing the concentration of methyl alcohol–water (1:1) stationary phase from 1 ml./Gm. of Celite to 0.5 ml./Gm. to improve recovery; (*c*) changing the isooctane–methylene chloride mobile phase to accommodate the polarity range of the two steroids; (*d*) packing the column with Celite support mixture in dry form rather than as a suspension in isooctane–methylene chloride mobile phase, and placing the sample on the column in a Celite mixture rather than in an isooctane–methylene chloride suspension. The Celite mixture technique proved more satisfactory in the authors' experience. Details of the modified assay procedure are as follows.

ASSAY PROCEDURE

Apparatus.—Chromatographic column, 15 mm. i.d., and 450 mm. long with Teflon stopcock.

Reagents.—Celite 545, methyl alcohol, isooctane, methylene chloride, glacial acetic acid, tetramethyl ammonium hydroxide (10% in water), blue tetrazolium (Dajac Laboratories), ethyl alcohol, sodium bicarbonate. All reagents are reagent grade.

Preparation of Column.—Use either glass wool or a fritted disk as the column support. Add 1 ml. of 8% sodium bicarbonate aqueous solution to 2 Gm. of Celite in a small beaker, mix well, and transfer to the column in three portions, gently packing each portion with a tamping rod made of glass or stainless steel. Add 3 ml. of methyl alcohol–water (1 + 1) to 6 Gm. of Celite, mix well, and transfer to the top of the bicarbonate column in several portions, packing gently after each portion.

Preparation of Sample.—Accurately measure or weigh a sample of mastitis preparation containing 2 mg. of steroid into a 150-ml. beaker containing 1 Gm. of Celite and mix thoroughly. Transfer this mixture to the column. Dry wash the beaker with 0.5 Gm. of Celite and add to the column.

Preparation of Standard.—Place into a 150-ml. beaker an aliquot equivalent to 2 mg. of the appropriate steroid reference standard dissolved in ethyl alcohol and evaporate to dryness on a steam bath. Dissolve the residue in 1 ml. of methyl alcohol, add 1 ml. of water, and mix well into 2 Gm. of Celite. Transfer to a column and treat standard and sample alike. This procedural standard gave assay values between 98 and 100% of a direct standard.

Elution of Column.—Add portions of methylene chloride–isooctane (1 + 9) to a sample beaker and transfer to the column. Collect 100 ml. of eluate with a flow rate of 1 drop/sec. Discard the eluate and use the same container to add methylene chloride to the column. Collect 100 ml. of eluate in a volumetric flask at the same rate. This methylene chloride fraction contains the steroid.

Spectrophotometric Assay.—Evaporate a 10.0-ml. aliquot of the methylene chloride fraction in a 50-ml. glass-stoppered conical flask to dryness on a steam bath under a gentle stream of air. Add 20.0 ml. of ethyl alcohol to the flask, swirl to complete solution, and add 2.0 ml. of blue tetrazolium solution (400 mg. in 100 ml. of ethyl alcohol) and 2.0 ml. of diluted tetramethyl ammonium hydroxide (1 ml. to 10 ml. with ethyl alcohol). After 45 min., stop the reaction by adding 1.0 ml. of glacial acetic acid. The color is then stable for 24 hr. Determine the absorbance of the solution against a reagent blank at 520 mμ on a suitable spectrophotometer, and compare with the standard treated in like fashion.

RESULTS AND DISCUSSION

Development of the Assay Procedure.—Initially the column was made with 1 ml. of methyl alcohol–water (1 + 1) per gram of Celite. Incomplete (80–85%) recoveries of steroid were obtained. A change to 0.5 ml. of stationary phase per gram of Celite improved the recoveries to over 95%. The initial mobile phase for removing the oil was a methylene chloride–isooctane mixture (2 + 8). Recovery experiments showed that this solvent was satisfactory for the hydrocortisone alcohol but not for hydrocortisone acetate because of some loss in the initial elution. A change to methylene chloride–isooctane (1 + 9) proved satisfactory, not only in removing the oil but also in yielding satisfactory recoveries of both steroids. In addition to the above steroids, satisfactory recoveries were obtained with prednisolone, prednisolone acetate, and dexamethasone alcohol during the investigational phase of this work. Some interfering substances

TABLE I.—INTERFERENCE IN DETERMINATION OF STEROIDS IN BLANK FORMULATIONS OF MASTITIS PREPARATIONS CONTAINING NO STEROID[a]

	Apparent Steroid Content, mg./Gm.				
	U.S.P. XVII Method for Hydrocortisone Ointment		New Method		
Mfr.	Anal. 1	Anal. 2	Anal. 1	Anal. 2	Anal. 3
1	0.29	0.63	0.10	0.00	0.01
2	0.44	0.78	0.17	0.10	0.10
3	0.53	0.59	0.10	0.04	...
4	0.17	0.35	0.07	0.06	0.02
5	0.01	0.00	0.07	0.01	...
6	0.25	0.29	0.16	0.05	0.07

[a] Usual concentration in commercial samples: 2–2.5 mg./Gm.

TABLE II.—RECOVERY OF STEROID ADDED TO BLANK FORMULATIONS

		Recovery, %				Recovery, %	
Mfr.	Hydrocortisone Added, mg.	Anal. 1	Anal. 2	Anal. 3	Hydrocortisone Acetate Added, mg.	Anal. 1	Anal. 2
1	2.00	98.1	97.2	...	2.02	98.0	98.5
	2.00	...	...	98.4	...	...	...
2	2.00	98.0	96.9	...	2.02	100.0	100.5
3	2.03	100.0	99.3	...	2.02	98.5	99.5
4	2.03	...	94.5	...	2.02	95.0	100.9
	2.00	...	...	94.5	...	...	...
5	2.03	97.7	98.1	...	2.02	96.5	95.0
	2.00	...	...	90.3	...	...	...

TABLE III.—DETERMINATION OF STEROIDS IN COMMERCIAL PREPARATIONS

Mfr.	Steroid	Label Claim, mg./Syringe[a]	Results	% of Label Claim
1	Hydrocortisone acetate	20	22.4	112.0
1	Hydrocortisone acetate	20	21.1	105.5
3	Hydrocortisone acetate	20	20.1	100.5
4	Hydrocortisone acetate	20	21.9	109.5
5	Hydrocortisone acetate	20	18.5	92.6
6, 1	Hydrocortisone	20	18.3	91.5
2	Hydrocortisone	20	18.4	92.2
3	Hydrocortisone	20	22.0	110.0
7	Prednisolone acetate	4	3.71	92.8

[a] Syringes tested ranged from 6 to 12 Gm. total content.

from the preparations being tested still passed through the column. On the assumption that the interference was caused by the procaine penicillin G and/or its degradation products, a sodium bicarbonate trap was placed on the bottom of the column. This removed almost all of the interference. A stronger alkaline trap would undoubtedly be satisfactory in holding back the interference but would encourage the degradation of the steroid. A comprehensive study of interference by antibiotics and other pharmaceutical ingredients in the various assay methods (2–4) is in progress.

Hydrocortisone and hydrocortisone acetate carried through this column procedure were examined by thin-layer chromatography (6). No degradation was observed.

Preliminary experiments indicate that this chromatographic system is also applicable to a large number of aqueous preparations, ointments, lotions, and creams containing antibiotics and steroids.

Application of the Assay Procedure.—The interferences caused by blank mastitis preparations, which contained all ingredients except the steroids (obtained through the courtesy of six manufacturers), when tested by the U.S.P. XVII method for hydrocortisone ointment and by the new procedure, are shown in Table I.

As shown in Table II, the recoveries of steroids added to blank mastitis ointments range from 90.3 to 100.0% for hydrocortisone and 95.0 to 100.9% for hydrocortisone acetate. These recovery data were based on direct standards rather than procedural standards.

Table III shows that the hydrocortisone and hydrocortisone acetate content of nine commercial preparations assayed by the new procedure ranged from 91.5 to 112% of label claim.

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