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RESIDUE DETERMINATION

A Thin-Layer Chromatographic Procedure for the Determination of Hydrocortisone Acetate and Alcohol Residues in Milk

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A combination thin-layer chromatographic-colorimetric procedure is presented for the determination of hydrocortisone alcohol and acetate residues in milk. After extraction of the steroid from the milk with a methylene chloride–hexane solvent mixture, the residue obtained on evaporation is subjected to thin-layer chromatography to separate the hydrocortisone derivatives. The zones are extracted from the adsorbent and the steroids determined colorimetrically by the Porter-Silber reaction with phenylhydrazine. Control and recovery analyses, carried out by this procedure, gave satisfactory results at all levels investigated with a limit of sensitivity of 10 p.p.b. Recoveries averaged 85.6% for the acetate and 75.1% for the alcohol at levels of 0.010 to 10.00 p.p.m. with control values of zero p.p.b. Related corticosteroids have also been separated by this chromatographic procedure.

Interest in the use of hydrocortisone derivatives as anti-inflammatory agents for the treatment of mastitis in dairy cattle prompted an investigation for the determination of these compounds in milk at the parts-per-billion (p.p.b.) levels. Methods available for the determination of hydrocortisone residues (*1, 4*) were investigated, and they were found inadequate in eliminating interferences of milk background or too time-consuming to be of practical use.

This report deals with the development of a reproducible, specific, and quantitative method for the determination of hydrocortisone derivatives in milk at parts-per-billion levels. The method utilizes a solvent extraction system that does not yield emulsions, with a recovery of 85 to 95% of the solvent; a separation of the hydrocortisone derivatives by thin-layer chromatography; and measurement of the separated components using the color reaction described by Porter and Silber (*3*).

Experimental

Reagents. PURIFIED METHYLENE CHLORIDE. Slurry 25 grams (approximately 150 ml.) of Nuchar C-190N activated carbon with 1 gallon of methylene chloride. Stir for 15 minutes using a mechanical stirrer. Filter through Whatman #12 fluted filter paper, or its equivalent. All of the methylene chloride used in this procedure must be purified in this manner.

SOLVENT MIXTURE. Mix four parts of purified methylene chloride with one part of ACS grade *n*-hexane.

SULFURIC ACID–ETHANOL SOLVENT. Mix two parts of 64% sulfuric acid in water with one part of absolute ethanol.

PHENYLHYDRAZINE HYDROCHLORIDE REAGENT. Dissolve 50 mg. in 50 ml. of sulfuric acid–ethanol solvent. This reagent must be prepared fresh daily.

SILICA GEL G AND HF_{254}. Distributed by Brinkman Instruments, Inc., Great Neck, L. I., N. Y. Prepare 22 2×8 inch plates with a layer of adsorbent 0.25 mm. thick as follows: Mix thoroughly 20 grams each of Silica Gel G and HF_{254}. Add 80 to 90 ml. of distilled water and shake vigorously for 30 seconds. Transfer to the adsorbent applicator and spread rapidly over the plates. Allow to air-dry overnight. When dry, the plates should be scored so that there are five strips on each plate, each strip 1 cm. wide (refer to Figure 1).

STEROID STANDARDS. Obtainable from: U.S.P. Reference Standards, 46 Park Ave., New York 16, N. Y. Weigh accurately 50 mg. of steroid and transfer to a 50-ml. volumetric flask. Dissolve in absolute ethanol, dilute to volume, and mix well. Label Solution A. Pipet 10 ml. of Solution A into a 100-ml. volumetric flask and dilute to volume with ethanol. Mix well and label Solution B. Pipet 10 ml. of solution B into a 100-ml. volumetric flask and dilute to volume with absolute ethanol. Mix well and label Solution C. Pipet 10 ml. of Solution C into a 100-ml. volumetric flask and dilute to volume with absolute ethanol. Mix well and label Solution D. This is the working standard containing 1.0 μg. of steroid per ml. of solution.

Apparatus. Funnels, separatory, 1000-ml., 125-ml., and 60-ml. capacity with polytetrafluoroethylene stopcocks.

Chromatographic equipment (Desaga), thin-layer, with 2×8 inch plates. Flasks, acetylation, 10-ml. capacity, Kimble Cat. No. K29425. Absorption cells, Arthur H. Thomas, Cat. No. 9102-M80, 0.7-ml. capacity, 1-cm. path length.

Vibrating shaker, Research Specialties Co., Richmond, Calif.

Analytical Procedure

Calibration Curve. Transfer aliquots of standard Solution D containing 2, 4, 6, 8, and 10 μg. of steroid to test tubes and evaporate to dryness under a stream of air in a water bath at 55° C. Using an

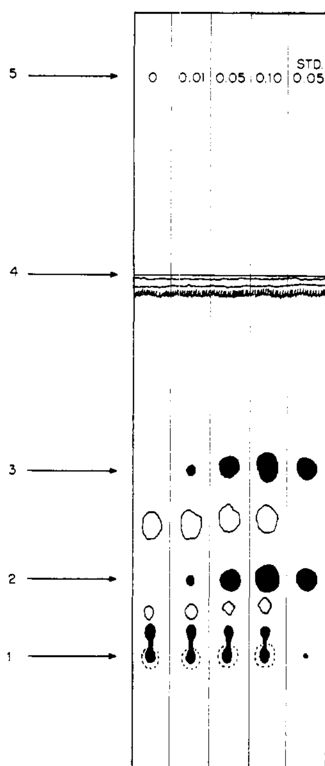

Figure 1. Chromatogram of hydrocortisone and alcohol from milk

Dark areas are absorbing spots. Enclosed white areas are fluorescent spots. 1, origin. 2, hydrocortisone alcohol. 3, hydrocortisone acetate. 4, solvent front, 10 cm. from origin. 5, p.p.m. added.

all-glass syringe (without needle), transfer 1 ml. of phenylhydrazine reagents to each and swirl to rinse the sides of the tube. Heat the mixtures for 1 hour in a water bath at 55° C. Allow the solutions to cool to room temperature and transfer them. using a glass pipet, to a 1-cm. absorption microcell. Read the absorbance of the solutions at 370, 410, and 450 mμ with the phenylhydrazine reagent as a reference. Calculate the corrected absorbance of the standards and plot it *vs.* the concentration of steroid in micrograms per milliliter of phenylhydrazine reagent, where the corrected absorbance equals:

$$A_{corr} = A_{410} - 1/2\,(A_{370} + A_{450})$$

The slope of the calibration curve for hydrocortisone acetate is 0.100 absorbance units per 4.5 μg. of steroid (a = 22.2) and for hydrocortisone alcohol is 0.100 units per 4.3 μg. (a = 23.2).

Analysis of Samples. Transfer four volumes of the solvent mixture (four times the volume of sample to be taken) to an Erlenmeyer flask of suitable size. Add a portion of milk containing no more than 20 μg. of each steroid to the flask while the solvent is being mixed vigorously with a mechanical stirrer. The speed of the stirrer should be such that the droplets of milk in the solvent are kept as small as possible. When the speed is insufficient, emulsions may occur. Continue to stir vigorously for one hour. After this time transfer the contents of the flask to a separatory funnel of suitable size and allow the layers to separate for 15 minutes. Draw the lower, solvent layer into a graduated cylinder and record the volume of solvent recovered. Transfer the solvent to a beaker of suitable size and allow to evaporate to dryness overnight in a hood.

Rinse the contents of the beaker into a 60-ml. separatory funnel with one 10- and two 5-ml. portions of *n*-hexane followed by 10 ml. of water. Shake vigorously for 1 minute; allow the layers to separate. Draw the lower aqueous layer into a second 60-ml. separatory funnel. Rinse the contents of the beaker with 10 ml. of distilled water and transfer the wash to the first separatory funnel. Repeat the above extraction and combine the aqueous layers in the second separatory funnel. If an emulsion forms, wash the aqueous layer and emulsion with 20 ml. of *n*-hexane, discarding the hexane layer.

Extract the steroid from the aqueous layer by shaking for 1 minute with 20 ml. of methylene chloride, allow the layers to separate, and draw the lower, solvent layer into a test tube. If an emulsion forms, draw the solvent layer and emulsion into another separatory funnel and shake vigorously for 5 to 10 seconds. Allow the layers to separate and draw the lower layer into the test tube. Evaporate the methylene chloride extract to dryness under a stream of air in a water bath at 55° C. Repeat the extraction of the aqueous layer with two additional 10-ml. portions of methylene chloride, combining the extracts in the test tube and evaporating to dryness.

After the evaporation, rinse the contents of the test tube into a 10-ml. acetylation flask with 2-, 2-, and 1-ml. portions of methylene chloride and again evaporate to dryness with heat and air. Add 100 μl. of methylene chloride to the 10-ml. flask; swirl to rinse the sides of the flask and dissolve the residue for spotting on the thin-layer plates.

Spot the entire solution on the thin-layer plate 3 cm. from the bottom of the plate using a 50-μl. pipet. A gentle stream of air may be used to aid in the evaporation of the solvent. Add 100 μl. of methylene chloride to the flask and swirl to rinse the flask thoroughly. Transfer the rinse to the same spot on the plate. Repeat the above rinse with another 50-μl. portion of methylene chloride. Spot standards containing 10 μg. each of the desired steroids to aid in locating the correct spot after chromatography. (The standards also aid in estimation of the level present in unknown samples, but are not used for quantitation and need not be extracted from the plate.)

Develop the chromatogram for a distance of 10 cm. from the origin with a solvent mixture of 7% methanol in methylene chloride. Allow the chromatogram to air dry and locate and mark the blue-absorbing spots by irradiating with a short-wavelength UV lamp (253 mμ). Note if the sample spots are larger or more intense than the standard.

For the determination of hydrocortisone alcohol, locate the correct spot using the appropriate standard as a reference. Scrape into a test tube an area of the plate to include the adsorbent 1 cm. in both directions from the center of the spot. Add 5 ml. of absolute ethanol and a glass bead to aid in agitation. Mix vigorously for 1 minute on a vibrating shaker and for 15 minutes on a wrist-action shaker. Filter the solution into a test tube using vacuum and a fritted glass funnel of medium porosity. Rinse the contents of the original tube into the funnel with 2-, 2-, and 1-ml. portions of ethanol. Rinse the side of the funnel with an additional 1 ml. of ethanol and filter completely with vacuum. Evaporate the ethanol to dryness in a water bath at 55° C. under a stream of air.

For the determination of hydrocortisone acetate, locate the correct spot using the appropriate standard as a reference. Scrape into a 60-ml. separatory funnel an area of the plate to include the adsorbent 1 cm. in both directions from the center of the spot. Add 10 ml. of 0.1*N* HCl and shake vigorously for 1 minute. Add 10 ml. of methylene chloride and shake vigorously for 1 minute. Allow the layers to separate; draw off the lower solvent layer into a 20 × 150 mm. test tube and evaporate to dryness in a water bath at 55° C. under a stream of air. Repeat the above extraction with two 10-ml. portions of methylene chloride, combining the extracts in the test tube and evaporating to dryness.

For a reagent blank, scrape an area 2 cm. long from the portion of the plate above the solvent front (12 to 14 cm. from origin) and extract in the same manner.

Rinse the residue from the test tube to a 60-ml. separatory funnel fitted with a polytetrafluoroethylene stopcock with 2-, 2-, and 1-ml. portions of methylene chloride. Using an all-glass syringe (without needle) transfer 1 ml. of phenylhydrazine reagent to the separatory funnel and shake vigorously for 1 minute. If the sample spots were larger or more intense than the standard on the chromatogram, add 2 ml. of reagent instead of 1 ml.

Allow the layers to separate for 10 minutes; draw off the lower layer into a small-diameter tube and place in a water bath at 55° C. for 1 hour. Allow the tube to cool to room temperature and transfer the solution to the 1-cm. absorption microcell using a glass pipet. Read the absorbance of the solutions at 370, 410, and 450 mμ, with the reagent

blank as the reference. Calculate the corrected absorbance of the sample and compare with the standard curve of the appropriate steroid.

Calculation.

$$A_{corr} = A_{410} - 1/2\ (A_{370} + A_{450})$$

$$\text{P.p.m. steroid} = \frac{\mu\text{g. from std. curve} \times \text{ml. phenylhydrazine reagent}}{\text{aliquot of Milk} \times \dfrac{\text{volume of solvent recovered}}{\text{volume of solvent used}}}$$

Recovery Determinations

Recovery experiments on hydrocortisone acetate were designed to cover the complete range of residue values that might possibly be encountered in a rate of disappearance study—that is, from 10 p.p.b. to 10 p.p.m. Four liters of milk from untreated cows were pooled to provide a common background for all the samples. Samples (200 ml.) were fortified with hydrocortisone acetate at levels of 10, 50, 100, and 500 p.p.b. and 1, 5, and 10 p.p.m. Four unmedicated control samples were also run. Appropriate aliquots were taken from these samples so that none contained more than 20 μg. of the steroid. Table I lists the individual recovery values obtained on the analysis of these samples. An average recovery of 85.6% was obtained on 13 determinations with four controls of zero p.p.b.

Hydrocortisone alcohol recoveries were carried out in the same manner as the acetate at levels of 10, 25, 50, 100, 250, and 500 p.p.b. An average of 75.1% recovery was obtained on 11 determinations with three control samples of zero p.p.b. The results of these analyses are presented in Table I.

Recovery determinations were also carried out by fortifying control milk with a combination of both hydrocortisone acetate and alcohol and determining each separately. The steroids were added at levels of 10, 25, and 50 p.p.b. of each form of the hydrocortisone; two unmedicated controls were also run. Table II lists the recovery obtained on the analysis of these samples. Average recoveries of 94.5% of the acetate and 73.3% of the alcohol were obtained with controls of zero p.p.b.

Discussion of Analytical Method

Peterson (*2*) and others (*1*) have demonstrated the complete partitioning of hydrocortisone from aqueous solution into methylene chloride. The use of this solvent system for the extraction of the steroid from whole milk resulted in the formation of very stable emulsions. Other solvents that were investigated, ether and chloroform, also gave the same type of emulsion. A solvent mixture of four parts of methylene chloride to one part of *n*-hexane afforded the easiest handling and best extraction of the steroid. Vigorous mixing of four volumes of this mixture to one volume of milk for 1 hour gave 85 to 95% recovery of the solvent used. This loss of solvent due to emulsification was small compared with other extraction procedures where as much as 60% of the solvent was lost. This procedure also was less time-consuming than extraction by dialysis as used by other workers.

Thin-layer chromatography for the separation of the steroid derivatives has the advantage of speed and specificity over paper and column chromatography as used earlier (*2*). The hydrocortisone acetate used in the treatment of mastitis is converted, in part, in the bovine udder to the alcohol form of the steroid. It was necessary to analyze the milk for each form of the steroid which was beyond the capabilities of the column method. A rapid thin-layer chromatographic procedure was therefore developed for this purpose.

The Porter-Silber colorimetric procedure (*3*) has been used by numerous investigators (*1, 2*) with slight modifica-

Table I. Recovery of Hydrocortisone Derivatives from Milk

Added, P.P.M.	Corr. Absorbance	From Std. Curve, μg.	Vol. of Reagent, Ml.	Milk Sample, Ml.	Found, P.P.M.	Recovery, %
		HYDROCORTISONE ACETATE				
0	0.000	0.00	1	200	0.00	. . .
0	0.000	0.00	1	200	0.00	. . .
0	0.000	0.00	1	200	0.00	. . .
0	0.000	0.00	1	200	0.00	. . .
0.01	0.043	1.90	1	200	0.0095	95
0.01	0.035	1.55	1	200	0.0078	78
0.05	0.189	8.50	1	200	0.043	86
0.05	0.204	9.20	1	200	0.046	92
0.10	0.171	7.25	2	200	0.078	78
0.10	0.192	8.70	2	200	0.087	87
0.50	0.217	9.60	1	20	0.480	96
0.50	0.189	8.50	1	20	0.430	86
1.00	0.172	7.80	2	20	0.780	78
1.00	0.185	8.33	2	20	0.833	83.3
5.00	0.187	8.45	1	2	4.23	84.6
10.0	0.183	8.25	2	2	8.25	82.5
10.0	0.192	8.65	2	2	8.65	86.5
					Average	85.6
		HYDROCORTISONE ALCOHOL				
0	0	0	1	200	0	. . .
0	0	0	1	200	0	. . .
0	0	0	1	200	0	. . .
0.010	0.045	1.90	1	200	0.0095	95.0
0.010	0.034	1.40	1	200	0.0070	70.0
0.025	0.087	3.70	1	200	0.0185	74.0
0.025	0.087	3.70	1	200	0.0185	74.0
0.050	0.185	7.57	1	200	0.0379	75.7
0.050	0.192	7.69	1	200	0.0385	76.9
0.050	0.211	8.35	1	200	0.0418	83.5
0.050	0.175	7.50	1	200	0.0380	76.0
0.100	0.028	1.2	1	20	0.060	60.0
0.250	0.080	3.4	1	20	0.170	68.0
0.500	0.166	7.1	1	20	0.355	71.0
					Average	75.1

Table II. Recovery of Hydrocortisone Acetate and Alcohol in Combination

Steroid Form	Added, P.P.M.	Corr. Absorbance	From Std. Curve, μg.	Vol. of Reagent, Ml.	Found, P.P.M.	Recovery, %
Acetate	0	0	0	1	0	. . .
	0.010	0.039	1.90	1	0.0095	95.0
	0.025	0.096	4.70	1	0.0235	94.0
	0.050	0.194	9.45	1	0.0473	94.6
					Average	94.5
Alcohol	0	0	0	1	0	. . .
	0.010	0.034	1.40	1	0.0070	70
	0.025	0.087	3.70	1	0.0185	74
	0.050	0.175	7.50	1	0.038	76
					Average	73.3

tions. Because of the inconsistent background encountered in the development of the color, many resorted to sample splitting with treatment of half the sample with a blank reagent. These methods proved cumbersome and reduced the sensitivity of the method considerably. Nelson (*1*) used the Porter-Silber reaction with a modification which he calls an absorption factor. This factor is actually a base line measurement of the color produced by the reaction of the steroid with the phenylhydrazine hydrochloride. This measurement technique was accurate and reproducible and was adapted for the authors' method.

In his work, however, Nelson carried out the colorimetric reaction on the residue after evaporation of the solvent. Although this technique was the easiest to handle, the solvent residue contributed to the absorption at 370 mμ, resulting in apparent low recovery of the standard material. When the steroid was partitioned from the solvent into the colorimetric reagent, as in the work of Silber (*4*), this effect was not encountered, and the characteristic curve shape was obtained.

Earlier procedures (*1*, *2*) required that aliquots of the standard solution be carried through all or part of the analytical method, thus correcting the results for losses due to handling in the procedure. The method reported here utilizes a standard curve for the quantitative measurement of the samples. The standards are subjected only to the colorimetric reaction. The results presented here are absolute recoveries and not corrected for losses due to the analytical procedure.

Methylene chloride from various suppliers was investigated for use in this procedure. The concentration of large volumes yielded a yellow-colored fluorescent residue which obscured the hydrocortisone on the thin-layer chromatograms. For this reason, it was necessary to purify the solvent before use. Of the various methods of purification studied, slurrying with Nuchar C-190N activated carbon proved most rapid and efficient.

Several lots of the silica gel adsorbent were used in these studies. In only one case was a reagent blank value encountered. Since the possibility of this blank does exist, it is recommended that a reagent blank of the adsorbent be run for each set of samples analyzed.

Florisil was also examined for use in the separation of the steroid forms from the other components of the milk extract and gave approximately the same R_f for the steroid. However, the silica gel, containing a fluorescent indicator, afforded a rapid and sensitive means for locating the steroid. The yellow-green fluorescent background enhanced the contrast of the blue-absorbing spot when irradiated with a short-wave (254 mμ) UV lamp. Under these conditions, less than 1 μg. can be located on the thin-layer plates.

The solvent mixture of 7% methanol in methylene chloride employed in the development of the chromatogram gave excellent resolution in separating combinations of the forms of the steroid from other components of the milk extract. Figure 1 is a graphic representation of the thin-layer chromatogram of the milk extract.

This method can also be used as a precise semiquantitative procedure for screening milk samples for steroid content. By spotting an appropriate series of standards on the thin-layer plate, rapid estimations of steroid content can be made.

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POROSITY OF FOOD PRODUCTS

Surface Areas and Densities of Freeze-Dried Foods

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The specific surface areas and densities of a variety of freeze-dried foods were determined. The specific surface areas calculated from low temperature (−195° C.) physical adsorption data using the standard Brunauer, Emmett, Teller (BET) treatment were less than 1 square meter per gram for many vegetable, fruit, meat, and fish products. True and apparent densities determined with helium, nitrogen, or dry air as the displaced medium showed that a micropore structure curtailing rapid gas diffusion through the dried mass was restricted to those layers which formed the outer surface of some foods.

Many problems encountered during the production, storage, and reconstitution of dehydrated foods may relate directly to their porosity and specific surface areas. Accurate values for these properties, established by independent means are, therefore, desirable.

No thorough study of the surface characteristics of a wide variety of dried foods as determined by gas displacement and nitrogen adsorption has yet been reported. Therefore, the authors' study of the physical features of dry milk (*3*, *4*) has been extended to include representative samples of freeze-dried foods now being developed for commerce.

This paper reports the specific surface areas of various foods as derived from the isotherms for the adsorption of nitrogen at −195° C. The calculations were made in accordance with methods described by Brunauer, Emmett, and Teller (*5*), hereafter BET method.

The true and apparent densities of these foods as determined by gas displacement techniques are also presented. From these density data an estimate of the relative porosities of the foods can be obtained.

Materials and Methods

Food samples were chosen so as to represent typical vegetable, fruit, meat, and fish products, freeze-dried whole or in diced form (Freez-Dri Products Co.,