High-Performance Liquid Chromatographic Determination of Hydrocortisone Cypionate: Method Development and Characterization of Chromatographic Behavior

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Abstract □ A reversed-phase high-performance liquid chromatographic method for hydrocortisone cypionate bulk drug and oral solution was developed that avoids the use of a heated column as described in USP XX. A study of the effect of the organic modifier concentration on the capacity factor suggests that mixed partition and adsorption phenomena are responsible for the retention of several steroids when acetonitrile is used in the mobile phase. Evidence is presented that hydrogen bonding of the solute molecule with silane hydroxyl groups may be responsible for the adsorption.

Keyphrases □ Hydrocortisone cypionate—high-performance liquid chromatographic analysis, bulk drug and oral solution, chromatographic behavior evaluated □ High-performance liquid chromatography—hydrocortisone cypionate in bulk and oral solution, chromatographic behavior evaluated □ Adsorption—effect of organic modifier on steroid retention, high-performance liquid chromatographic analysis of hydrocortisone cypionate

Hydrocortisone cypionate[1] (21-cyclopentylpropionyl ester of hydrocortisone, I) is described in USP XX (1) and is marketed as an oral suspension[2]. The compendial assay for potency requires a 1-m column packed with macroparticulate octadecylsilane and heated to 60°. Under the conditions employed in this method [mobile phase of 25% (v/v) methanol in water], the analyte does not elute in a convenient time at 25°. Although heating to 60° allows for an acceptable analysis time, it is inconvenient since a column oven capable of accepting a 1-m column is necessary.

A stability-indicating assay was developed that utilizes a microparticulate octadecylsilane column at ambient temperatures. The chromatographic behavior of I along with other nonpolar steroids was examined. Evidence is provided that suggests that the separations are not governed totally by partition mechanisms.

BACKGROUND

Hydrocortisone cypionate (I) is derived from the condensation of hydrocortisone alcohol and a cyclopentylpropionic acid derivative. Consequently, hydrocortisone represents a potential process impurity. Furthermore, hydrocortisone is a potential degradation product arising from ester hydrolysis. The C-11 oxidation product, cortisone cypionate (II), is another potential degradation product. The conversion of 11β-ols to 11-ones by air oxidation in the crystalline state first was observed in C-20-epimeric 11β-hydroxy-17,20-acetonido-21-acetates (III) (2).

In this study, it was observed that the 21-acetoxy group was necessary for air oxidation of the 11β-ol since the corresponding 21-ol was stable. This phenomenon also was observed in samples of hydrocortisone 21-*tert*-butyrate, hydrocortisone 21-valerate, hydrocortisone 21-stearate, and hydrocortisone 21-cypionate (3). However, oxidation of the 11β-ol was not observed or induced in aged samples (15–17 years old) of the 21-pivalyl, acetyl, and propionyl esters of hydrocortisone. The crystalline steroid form played a major role in sensitivity to air oxidation. Consequently, a stability-indicating assay should distinguish between hydrocortisone cypionate and its possible degradation products, hydrocortisone and cortisone cypionate.

The retention of nonpolar steroids, such as I and II, in reversed-phase high-performance liquid chromatographic (HPLC) systems generally is held to be due to the distribution of the analyte between the stationary and mobile phases. In such cases, the capacity factor, k' (also called the capacity ratio), is given by:

$$k' = \frac{[X]_s}{[X]_m}\frac{V_s}{V_m} \qquad \text{(Eq. 1)}$$

where $[X]_s$ and $[X]_m$ represent the analyte concentration in the stationary and mobile phases, respectively, and V_s and V_m are the total volumes of each phase in the column. This equation assumes ideal behavior in dilute solutions and, hence, neglects activity coefficients. Solubility parameter theory (4, 5) states that the distribution of the analyte between the two phases is related to the Hildebrand solubility parameters of the analyte (δ_x), stationary (δ_s), and mobile (δ_m) phases and to the total volume of the analyte ($\overline{V}_x$) as shown by:

$$\log\frac{[X]_s}{[X]_m} = \overline{V}_x\frac{[(\delta_x - \delta_m)^2 - (\delta_s - \delta_x)^2]}{2.3RT} \qquad \text{(Eq. 2)}$$

where R is the gas constant and T is the absolute temperature.

The δ values vary linearly with the solvent composition for binary solvent mixtures. Thus, the solubility parameter for the mobile phase can be expressed as:

$$\delta_m = \delta_a + (\delta_b - \delta_a)C \qquad \text{(Eq. 3)}$$

where δ_a and δ_b are the solubility parameters of the two components of the solvent mixtures and C is the concentration (mole fraction) of solvent b in this mixture (solvent b is the stronger solvent in terms of eluting power). Substitution of Eq. 3 into Eq. 2 gives:

$$\log k' = \log\frac{V_s}{V_m} + \frac{\overline{V}_x}{2.3RT}[(\delta_x - \delta_a)^2 - (\delta_s - \delta_x)^2] - \frac{2\overline{V}_x}{2.3RT}(\delta_x - \delta_a)(\delta_b - \delta_a)C + \frac{\overline{V}_x}{2.3RT}(\delta_b - \delta_a)^2C^2 \qquad \text{(Eq. 4)}$$

By assuming that the term containing the C^2 term can be ignored, a first-order approximation can be written as:

$$k' = k_0'10^{-nc} \qquad \text{(Eq. 5)}$$

where k_0' and n are constants that contain the terms V_s, V_m, $\overline{V}_x$, δ_x, δ_a, δ_s, and $2.3RT$ (6).

Equation 5 suggests that a plot of $\log k'$ *versus* C will be linear with a slope of $-n$ and an intercept of $\log k_0'$. This behavior was observed in a study of the elution of 2-ethyl-9,10-anthraquinone, chloronaphthalene, halogenated benzenes, fused-ring aromatics, and phthalate esters using methanol–water and dioxane–water mobile phases (7).

EXPERIMENTAL

Materials—Medroxyprogesterone acetate, hydrocortisone cypionate (I), hydrocortisone, hydrocortisone acetate, and methylprednisolone acetate were USP or NF quality. Cortisone cypionate (II) was generated *in situ* from an aged sample of I. Fractions of the eluate containing the II peak were collected and dried. Electron-impact mass spectrometry of the residue gave a molecular ion at m/z 484, consistent with that of II. Methanol and acetonitrile were glass distilled[3]. Deionized water was used for all mobile phases.

Mobile Phases—All mobile phases were prepared by measuring the

[1] Currently described as cortisol cypionate in USP XX.
[2] Fluid CORTEF, The Upjohn Co.
[3] Burdick & Jackson, Muskegon, Mich.

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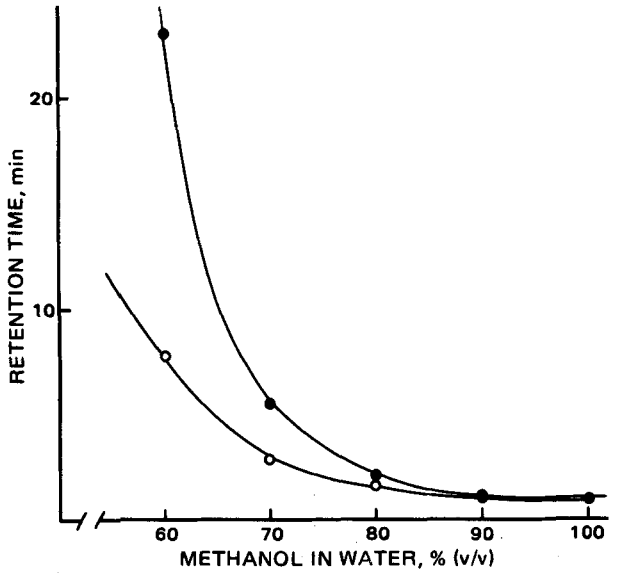


Figure 1—*Effect of the methanol concentration (percent volume to volume) in the mobile phase on the retention times of hydrocortisone cypionate (●) and medroxyprogesterone acetate (○).*

required quantity of the organic modifier in a 1-liter graduated cylinder followed by the addition of water to ~95% of the final volume. The mixtures then were allowed to equilibrate to room temperature before the final adjustment to volume was made. Degassing of the mobile phases was not necessary. To determine the mole fraction of the organic modifier in the mobile phase, water was added from a container of known volume so that volume changes due to mixing could be taken into account.

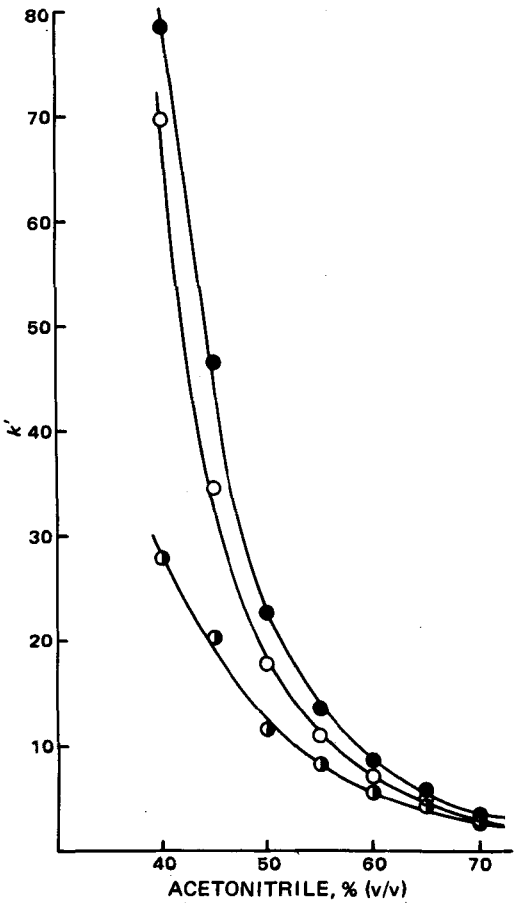
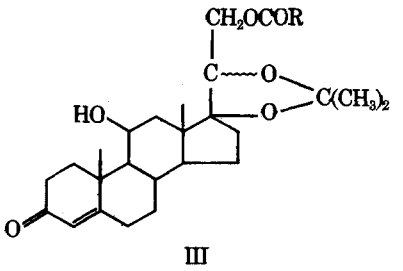


Figure 2—*Effect of the acetonitrile concentration on the capacity factors for medroxyprogesterone acetate (◑), hydrocortisone cypionate (○), and cortisone cypionate (●).*



III

Instrumentation—The HPLC system was described previously (8).

Quantitative Procedure for I in Oral Suspensions—A volume of the suspension, equivalent to 12–15 mg of I, was transferred accurately to a suitable container. The internal standard solution (15.0 ml, 0.4 mg of medroxyprogesterone acetate in acetonitrile/ml) was added, and the solution was shaken for 90 min. The phases were allowed to separate, and 25-μl aliquots of the clear supernate were chromatographed using a mobile phase of 50% (v/v) acetonitrile in water at a flow rate of ~2.0 ml/min. Samples of bulk drug were analyzed by mixing an accurately weighed quantity of drug (~15 mg) with 15.0 ml of the internal standard. Calculations are based on the measurement of the peak height ratios of a sample preparation to those of a standard preparation.

RESULTS AND DISCUSSION

The initial purpose of this study was to develop a stability-indicating method that did not require a 1-m column heated to 60°. A study of the retention times of medroxyprogesterone acetate and I as a function of the methanol concentration in the mobile phase (at ambient temperatures) is shown in Fig. 1. While adequate resolution of the internal standard (medroxyprogesterone acetate) and I could be achieved at methanol concentrations of 65–70% (v/v), II was not even partially resolved from I until the methanol concentration was lowered to 50% (v/v). However, analysis times became prohibitively long with the microparticulate (10 μm) octadecylsilane[4] column (30 cm × 4 mm). At this point, methanol as an organic modifier was abandoned in favor of acetonitrile.

Acetonitrile gave adequate resolution of I, II, and the internal standard at a 50% (v/v) concentration (the initial concentration examined). A more

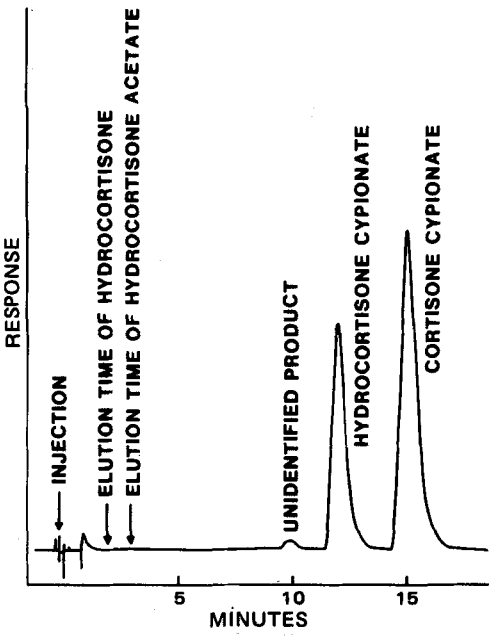


Figure 3—*Chromatographic tracing of hydrocortisone cypionate and cortisone cypionate showing the elution times of hydrocortisone and hydrocortisone acetate. Under these conditions, medroxyprogesterone acetate eluted at 8.8 min.*

[4] μBondapak C_{18}, Waters Associates, Milford, Mass.

Table I—Linear Regression Data for Plot of log k' *versus* log C for Medroxyprogesterone Acetate, Hydrocortisone Cypionate (I), and Cortisone Cypionate (II)

Compound	Concentration of Acetonitrile in Mobile Phase					
	Percent (Volume to Volume)			Mole Fraction		
	Slope	Intercept	Correlation Coefficient	Slope	Intercept	Correlation Coefficient
Medroxyprogesterone acetate	−4.08	8.00	0.9981	−2.60	−0.489	0.9986
I	−5.40	10.47	0.9985	−3.44	−0.780	0.9948
II	−5.56	10.82	0.9991	−3.55	−0.763	0.9981

complete study on the effect of the acetonitrile concentration indicated that adequate separations and reasonable retention times could be achieved between 45 and 55% (v/v) (Fig. 2). For example, under the final analytical conditions chosen (50% acetonitrile–water), II eluted within 20 min (Fig. 3). A plot of log k' *versus* acetonitrile concentration (percent volume to volume) shows nonlinear behavior (Fig. 4). Equation 5 predicts that this plot should be linear. A similar plot using mole fraction on the x-axis displayed the same behavior. The data then were plotted in a log–log fashion. Excellent linearity was achieved (Table I) for acetonitrile expressed both as percent (volume to volume) and mole fraction. The same linear correlation of log k' *versus* C was obtained on two different columns from the same manufacturer.

To characterize further the correlations observed, a similar study was performed on three more polar steroids: hydrocortisone, hydrocortisone acetate, and methylprednisolone acetate. The plot of log k' *versus* acetonitrile concentration (percent volume to volume) in the mobile phase for these three steroids is shown in Fig. 5. Once again, distinct curvature is observed whereas the data plotted as log k' *versus* log C give an excellent linear fit (Table II). These data suggest mechanisms other than retention by partitioning between the two phases.

A study using methanol as the organic modifier was performed to make sure that the observed behavior was not an artifact of the columns. In this case, excellent correlations of log k' *versus* methanol concentration (percent volume to volume) were obtained (Fig. 6 and Table III). This behavior is as predicted by Eq. 5.

The behavior observed for acetonitrile is similar to many instances observed for adsorption chromatography. In adsorption chromatography, the distribution coefficient in a binary solvent system has been described as:

$$\log D_{ab} = \log D_a + \bar{\alpha} A_s(\epsilon_a^0 - \epsilon_{ab}^0) \qquad \text{(Eq. 6)}$$

where D_{ab} is the distribution coefficient in the binary solvent system, D_a is the distribution coefficient in a single solvent system a, $\bar{\alpha}$ is a measure of the adsorbent activity, A_s is the molecular area of the adsorbed sample molecule on the adsorbent surface, and ϵ^0 is the solvent strength parameter describing the influence of the solvent on adsorption (9). The term ϵ^0 is independent of the nature of the adsorbent and the sample. Solvent b is assumed to be a much stronger eluent than a ($\epsilon_b^0 > \epsilon_a^0$).

Equation 6 was manipulated and simplifed to give:

$$k' = k_0' C^{-n} \qquad \text{(Eq. 7)}$$

where k' is the capacity factor in the binary solvent system, k_0' is the capacity factor in solvent system b, and n is A_s/n_b (n_b is the effective molecular area of an adsorbed solvent molecule b). The relationship expressed in Eq. 7 was verified for lumisterol, tachysterol, calciferol, and ergosterol on Lichrosorb ALOX T with n-propanol–n-heptane mobile phases (10); indole alkaloids on silica with methylene chloride–diethylamine, chloroform–methanol, n-hexane–tetrahydrofuran, and tetrahydrofuran–methanol mobile phases (11); and steroids on silica with mobile phases consisting of 21 different binary mixtures (12).

Table II—Linear Regression Data for Plot of log k' *versus* log C[a] for Hydrocortisone, Hydrocortisone Acetate, and Methylprednisolone Acetate

Compound	Slope	Intercept	Correlation Coefficient
Hydrocortisone	−2.73	4.50	0.9984
Hydrocortisone acetate	−3.53	6.23	0.9993
Methylprednisolone acetate	−4.05	7.20	0.9995

[a] Concentration of acetonitrile in the mobile phase (percent volume to volume).

Table III—Linear Regression Data for Plot of log k' *versus* C[a] for Hydrocortisone, Hydrocortisone Acetate, and Methylprednisolone Acetate

Compound	Slope	Intercept	Correlation Coefficient
Hydrocortisone	−0.047	2.79	0.9982
Hydrocortisone acetate	−0.057	3.31	0.9997
Methylprednisolone acetate	−0.054	3.71	0.9987

[a] Concentration of methanol in the mobile phase (percent volume to volume).

The data presented in this paper suggest that the separation of steroids on certain microparticulate octadecylsilanes may not be due entirely to partitioning mechanisms and that adsorption phenomena may play a role. If so, adsorption phenomena would influence the degree of resolution obtained from such columns with differing carbon coverages of the silica surface. Therefore, different methods of bonding alkyl chains to the surface may give different chromatographic behavior for the same analytes with the same mobile phase when columns from different manufacturers are used. Thus, it is expected that the behavior observed in this study may not be found with columns that have a higher percentage of the silanol sites derivatized. The observed behavior is probably typical of column materials with a low carbon load (<10%).

The facile separation of I and II in mobile phases containing acetonitrile can be rationalized by considering an adsorption phenomenon. In some bonded octadecylsilane columns, only a small percentage of the silane hydroxyl groups on the particle surface are bonded covalently to the octadecylsilane group. Consequently, there may be numerous free silanol groups that can interact by hydrogen bonding with the mobile phase and analytes. In methanol–water mobile phases, all solvent molecules have the capacity for hydrogen bonding both as donors and acceptors. Thus, it is expected that interaction of the analyte molecules with the hydroxyl group is essentially swamped by the interaction of the mo-

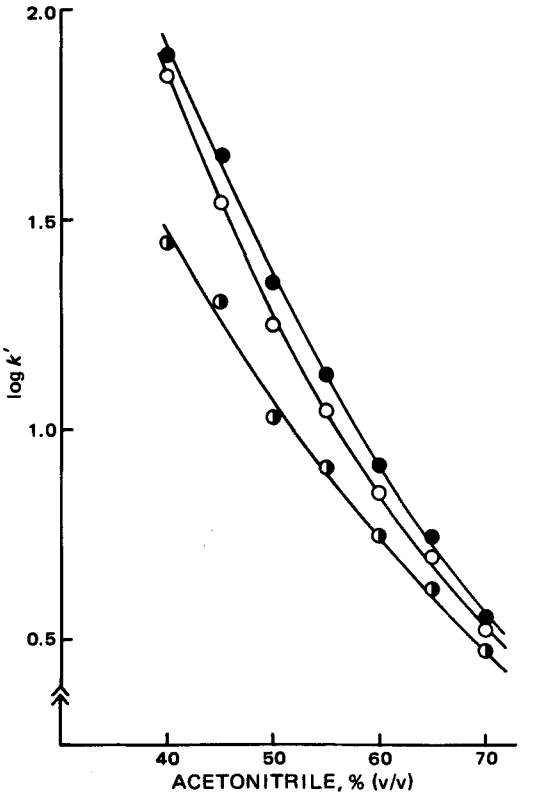


Figure 4—*Relationship of log* k′ *(capacity factor) and acetonitrile concentration for the steroids described in Fig. 2.*

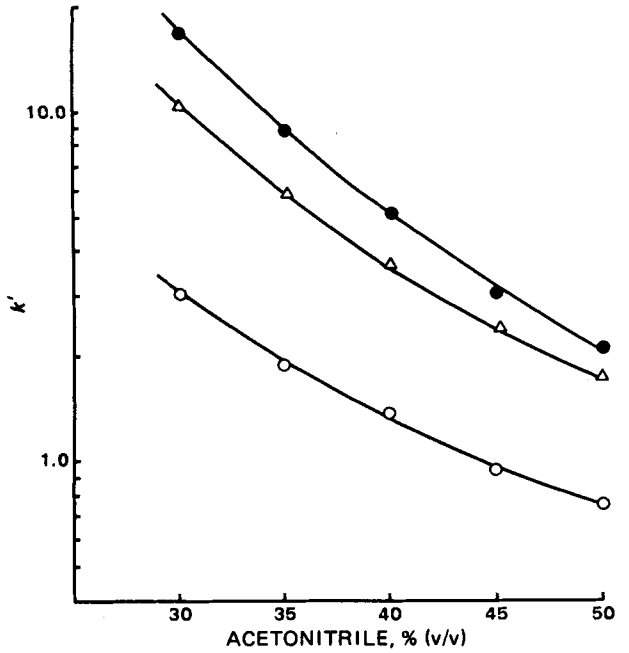


Figure 5—*Relationship of log* k′ *and acetonitrile concentration for hydrocortisone (○), hydrocortisone acetate (△), and methylprednisolone acetate (●).*

bile phase and these hydroxyl groups. Consequently, with methanol–water mobile phases, retention by adsorption mechanisms should not be evident. This behavior was confirmed for hydrocortisone, hydrocortisone acetate, and methylprednisolone (Fig. 7).

On the other hand, acetonitrile is an aprotic solvent and does not participate in hydrogen bonding. At high acetonitrile concentrations, a solvent environment can be created such that hydrogen bonding of the analytes with the silane hydroxyl groups will not be overcome by interactions with the solvents. This is apparently the case for I and II in the mobile phases that contain high concentrations of acetonitrile (in water). In Fig. 4, the curvature is more distinct at higher acetonitrile concentrations. Interaction of both I and II with the silane hydroxyl groups favor their separation since I contains a hydroxyl group at the 11-position while II possesses a keto group at the 11-position. While the 11-hydroxyl group of I can act as a hydrogen bond acceptor, it is expected that the carbonyl oxygen of the 11-keto group of II may be a stronger hydrogen bond acceptor because of the partial polarization of the carbonyl double bond. Thus, the greater retention of II in acetonitrile–water mixtures is reasonable based on these considerations.

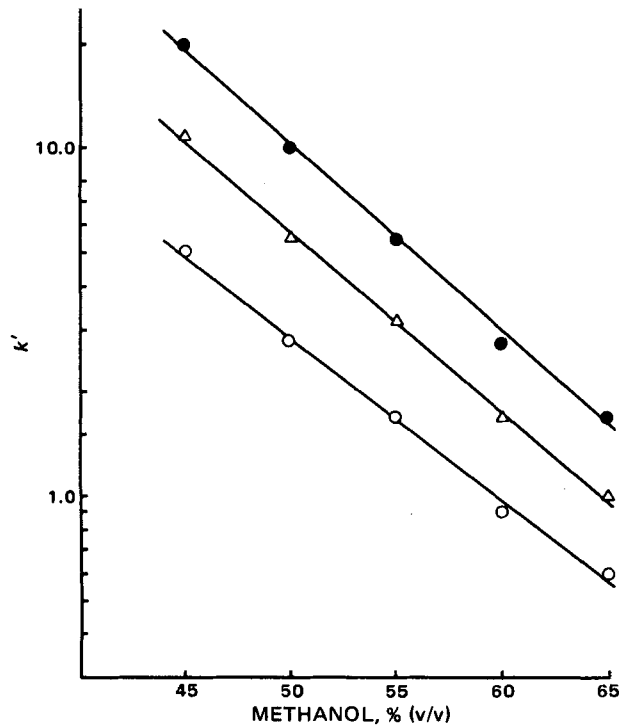


Figure 6—*Relationship of log* k′ *and the methanol concentration for the steroids described in Fig. 5.*

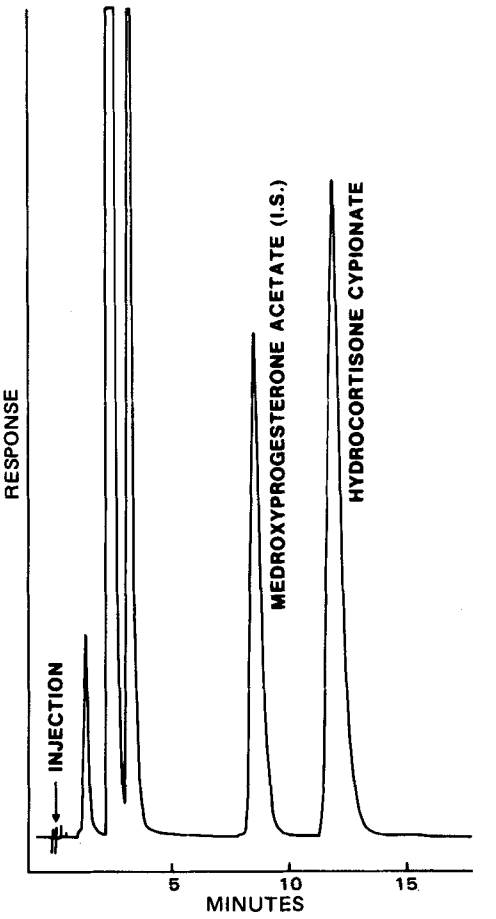


Figure 7—*Chromatographic tracing of typical sample preparation obtained from the oral suspension.*

To develop and validate an analytical method for I, a mobile phase of 50% (v/v) acetonitrile in water was chosen. This mobile phase gave adequate resolution of the analyte, internal standard, potential impurities, and degradation products within a reasonable time (Fig. 3). The unidentified product was present only in the aged sample of I and would not interfere with the internal standard peak that eluted at 9 min.

A chromatographic tracing of a typical sample preparation is shown in Fig. 7. The excipient peaks eluted early and did not interfere. Recovery from a spiked placebo was excellent (Table IV). The spiking levels ranged from 68 to 131% of the labeled amount of I. Replicate analyses of a single lot of oral suspension gave a mean value of 13.3 mg/5 ml with a relative standard deviation of 0.6% (n = 6). Several lots of an aged product (>5 years old) were assayed and were in good agreement with theory (97–101%). Cortisone cypionate was not detected in any sample (detection limit of 0.3%), indicating that conversion by oxidation does not occur in an aqueous environment.

Table IV—Recovery of Hydrocortisone Cypionate (I) from Spiked Placebo

Amount Added, mg/5 ml	Amount Found, mg/5 ml	Recovery, %
9.05	9.11	100.7
10.39	10.39	100.0
13.52	13.50	99.8
15.11	15.03	99.5
17.57	17.42	99.1
Mean		99.8

This method was applied to bulk drug samples, giving a relative standard deviation of <0.4% ($n = 5$).

In summary, the method presented here is rapid, stability indicating, precise, and accurate, and it is experimentally simpler than current compendial assays.

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Initial Rate Studies of Hydrolysis and Acyl Migration in Methylprednisolone 21-Hemisuccinate and 17-Hemisuccinate

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Abstract □ The degradation of methylprednisolone 21-succinate in aqueous solution was examined as a function of pH at 25° by monitoring the initial rates of product formation. In addition to hydrolysis, acyl migration from the 21-hydroxyl group to the 17-hydroxyl group was found to be an important reaction. The 17-succinate was isolated, and its decomposition to the 21-succinate was followed by the initial rate method. Direct hydrolysis of the 17-ester was much slower than the 17 → 21 acyl migration under alkaline conditions. From the rate constants for the forward and reverse acyl migration, it may be concluded that the 21-ester is thermodynamically more stable, even though its alkaline hydrolysis is faster. The hydrolysis of the 21-ester and the reversible rearrangement are subject to intramolecular catalysis by the terminal carboxyl group, for which a kinetic pKa value of 4.5–4.6 was estimated.

Keyphrases □ Methylprednisolone—17- and 21-succinates, hydrolysis and acyl migration, initial rate studies □ Hydrolysis—methylprednisolone 17- and 21-succinates, initial rate studies □ Degradation—methylprednisolone 17- and 21-succinates, hydrolysis and acyl migration, initial rate studies

Methylprednisolone 21-succinate (sodium salt[1]) is a soluble prodrug of methylprednisolone used as an injectable corticoid in acute hypersensitivity and dermatological conditions. Solubilization is achieved through the use of the ionizable hemisuccinate moiety, which is cleaved *in vivo* to release the active parent compound.

Previous studies of the kinetic behavior of this compound and similar compounds in aqueous solution generally focused on the hydrolytic cleavage of the ester linkage. Hydrolysis usually is regarded as the major degradation pathway of 21-steroid esters (1–3), followed by further decomposition *via* several routes (2, 4, 5). These kinetic studies were based on the results of nonspecific analytical methods such as the consumption of base (1) or extraction coupled with the blue tetrazolium assay (2) for measuring reaction rates.

In this study, the kinetics of methylprednisolone 21-hemisuccinate degradation were reexamined using a highly sensitive and specific high-performance liquid chromatographic (HPLC) technique. By monitoring the initial rates of product formation, data could be obtained easily at room temperature (25°) and at pH values where the reactions are quite slow. As a result of the improved analytical methodology, the role of acyl migration in the aqueous solution degradation of this steroid 21-ester and the isomeric 17-ester could be explored in addition to the hydrolysis reaction.

EXPERIMENTAL

HPLC Analysis—A modular high-performance liquid chromatographic system consisting of an automated sample injector[2], a constant-flow pump[3] operated at 0.9–1.4 ml/min, a reversed-phase column[4] packed with 10-μm Lichrosorb RP-18[5], a variable-wavelength UV detector[6] operated at 248 nm, and a digital integrator[7] was used for all kinetic studies. The mobile phase contained 33% acetonitrile[8] and 67% water buffered at pH 5.2–5.4 with 0.05 *M* acetate buffer.

Under these chromatographic conditions, methylprednisolone 17-hemisuccinate was eluted first, followed by methylprednisolone and then by methylprednisolone 21-hemisuccinate. As will be discussed later, the retention times of the esters changed dramatically with small changes in the pH. Detector response, measured either as the peak area or peak height, was linear for all solution components over the concentration range of interest.

[1] Solu-Medrol (methylprednisolone sodium succinate), The Upjohn Co.

[2] Wisp model 710A, Waters Associates, Milford, Mass.
[3] Model 110A, Altex Scientific, Berkeley, Calif.
[4] Brownlee Laboratories, Berkeley, Calif.
[5] E. Merck, Darmstadt, West Germany.
[6] Altex/Hitachi model 153-00, Altex Scientific, Berkeley, Calif.
[7] Model 3380A, Hewlett-Packard, Avondale, Pa.
[8] Burdick & Jackson Laboratories, Muskegon, Mich.

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