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▲ To whom inquiries should be directed.

Analysis of Adrenocortical Steroids in Pharmaceutical Preparations by High-Pressure Liquid–Liquid Chromatography

MICHAEL C. OLSON

Abstract □ The adrenocortical steroids hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate were separated by liquid–liquid chromatography. A commercially prepared reverse-phase cyano ethyl silicone column was used in conjunction with a UV precision photometer. Linearity studies were carried out using the internal standard technique and peak height measurements. Responses of each steroid were linear over the working range when *equal* injection volumes were used. The results of a study of the controlled decomposition of hydrocortisone in basic solution indicated a good separation of drug from the degradation products. Samples of adrenocortical steroids in various dosage forms, primarily creams and ointments, were analyzed by the proposed procedure, yielding a single steroid assay value and an identification and quantitation of each foreign steroid. The sample preparation was a simple dissolution of the steroid in alcohol plus the addition of an internal standard prior to injection.

Keyphrases □ Adrenocortical steroids in pharmaceutical preparations—analyzed by high-pressure liquid–liquid chromatography □ Steroids (hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate)—analyzed by high-pressure liquid–liquid chromatography □ High-pressure liquid–liquid chromatography—analysis of adrenocortical steroids □ Chromatography, high-pressure liquid–liquid—analysis of adrenocortical steroids

Liquid–liquid chromatography is actively being investigated for drug analysis (1–9). Studies of various groups of steroids have demonstrated that both qualitative detection (10–16) and quantitative measurements (17–19) may be performed. Except for these particular works (17–19), no other reports appear to have been published dealing with the quantitative analysis of adrenocortical steroids.

The official compendial approaches (20, 21) to the assay of four adrenocortical steroids—hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate—employ well-established methods. The steroids are either extracted with alcohol for total steroid content or separated by TLC to isolate a single steroid, after which the actual measurement is carried out by blue tetrazolium colorimetry. When tablets are assayed, the foreign steroid content is determined as the difference between the results of the single steroid and the total steroid assays. The problems with the blue tetrazolium color reaction—*viz.*, its nonspecificity for individual steroids and the many compounds that interfere with it, have been thoroughly documented (22–26).

Other analytical methods for steroid analysis are available (27–31). These include spectrophotometry and colorimetry, approaches that possess the same problems in varying degrees as does the blue tetrazolium color reaction, and GLC where the problem is the labile nature of the corticosteroids. If steroid derivatives are formed to ensure thermal stability, complete and specific derivatization must be achieved.

This work describes a study of a liquid–liquid chromatographic method for the analysis of hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate and the basic aqueous decomposition of hydrocortisone. The linearity of the detector response to the concentration of the steroid was confirmed for each steroid and actual commercial products; creams, ointments, lotions, and suppositories were analyzed for the presence, identification, and quantity of foreign steroids and for the main steroid component in terms of the single steroid assay value. This method was fast, accurate, and specific for each individual steroid studied.

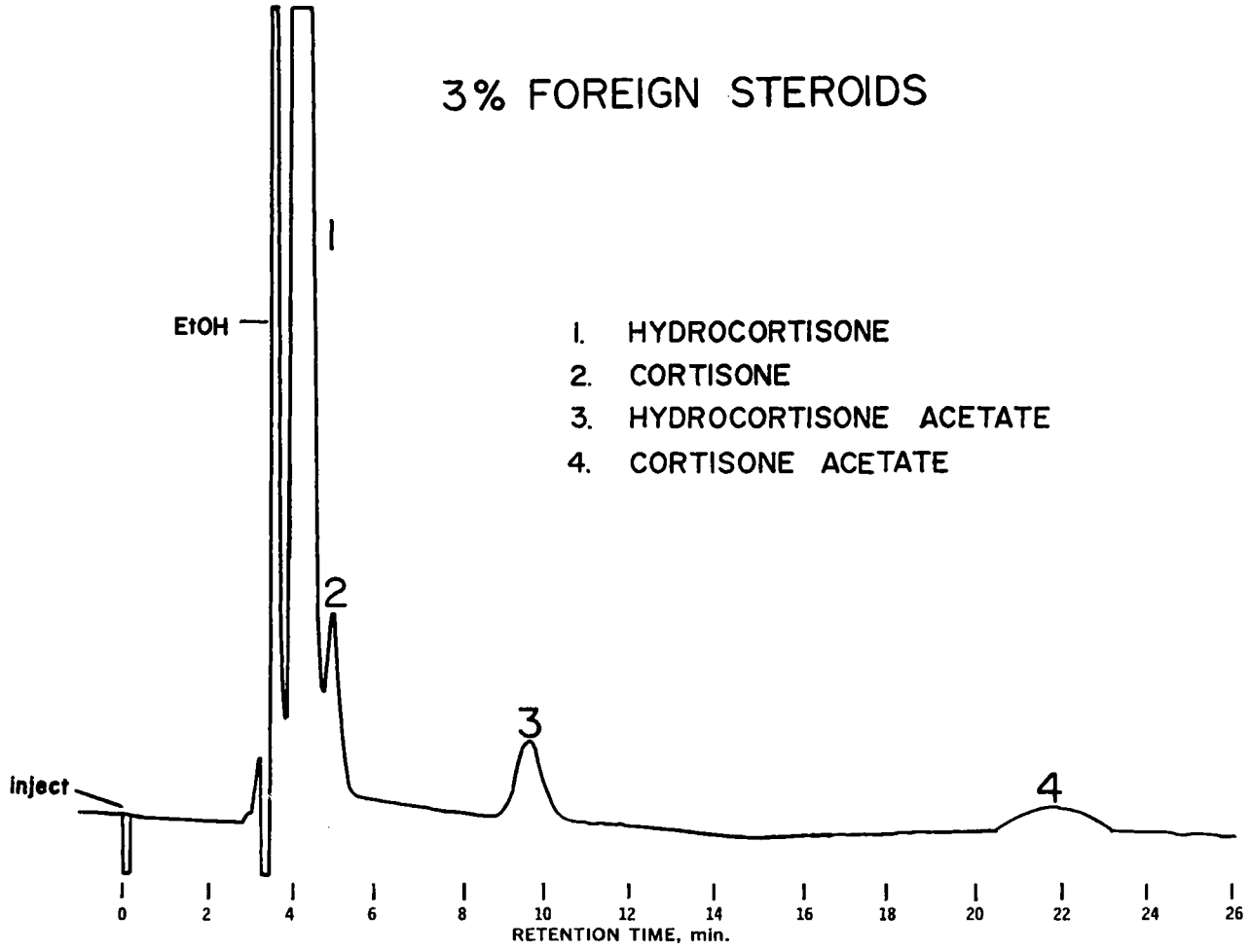

Figure 1—*Chromatogram of standards, 3% (w/w) cortisone, hydrocortisone acetate, and cortisone acetate, based on 6 mg. of hydrocortisone on the cyano ethyl silicone column illustrating foreign steroids test. Mobile phase = 1% methanol in water, attenuation = 0.02 aufs., pressure = 500 psig., flow rate = approximately 0.5 ml./min., and temperature = ambient.*

EXPERIMENTAL

Chemicals, Apparatus, and Instrumentation—Solvents were at least reagent grade and the steroid standards were either official grade (USP or NF) or working grade with a purity greater than 99.0%. Ethanol refers to 95% ethanol (USP grade).

All injections were made with either a 5-μl. high-pressure syringe[1] or a 10-μl. syringe[1] equipped with a Kel-F guide[1].

A liquid–liquid chromatograph[2] equipped with a precision UV photometer set at 254 nm. was used. The columns were precision-bore stainless steel, 1 m. × 2.1 mm. i.d., commercially[3] packed with either a cyano ethyl silicone[4] coated 1% on Zipax or an octadecyl silane[5] permanently bonded to Zipax.

Unless otherwise noted, the instrument parameters for the cyano ethyl silicone column were as follows: pressure, 500 psig. (flow rate approximately 0.5 ml./min.); temperature, ambient (approximately 25°); and mobile phase, water containing about 1% methanol.

Method for Linearity Study—The four steroids were studied on the cyano ethyl silicone column. It was convenient to prepare only two solutions (I and II), both in ethanol, containing all of the steroids in combination with the chosen internal standard; the latter, for convenience, was one of the steroids itself. The concentration range (in micrograms per milliliter) over which each steroid was studied was: Solution I—hydrocortisone, 500–15.6; cortisone 500–15.6; and hydrocortisone acetate, 1500–46.9; and Solution II—hydrocortisone acetate, 1000–31.2; and cortisone acetate, 2000–62.5.

Solution II was injected at a higher pressure, 1200 psig., to shorten the retention time of the latest eluting compound, cortisone acetate. Varied amounts of each dilution of the two solutions were injected into the liquid chromatograph in a pattern such that the concentration level of each solution was half of the concentration of its predecessor. As a result, to maintain a relatively constant detector response, a reciprocal attenuator setting was chosen for the recorder. The actual injection scheme for each solution is presented in Table I. The peak heights were measured for each compound.

Decomposition of Hydrocortisone—A 1.00-ml. volume of a 1-mg./ml. solution of hydrocortisone standard in ethanol was pipeted into 11 10-ml. volumetric flasks. Then 1.00 ml. of a 0.05 *N* aqueous sodium hydroxide solution was added. After sitting at room temperature for specific periods, 1.00 ml. of 0.05 *N* aqueous hydrochloric acid was added to quench the reaction. The solutions were diluted to volume with ethanol, and multiple injections of each solution were made on the cyano ethyl silicone column. As an index of steroid remaining, the hydrocortisone peak height was measured for each injection and the average peak height for each decomposition time was calculated.

Sample Analysis: Foreign Steroids and Assay—Random samples of creams, ointments, suppositories, and lotions containing hydrocortisone, hydrocortisone acetate, and cortisone acetate were obtained. The sample preparation was identical for each dosage form, with the only differences being the sample size for each different steroid and their respective internal standards. The following was the generalized sample preparation procedure used.

A sample equivalent to 6 mg. of hydrocortisone or cortisone, 12 mg. of hydrocortisone acetate, and 25 mg. of cortisone acetate

[1] Hamilton Co., Whittier, CA 90608
[2] Du Pont model 820.
[3] Instrument Products Division, E. I. Du Pont de Nemours & Co., Wilmington, DE 19898
[4] ANH.
[5] ODS.

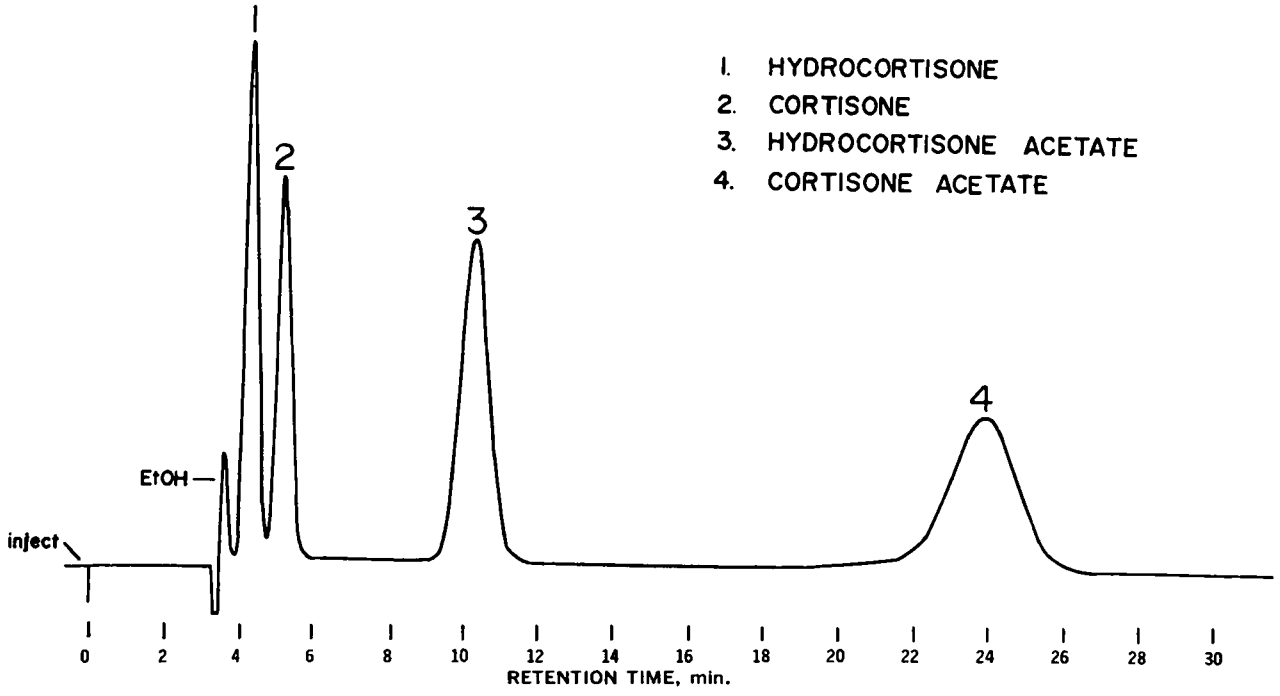

Figure 2—*Chromatogram of standards, hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate, on the cyano ethyl silicone column. Mobile phase = 1% methanol in water, pressure = 500 psig., flow rate = approximately 0.5 ml./min., and temperature = ambient.*

was accurately weighed into a 50-ml. glass-stoppered centrifuge tube. A 30-ml. volume of hot ethanol was added and the tube was shaken by hand or held on a mixer[6] for a few minutes, heated on a steam bath, and mixed again. If the sample dissolved totally or formed a cloudy suspension without any solid material remaining on the bottom of the tube, it was ready for the next step of the procedure. Further treatment was required if the ethanol was not dispersed into the semisolid base. This additional treatment included reheating, additional shaking, or ultrasonic agitation. After the solubilization step, the sample was allowed to cool, overnight if convenient, to allow the excipient materials to settle out; the tube was then centrifuged at about 1500 r.p.m. for 20 min.

To determine the foreign steroids content, this solution was injected onto the cyano ethyl silicone column at a recorder attenuation of 0.02 absorbance unit full scale (0.02 aufs.), a value four times as sensitive as the one used for the assay procedure (0.08 aufs.). A foreign steroid standard was prepared, which in the case of hydrocortisone was based on a sample size equivalent to 6 mg. of this steroid, containing each contaminant 3% by weight of the subject steroid. The chromatogram of this standard is shown in Fig. 1. In preparing the standards for the other three steroids, it was just a matter of varying the amounts of each of the four steroids, since they are cross-contaminants for each other. By using the standard chromatogram as a guide, the approximate level of each contaminant was determined.

If it appeared that the foreign steroids level was excessive, a more accurate quantitative determination could be made by increasing the sample size or by injecting the sample at a higher sensitivity. (The maximum sensitivity for the instrument used in these studies was 0.005 aufs.).

To carry out the assay, equal amounts of the appropriate internal standard were added to both the sample and a standard preparation and equal volumes of both solutions were injected. If the steroid to be used as an internal standard is found to be a foreign steroid contaminant, another steroid may be chosen as an internal standard. The assay value was calculated using peak height measurements and Eq. 1:

$$\text{percent steroid in sample} = \frac{PH_{spl}}{PH_{std}} \times \frac{PH^{I.S.}_{std}}{PH^{I.S.}_{spl}} \times \frac{wt_{std}}{wt_{spl}} \times 100 \quad \text{(Eq. 1)}$$

where:

PH_{spl} = peak height of sample
PH_{std} = peak height of reference standard
$PH^{I.S.}_{std}$ = peak height of internal standard in the standard
$PH^{I.S.}_{spl}$ = peak height of internal standard in the sample
wt_{std} = weight of standard
wt_{spl} = weight of sample

RESULTS AND DISCUSSION

Column Selection—The principal task was to find a column that would yield the best separation of the four corticosteroids in the shortest time. The first column examined, a cyano ethyl silicone column, satisfied these criteria (Fig. 2). The numbers of theoretical plates calculated for each compound were: hydrocortisone, 1254; cortisone, 1002; hydrocortisone acetate, 907; and cortisone acetate, 741. Of the four compounds, hydrocortisone had the lowest affinity for the column, which functions on the reverse-phase principle (10, 32); the most polar mobile phase, water, was required for retention of this compound. Lowering the polarity of the mobile phase by adding alcohol produced shorter retention times for all four compounds, indicating that the optimum mobile phase was water. However, the addition of a small amount of methanol (about 1%) was necessary for column stability.

Table I—Injection Scheme for Linearity Study

Injection Volume, μl.	Recorder Attenuation, aufs. 0.64	0.32	0.16	0.08	0.04	0.02
	Solution I					
5	x[a]	x	x			
3	x	x	x	x	x	x
1	x					
	Solution II					
10	x	x	x			
8	x	x	x			
6	x	x	x	x	x	x
4	x	x	x	x	x	x
2	x	x	x	x	x	x

[6] Vortex, Thomas Scientific Apparatus, Philadelphia, PA 19105

[a] x denotes an injection.

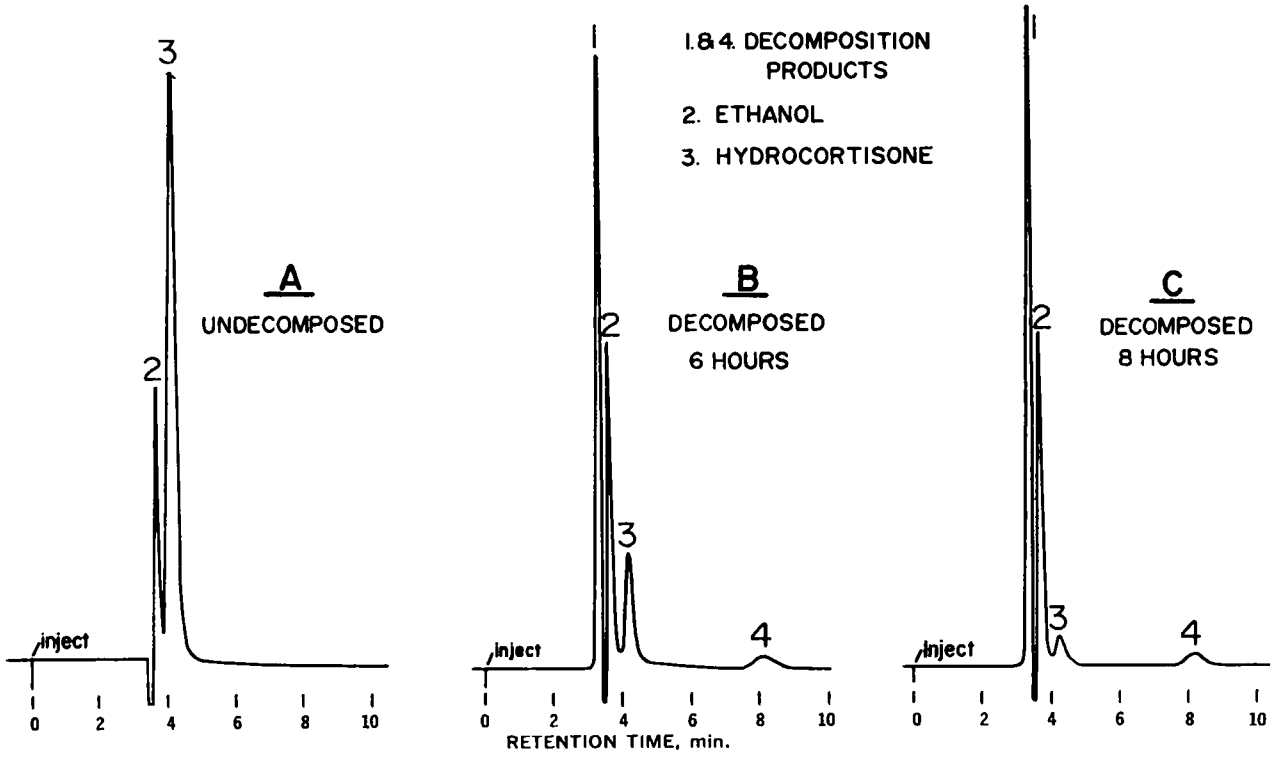

Figure 3—*Decomposed standards on the cyano ethyl silicone column. Conditions were the same as in Fig. 2, and attenuation = 0.08 aufs.*

The second column material evaluated was octadecyl silane[7]. This material, which also functions by reverse-phase chromatography, yielded the same elution order as the cyano ethyl silicone column with one exception; hydrocortisone and cortisone were not resolved. As before, water was the best mobile phase, in conjunction with a column temperature of 60° as recommended (33) and a pressure of 700 psig. Due to the need for elevated temperatures, a capability that not all instruments possess, and its inability to separate two steroids, this column was considered as second choice to the cyano ethyl silicone column for this study.

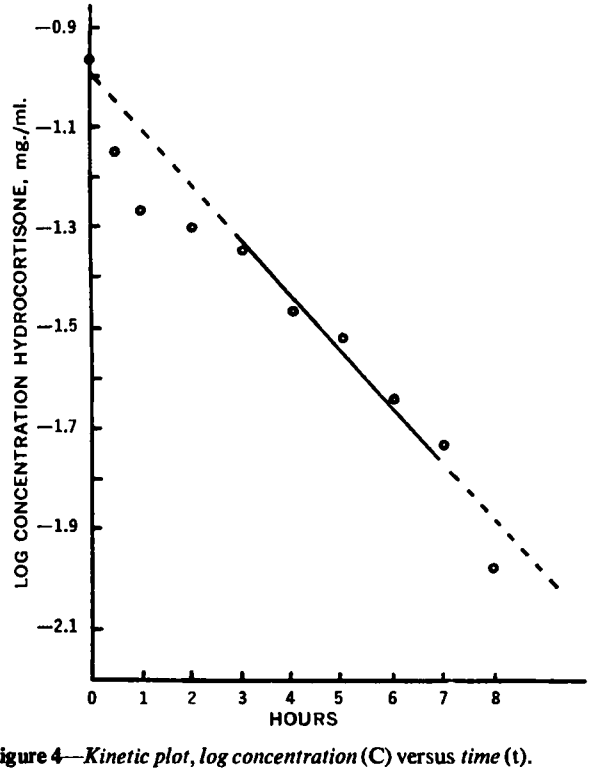

Figure 4—*Kinetic plot, log concentration* (C) versus *time* (t).

Peak Height Measurement and Linearity of Response—In all of the work presented, the quantity of active ingredient and foreign steroids was determined by using peak height measurement. While this property is most frequently used, it is recognized that a better measurement of material eluted is represented by the area under the peak. The use of peak heights is made assuming that the peak widths at half-height are equal for the same compound under the same instrumental conditions, thereby establishing the proportionality of the area to peak height. The peak height measurement was used for convenience and because the early eluting peaks have very narrow widths at half-height, making measurement of this band difficult and susceptible to large relative errors.

The relationship of detector response to the quantity of steroid was determined. This task was made more complex since the use of an internal standard in the assay procedure requires that linearity of detector response be established both for the test compound and the internal standard. To simplify the problem, the steroids were used as the internal standards for each other in the following arrangement: for hydrocortisone, cortisone, and cortisone acetate, the internal standard was hydrocortisone acetate; for hydrocortisone acetate, the internal standard was cortisone. By examining the chromatograms of Solutions I and II, it was simply a matter of measuring the peak heights for each compound and calculating the ratios of the subject steroid to the internal standard for the different pairs at each injection.

To simplify the experimental manipulations, serial dilutions of Solutions I and II were made according to the scheme summarized in Table I. The subsequent dilutions of the parent solution should have produced the same ratios for each pair if Beer's law is followed at 254 nm. over the concentration range studied. The calculated ratios are presented in Table II, columns A–F. It is apparent from columns C and E that different injection volumes of the same solution produce varying peak height ratios. When the injection volume (column C) was kept constant and the concentration was varied (column D), a relatively constant value was obtained (column E); the relative average deviation of the ratios for the different concentrations at a constant injection volume are shown in column F. From the data in

[7] Permaphase.

Table II—Results of Linearity Study

	A Subject / Internal Standard	B Retention	C Injection Volume, μl.	D Number of Different Concentrations Studied	E Average of Ratios of Peak Heights	F Reproducibility of Ratios for a Single Injection Volume in Relative Average Deviation Units, %[c]
1[a]	Hydrocortisone / Hydrocortisone acetate	55 mm. / 122 mm.	5	3	0.80	0.6
			3	6	0.92	1.2
			1	1	1.02	
2[a]	Cortisone / Hydrocortisone acetate	64 mm. / 122 mm.	5	3	0.64	1.6
			3	6	0.67	0.9
			1	1	0.69	
3[b]	Cortisone acetate / Hydrocortisone acetate	112 mm. / 50 mm.	10	3	0.82	
			8	3	0.82	
			6	6	0.82	
			4	6	0.81	
			2	6	0.80	
4[b]	Hydrocortisone acetate / Cortisone acetate	50 mm. / 112 mm.	10	3	1.21	0.9
			8	3	1.22	0.4
			6	6	1.22	0.5
			4	6	1.23	0.2
			2	6	1.25	0.3

[a] Ratios 1 and 2 were calculated from the injections of Solution I. [b] Ratios 3 and 4 were taken from the same chromatogram and are reciprocals of each other. Solution II was used in this case and the chromatogram was recorded at 1200 psig. pressure. [c] Relative average deviation =

$$\sum_{i=1}^{n} \frac{[\bar{x} - x_i]}{(n)(\bar{x})} \times 100$$

where $\bar{x}$ = mean, n = number of data, and x_i = datum.

column F, it is apparent that for identical injection volumes the compounds consistently exhibit a linear detector response for the quantity of material being measured over the range examined (35).

Decomposition of Hydrocortisone, Product Separation, and Kinetics—The decomposition of hydrocortisone in basic aqueous medium was studied to ensure the specificity of the column for this compound and its decomposition products. A study was reported (17) on flumethasone pivalate utilizing liquid–liquid chromatography. The decomposition of prednisolone was studied (34) in alkaline solution using chloroform extraction followed by UV spectroscopy. This study is cited because of the structural similarities between prednisolone and hydrocortisone, differing as they do only by a double bond in the A ring.

While the decomposition reaction for hydrocortisone is complex, the major site of reaction is at the C_{17} side chain; reaction rates and decomposition products similar to those found in prednisolone would be expected. The chromatograms obtained by injecting samples of the degraded solutions onto the cyano ethyl silicone column are shown in Fig. 3. Guttman and Meister (34) reported two classes of decomposition products in their study: a small amount of neutral material and a larger amount of acidic material. Chromatograms B and C in Fig. 3 show two additional peaks; peak 1, unretained by the column, is probably the large quantity of acidic material, and peak 4 may correspond to the smaller amount of neutral material. A hydrocortisone standard that was degraded for 72 hr. yielded no peaks at the hydrocortisone retention time using the cyano ethyl silicone column, proving that the decomposition products are separated from the parent steroid. Identification of decomposition peaks was not attempted.

The quantitative data available from the chromatograms (Fig. 3) were interpreted using familiar kinetic concepts. The average peak height for each time was used to calculate concentration (C). Since the molar ratio of sodium hydroxide to hydrocortisone is 1.81×10^4 and since previous work (34) showed the decomposition of prednisolone to be pseudo-first order, a plot of the data was made in terms of log C *versus* time (Fig. 4). A straight line is drawn in the region where five points appear to be linear. When this line is extrapolated back to the axis, the intersection is very close to the experimental value for an undecomposed solution. Similarly, the slope of the straight-line segment yields a rate constant of 0.252 $hr.^{-1}$ at room temperature. This is compared to prednisolone, at the same concentration and in a solution of the same pH as in this work, for which a rate constant of 0.293 $hr.^{-1}$ was reported (34) at 35°, a comparable but higher value than that for hydrocortisone, to be expected for a higher temperature.

The scatter of points in Fig. 4 introduces some doubt as to the reaction order. The main scatter is at the initial times where the concentrations are changing most rapidly; these points are not considered in the construction of the straight line. On the other hand, the extrapolated value (−0.99) resulting from the straight line agrees well with the experimental result (−0.97), and the agreement of the slope with that reported (34) indicates that the line is not of fortuitous origin. It may be that additional work could result in a more linear overall response, but, in any case, the method separates the decomposition products from the parent molecule effectively, a desirable attribute for any analytical approach.

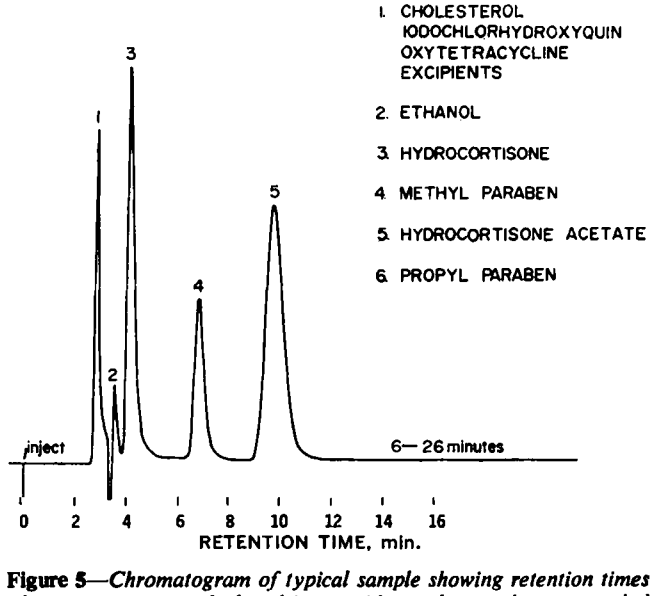

Figure 5—*Chromatogram of typical sample showing retention times of common compounds found in steroid samples on the cyano ethyl silicone column under same conditions as in Fig. 3.*

Table III—Assay Results of Commercial Samples

Number	Active Ingredient, Hydrocortisone, %	Product Type	Other Active Ingredients	Assay, % of Declared: Liquid–Liquid Chromatography	Assay, % of Declared: Other Methodology
1	0.25	Ointment		97.6	
2	0.25	Cream	Iodochlorhydroxyquin	79.8	78.2[a], 77.2[b]
3	0.25	Cream		100.8	
4	0.25	Cream		100.0	102.0[c]
5	0.5	Ointment		99.9	
6	0.5	Ointment		94.6[d]	91.8[e], 82.0[e]
7	0.5	Cream	Iodochlorhydroxyquin	102.0	
8	0.5	Cream	Iodochlorhydroxyquin	93.2	
9	0.5	Cream	Iodochlorhydroxyquin	94.6	91.6[f]
10	0.5	Cream	Iodochlorhydroxyquin	96.1	
11	0.5	Cream	Diiodohydroxyquin	96.8	
12	0.5	Cream		98.2	
13	0.5	Cream		99.0	105.0[c]
14	0.5	Lotion		106.2	
15	1	Ointment	Oxytetracycline	97.0	
16	1	Ointment	Oxytetracycline	99.4	
17	1	Ointment		98.4	
18	1	Ointment		94.4	
19	1	Ointment		105.7	101.0[g]
20	1	Cream		103.9	
21	1	Cream		97.5	
22	1	Cream	Iodochlorhydroxyquin	104.9	
23	1	Cream	Iodochlorhydroxyquin	91.0	
24	1	Cream	Iodochlorhydroxyquin	95.5	
25	2.5	Ointment		103.2	100.3[c]
26	25 mg.	Hydrocortisone acetate suppositories		98.4	92.5[f]
27	1.5	Cortisone acetate ointment		100.7	104.7[f]

[a] Alcohol extraction, blue tetrazolium (average of two). [b] Official NF XIII procedure. [c] Blue tetrazolium (average of three in Ointment 6). [d] Average of five results. [e] Red tetrazolium. [f] Isoniazid. [g] Fluorometric.

Procedural Format—The procedure for foreign steroids and assay covers situations where both detection of undesirable steroids and measurement of the major component are accomplished. Because of the nature of the assay and its use of internal standards, the procedure should be followed as written. The determination of foreign steroids not only accomplishes the accurate detection of the related but undesirable steroidal impurity, but it assures the analyst that the elution region in which the internal standard is to emerge is vacant and not occupied by some unanticipated compound. Clearly, the foreign steroids procedure provides for the validity of the assay which follows.

Analysis of Dosage Forms—A number of pharmaceutical dosage forms were analyzed by the proposed method. This procedure is capable of identifying and quantitatively measuring the main component and each foreign steroid, if present. By using the cyano ethyl silicone column, it was confirmed that none of the products in Table III had excessive foreign steroid levels based on the tolerances in the official compendia for tablet formulations. In most cases, however, some foreign steroids were found at the 1% level. When samples were analyzed which were manufactured by the same company and differed only in the strength of the active ingredient, it was observed that the same foreign steroids were present in each product at identical levels, indicating that the steroid raw material for the products probably came from the same lot or process. This procedure would be most effective for certifying the purity of raw material steroids.

The internal standards were used to monitor and determine the sample preparation content. In determining the ratio of internal standard to steroid needed for equivalent responses, the approximate peak height response ratio used was 1:1:3:6 for hydrocortisone, cortisone, hydrocortisone acetate, and cortisone acetate, respectively. If internal standards are not available, the assay procedure may be altered to require dilution to a definite volume and multiple injections. The average peak heights would then be used to determine the assay value. This procedure would probably be less accurate but would suffice in most cases.

Assay results for the products analyzed on the cyano ethyl silicone column are given in Table III. Results achieved by other analytical methods for some samples are included for comparison. Only Sample 2 fell outside of the official limits; it was assayed by two other procedures that showed very good correlation with the proposed procedure. Sample 6 was analyzed by two different chemists using the blue and red tetrazolium procedures which yielded erratic results. This sample was assayed five times by liquid–liquid chromatography using five different sample weights from three tubes of the same code, with the results of 92.6, 96.6, 91.6, 95.6, and 97.2% of declared. The average deviation of these five results is 2.1%, which can be attributed in part to slight sample inhomogeneity but proves that it is not at the high level indicated by the tetrazolium results. One sample of hydrocortisone tablets was assayed on the octadecyl silane column and yielded no extraneous peaks other than the product peak. Due to the similarity of this and the cyano ethyl silicone columns, *i.e.*, they are both nonpolar and operate on the reverse-phase principle, it would be safe to assume that tablet formulations would provide no interferences on the latter column.

Interferences—The main problem in any chromatographic procedure is interfering compounds. In analyzing all of the products presented in Table III, none of the active ingredients in combination with the steroids interferes with the steroid peak but a problem may be encountered because of the peak ascribable to the cream, ointment, suppository, and lotion *base* or other formulation components. Most of this material is unretained by the column. In some cases where a large amount of excipient material comes through the extraction procedure, a broad peak is obtained. This results in a subsequent loss of resolution between the hydrocortisone and excipient peak. When this situation arises, care must be taken in constructing the baseline for the hydrocortisone. Products containing the other steroids do not present this problem owing to the longer retention time. Figure 5 presents a general chromatogram indicating the elution behavior of various compounds found in combination with the steroid.

This procedure appears to be applicable to other steroids that are accompanied by foreign steroid contamination. With this type of procedure, not only may the main steroid be quantitatively measured but each individual contaminant can be identified and quantitatively analyzed.

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PHARMACEUTICAL TECHNOLOGY

Tooling as a Factor in Tablet Weight Variation and Control

WILFRED C. LING

Abstract □ The importance of punch length uniformity to the control of tablet weight variation was demonstrated in a rotary tablet press. An assessment of the weight variation of single-station compression on the multiple-station tablet press was obtained by means of a special isolation technique involving a force-actuated weight control device. Analysis of the relation between punch length and compression force pointed to the uncertainty of the relationship between tablet weight and compression force when variations of upper and lower punch lengths are considered. Control of tablet weight based on compression force signal suffers from this uncertainty, as demonstrated by experimental data.

Keyphrases □ Tablet weight variation—statistical contribution of individual tool parameters of rotary press □ Weight variation, tablets—statistical contribution of individual tool parameters of rotary press □ Tool parameters of rotary press—effect on tablet weight variation □ Punch length uniformity, rotary tablet press—effect on tablet weight variation

A foremost concern in the manufacture of tablets is the assurance of tablet weight uniformity. Studies of tablet weight variation on single-punch tablet machines (1, 2) have generally attributed the weight variation to nonuniform filling of the dies caused by variations in such parameters as granule size and distribution, bulk density, and flow properties of the granulations. In the case of the multiple-station rotary press, however, an