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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ACKNOWLEDGMENTS

The authors thank W. F. Beyer for the hydrocortisone cypionate sample that contained cortisone cypionate.

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

BRADLEY D. ANDERSON ˣ **and VANESSA TAPHOUSE**

Received April 7, 1980, from *The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication July 25, 1980.

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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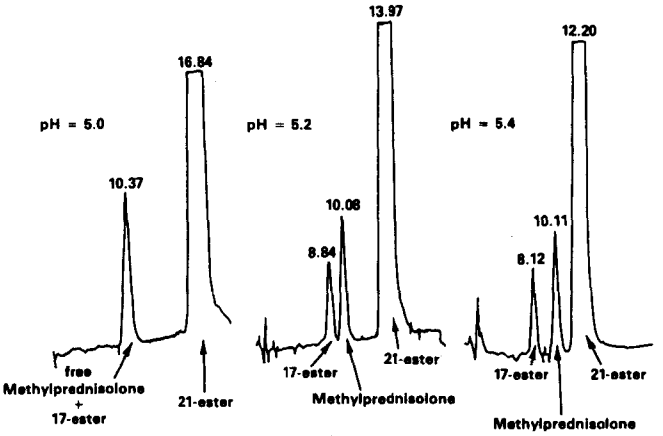


Figure 1—*Effect of mobile phase pH on the resolution of methylprednisolone and methylprednisolone 17-hemisuccinate in the presence of high concentrations of 21-hemisuccinate. The flow rate was 1.3 ml/min, and the detector sensitivity was 0.16 absorbance unit/10-mv output. Other chromatographic conditions are reported in the text.*

Standard Solutions—Standard solutions of methylprednisolone and methylprednisolone 17- and 21-succinates were prepared at concentrations of 1.0×10^{-6}–1.0×10^{-5} *M*, depending on the samples being analyzed. These solutions were prepared at pH 3–4 in 0.01 *M* ionic strength buffer (6) and were stable for at least 1 day at 25°.

Kinetic Studies—Solutions of methylprednisolone 21- or 17-succinate were prepared, at concentrations in the range of 1.0–6.0×10^{-4} *M* in 0.01 *M* ionic strength buffers (6), by one of the following two methods:

1. An amount of the ester stock solution in dimethylformamide of known concentration and density was weighed accurately into a 25-ml volumetric flask and diluted with buffer to 25 ml.

2. An amount of the ester was weighed accurately, transferred to a 25-ml volumetric flask, dissolved in 200 μl of dimethylformamide, and then diluted to 25 ml with buffer.

All solutions contained <1% dimethylformamide. The time of preparation and the exact pH were recorded. Samples were maintained at 25° in a constant-temperature water bath or in the sample compartment of the automatic sampler, which was modified to maintain a constant temperature of 25 ± 0.6° in the compartment.

Samples for HPLC analysis were injected directly using the conditions described previously. In general, at least five data points prior to 3% degradation of the starting material were obtained. Product concentrations generally were determined using the ratio of the peak areas of the sample and standard. However, when very high precision was desired, peak heights were preferable.

Isolation of Methylprednisolone 17-Hemisuccinate—Although an additional impurity in methylprednisolone 21-succinate solutions had been observed previously by HPLC and some evidence suggested that it was the 17-succinate[9], the compound had not been isolated in sufficient quantity for conclusive structural elucidation. Furthermore, relatively pure 17-hemisuccinate was needed for use as a standard for the kinetic studies.

Methylprednisolone 21-hemisuccinate (3 g) was dissolved in 100 ml of deionized water by adjusting the pH to ~7.5 with dilute sodium hydroxide. The solution was heated to 60–70°, and the pH was maintained at 7.5 by occasional addition of dilute sodium hydroxide. Formation of the unknown product and free methylprednisolone were monitored by HPLC. After 1 day, the unknown appeared to reach a maximum concentration of ~12% of the initial starting material (by peak areas).

After cooling, the solution was acidified (acetic acid) and extracted with three 100-ml portions of ethyl acetate. The organic layers were combined, and the solvent was removed on a rotary evaporator. The viscous residue was redissolved in a solution of 40% acetonitrile and 60% aqueous buffer containing 0.05 *M* acetic acid, which was adjusted to pH 5.2 with potassium hydroxide. This solution was injected onto a reversed-phase preparative system[10] and eluted with a mobile phase of the same composition as the sample solvent. At a flow rate of 18 ml/min, the fraction containing the desired product eluted in ~13 min. This fraction was acidified (acetic acid) and extracted with three equal-volume portions of ethyl acetate.

The organic extracts were combined and dried (anhydrous sodium sulfate), and the solvent was evaporated to a few milliliters. Several portions of acetonitrile were added and evaporated, using a rotary evaporator to pull off residual acetic acid. The final residue was dissolved in a small volume (~20 ml) of ethyl acetate, filtered, and added dropwise to a large volume of hexane (~500 ml), with rapid stirring, to give 175 mg of a white precipitate[11]. This material was collected and dried overnight at 60° in a high vacuum and was shown by HPLC to contain no detectable methylprednisolone and <1% 21-succinate, mp 143° dec.[11].

Anal.—Calc. for $C_{26}H_{34}O_8$: C, 65.8; H, 7.22. Found: C, 65.69; H, 7.57.

The mass spectrum of the trimethylsilylated derivative of the unknown was reasonable for the 17-hemisuccinate but was not conclusive. A peak at *m/z* 587 corresponding to loss of $CH_2OSi(CH_3)_3$ from the molecular ion would not be expected from the 21-hemisuccinate or from an intermediate hemi-ortho ester unless decomposition occurred, but this peak is reasonable for the 17-ester, which has a CH_2OH group.

Noise-decoupled ^{13}C-NMR spectroscopy showed a downfield shift in the quaternary C-17 from δ 88.68 for the 21-hemisuccinate to δ 94.48 for the unknown and an upfield shift of the 21-carbon from δ 67.68 to 65.58. Moreover, no quaternary carbons were observed in the region expected for a carbon bonded to three oxygens, eliminating the unlikely possibility that the unknown was the hemi-ortho ester, the existence of which has been postulated (7).

The kinetic behavior of the unknown strongly supports the assigned structure.

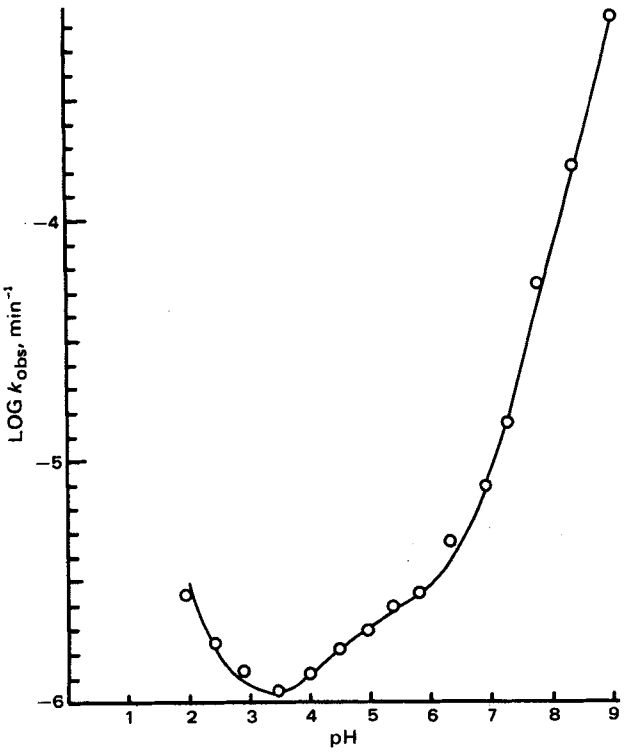


Figure 2—*Plot of hydrolysis rate as a function of pH for methylprednisolone 21-hemisuccinate in aqueous buffers at 25°. Key: O, experimental data; and —, theoretical curve.*

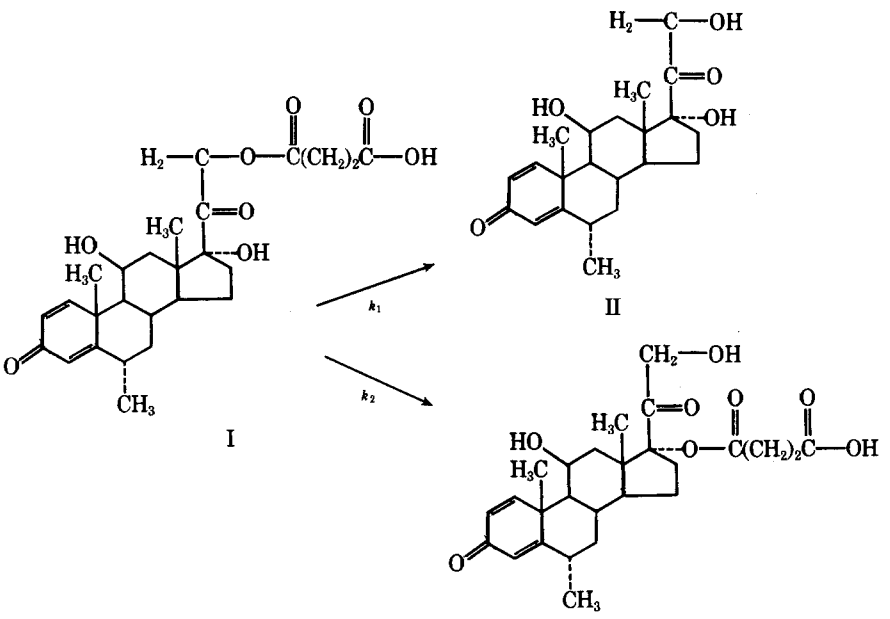
RESULTS AND DISCUSSION

Reversed-Phase HPLC Determination of Methylprednisolone 21-Hemisuccinate and Its Degradation Products—Previous studies of soluble steroid degradation generally focused on the hydrolysis reaction and failed to address acyl migration. Presumably, the analytical methods used were not specific for the 17-ester in the presence of the 21-ester. Although normal-phase chromatographic methods for the separation of methylprednisolone (II) and the 17-hemisuccinate (III) from methylprednisolone 21-hemisuccinate (I) (Scheme I) have been developed (8), a reversed-phase method was considered as more desirable for

[9] H. J. Rodriguez, L. E. Fox, and W. F. Beyer, The Upjohn Co., Kalamazoo, MI 49001, internal communications.

[10] Two size B RP-8 Lobar (E. Merck, Darmstadt, West Germany) columns were employed.

[11] In a repetition of the synthesis, crystallization occurred in ethyl acetate prior to the precipitation step. The crystallized material exhibited a melting point of 175.0–178.5°.



Scheme I—Initial reaction pathways in the degradation of methylprednisolone 21-hemisuccinate

kinetic studies since it would allow direct injection of an aqueous solution without time-consuming extraction during which further degradation could occur.

A recent report that described a reversed-phase HPLC procedure for the components of interest failed to mention the 17-hemisuccinate (9). Those investigators reported the use of a mobile phase containing 2% acetic acid. In the present study, it was found that the pH of the mobile phase is important. Figure 1 shows the effects of relatively small changes in pH on the resolution of the three solution components. As expected, the retention times of the hemisuccinate isomers can be varied relative to that of free methylprednisolone. At pH 5.0, the 17-hemisuccinate peak merged with the free methylprednisolone peak. In the previously reported method (9), the peaks for the 17-ester and free methylprednisolone probably merged.

Kinetic Studies—*Advantage of Initial Rate Method*—Because the reaction rates are very slow at room temperature and at pH values near neutrality, most earlier studies were carried out at high temperature and at pH values where reaction rates were well above the minimum in the pH–rate profile so that reactions could be followed conveniently for several half-lives. When the reactions are followed for several half-lives, other competing side reactions, such as oxidation of the dihydroxyacetone side chain (4) and D-homo rearrangement (10), complicate the kinetics.

To obtain the complete pH–rate profile at room temperature in a reasonable time and to avoid the mathematical complications brought about by consecutive reactions when following kinetics over several half-lives, initial rates of product formation were monitored. Prior to 2–3% degradation, the concentration of the reactant can be considered constant so that product formation rates are constant. The free alcohol and 17-ester form at rates that are independent of the presence of each other in initial rate studies.

Determination of Reaction Order—Both the hydrolysis reaction and the acyl migration from the 21-position were shown to be first order with respect to methylprednisolone 21-hemisuccinate. In the initial rate studies, the reactions shown in Scheme I occur. In Scheme I, k_1 and k_2 represent pseudo-first-order rate constants. Although the 21 → 17 rearrangement is reversible, the reverse reaction rate is negligible early in the approach to equilibrium. Since I can be considered constant during the time frame of interest, the reaction order can be determined by varying the initial concentration of I. Reaction rates were found to be a linear function of the initial concentration of I, indicating that these reactions are first order in I. Rate constants at various concentrations of I and at three pH values are shown in Table I. As expected, the rate constants do not change significantly with the concentration of I in the dilute region.

Table I—Rate Constants at Various Dilute Concentrations of Methylprednisolone 21-Hemisuccinate (I)

pH	Concentration, $M \times 10^4$	k_1, min^{-1}	k_2, min^{-1}
7.30	1.94	$(1.5 \pm 0.8) \times 10^{-5}$	$(2.4 \pm 0.9) \times 10^{-5}$
7.30	4.10	$(1.6 \pm 0.3) \times 10^{-5}$	$(2.9 \pm 0.3) \times 10^{-5}$
7.30	5.91	$(1.5 \pm 0.1) \times 10^{-5}$	$(2.6 \pm 0.2) \times 10^{-5}$
5.82	2.08	$(3.6 \pm 0.6) \times 10^{-6}$	$(1.3 \pm 0.2) \times 10^{-5}$
5.82	3.24	$(2.7 \pm 0.2) \times 10^{-6}$	$(1.1 \pm 0.1) \times 10^{-5}$
5.82	4.15	$(3.0 \pm 0.3) \times 10^{-6}$	$(1.2 \pm 0.03) \times 10^{-5}$
4.02	0.968	$(1.6 \pm 0.2) \times 10^{-6}$	$(3.7 \pm 0.3) \times 10^{-6}$
4.02	1.28	$(1.7 \pm 0.1) \times 10^{-6}$	$(3.6 \pm 0.1) \times 10^{-6}$
4.02	2.00	$(1.4 \pm 0.1) \times 10^{-6}$	$(3.2 \pm 0.3) \times 10^{-6}$

Hydrolysis of Methylprednisolone 21-Hemisuccinate—The pH–rate profile for the hydrolysis of methylprednisolone 21-succinate is shown in Fig. 2. From the shape of the curve, it is evident that more than one mechanism must be invoked to account for the hydrolysis at different pH values. The line drawn in Fig. 2 represents the theoretical curve based on the relationship:

$$k_1 = \frac{k^{hyd}_{H^+}[H^+]^2 + k^{hyd}_{H_2O}[H^+] + k^{hyd}_{OH^-}K_w + k'^{hyd}_{OH^-}[OH^-]K_a}{[H^+] + K_a} \quad \text{(Eq. 1)}$$

Values for the rate constants at 25° were estimated using a least-squares computer-fitting technique (11) and are listed in Table II with values representing 95% confidence limits. A pKa of 4.54 was assumed based on the results of curve fitting of the acyl migration data (to be presented later).

The value obtained for $k'^{hyd}_{OH^-}$ is close to that calculated for a similar system (1). The $k'^{hyd}_{OH^-}$ value represents specific hydroxide-ion-catalyzed hydrolysis of the succinate anion and is the most important term at pH values above 6.5.

Between pH 4.1 and 6.5, the dominant term in the hydrolysis is hydroxide-ion attack on the unionized ester or the kinetically equivalent water attack on the anion. Mechanistically, the increase in the rate over that predicted from $k'^{hyd}_{OH^-}$ as the pKa is approached can be interpreted as being due to one or more of the following reactions: (*a*) intramolecular nucleophilic catalysis by the anion, (*b*) intramolecular general base catalysis of attack by water, (*c*) intramolecular general acid catalysis of

Table II—Rate Constants for Hydrolysis of Methylprednisolone 21-Hemisuccinate in Water at 25°

Parameter	Rate Constant	95% Confidence Limit
$k'^{hyd}_{OH^-}$	74 liters/mole/min	±14 liters/mole/min
$k'^{hyd}_{OH^-}$	7.2×10^3 liters/mole/min	$\pm 1.7 \times 10^3$ liters/mole/min
$k^{hyd}_{H_2O}$	1.0×10^{-6} min^{-1}	$\pm 0.34 \times 10^{-6}$ min^{-1}
$k^{hyd}_{H^+}$	2.0×10^{-4} liter/mole/min	$\pm 1.2 \times 10^{-4}$ liter/mole/min

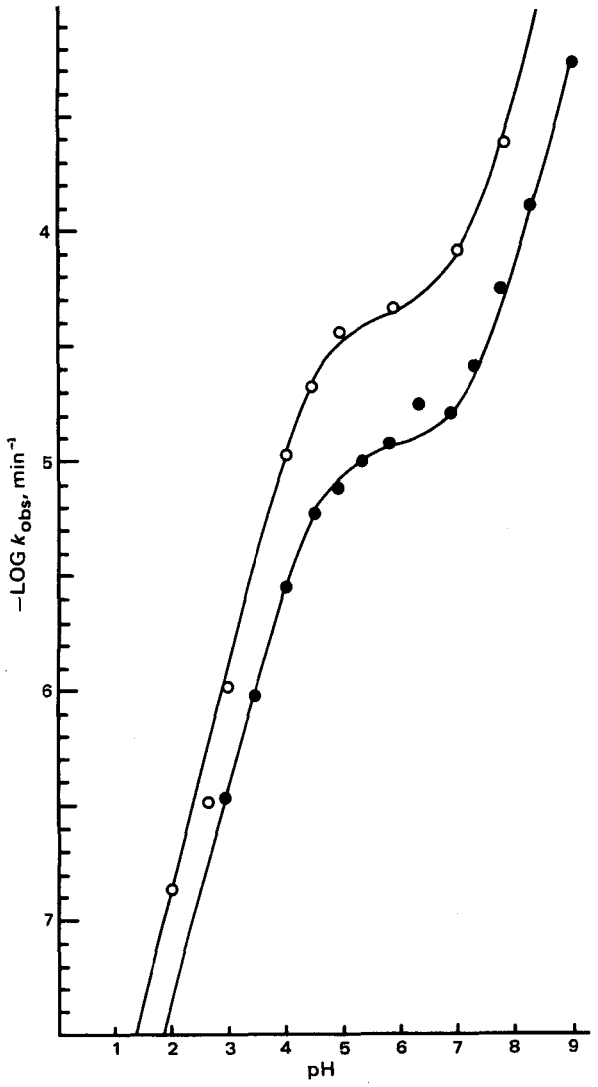
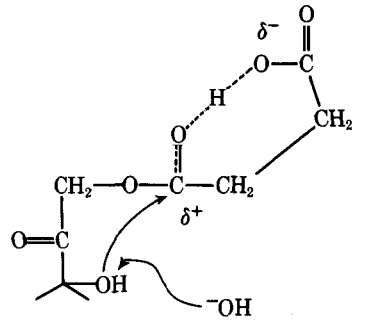


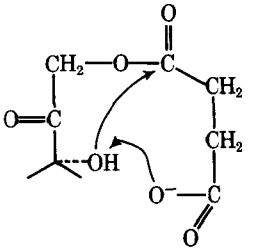
Figure 3—*Plot of rearrangement rate as a function of pH for the migration of the hemisuccinate moiety from the 17 → 21 position (○) and from the 21 → 17 position (●) in aqueous buffers at 25°.*

attack by hydroxide ion, and (*d*) repulsion of hydroxide ion by the negatively charged carboxylate which decreases as the pKa is approached. The present study does not differentiate between these kinetically equivalent mechanisms.

Although intramolecular nucleophilic attack by the terminal carboxylate was shown to occur in some monoesters of dicarboxylic acids (12), evidence supporting this mechanism in the present case is not available. Fersht and Kirby (13) showed that the borderline between intramolecular general base and nucleophilic catalysis lies in the region where the pKa of the leaving group is 5.5–6.5 units more basic than the pKa of the attacking nucleophile. Based on the estimations that the apparent pKa of the C-17 side chain of methylprednisolone probably is near 11 due to enolization (14) and that the pKa of the terminal carboxylic acid group is 4.5, methylprednisolone 21-succinate apparently lies on the borderline between the general base and nucleophilic mechanisms. Intramolecular



Scheme II—General acid-specific base catalysis



Scheme III—General base catalysis

general acid catalysis also cannot be ruled out. In fact, this mechanism appears to be the most reasonable mode of catalysis of acyl migration.

Acyl Migration in Methylprednisolone Hemisuccinate—*21 → 17 Acyl Migration*—Although acyl migration in steroid esters was reported previously (15, 16), the present investigators are not aware of any quantitative data on acyl migration from the 21-hydroxyl group to the highly hindered 17-hydroxyl group. In spite of the fact that the 17-hydroxyl group is in a sterically crowded environment, the 21 → 17 rearrangement is quite facile. The pH–rate profile for the rearrangement of the hemisuccinate moiety from the 21-hydroxyl group to the 17-hydroxyl group is shown in Fig. 3. The initial rate of 21 → 17 acyl migration is faster than hydrolysis at pH values between 3.6 and 7.4. The theoretical curve drawn in Fig. 3 represents the following relationship:

$$k_2 = \frac{k_{OH^-}^{21\rightarrow 17} K_w + k_{OH^-}^{\prime 21\rightarrow 17} K_a [OH^-]}{[H^+] + K_a} \qquad \text{(Eq. 2)}$$

Estimates of the second-order rate constants $k_{OH^-}^{21\rightarrow 17}$ and $k_{OH^-}^{\prime 21\rightarrow 17}$ and the acid dissociation constant K_a were obtained by applying a least-squares computer-fitting technique (11) to the rate data *versus* pH. These values are listed in Table III along with 95% confidence limits.

Intramolecular catalysis represents an acceleration of ~600-fold in the rearrangement. This acceleration is significantly greater (by a factor of ~6) than the intramolecular catalysis observed previously in the hydrolysis and accounts for the fact that the initial rate of acyl migration is faster than hydrolysis between pH 3.6 and 7.4. Two possible mechanisms may account for this acceleration (Schemes II and III). Intramolecular general acid catalysis involves the formation of a six-membered ring (assuming a linear hydrogen bond), while general base catalysis requires the formation of a 10-membered ring. On this basis, general acid catalysis is more likely.

Repulsion of hydroxide ion by the anion probably can be ruled out due to the magnitude of the rate enhancement. Nucleophilic catalysis, which is a possible mechanism in hydrolysis, is not possible in the rearrangement.

17 → 21 Acyl Migration—Also shown in Fig. 3 is the pH–rate profile for the rearrangement of the succinate from the 17-hydroxyl group to the

Table III—Kinetically Determined pKa Values for Methylprednisolone Succinate Esters and Rate Constants for 21 → 17 and 17 → 21 Acyl Migration in Water at 25°

Compound	Parameter	Parameter Value	95% Confidence Limit
21-Succinate	pKa	4.54	±0.19
	$k_{OH^-}^{21\rightarrow 17}$	3.5×10^4 liters/mole/min	$\pm 0.9 \times 10^4$ liters/mole/min
	$k_{OH^-}^{\prime 21\rightarrow 17}$	59 liters/mole/min	±16 liters/mole/min
17-Succinate	pKa	4.62	±0.13
	$k_{OH^-}^{17\rightarrow 21}$	1.2×10^5 liters/mole/min	$\pm 0.2 \times 10^5$ liters/mole/min
	$k_{OH^-}^{\prime 17\rightarrow 21}$	290 liters/mole/min	±100 liters/mole/min

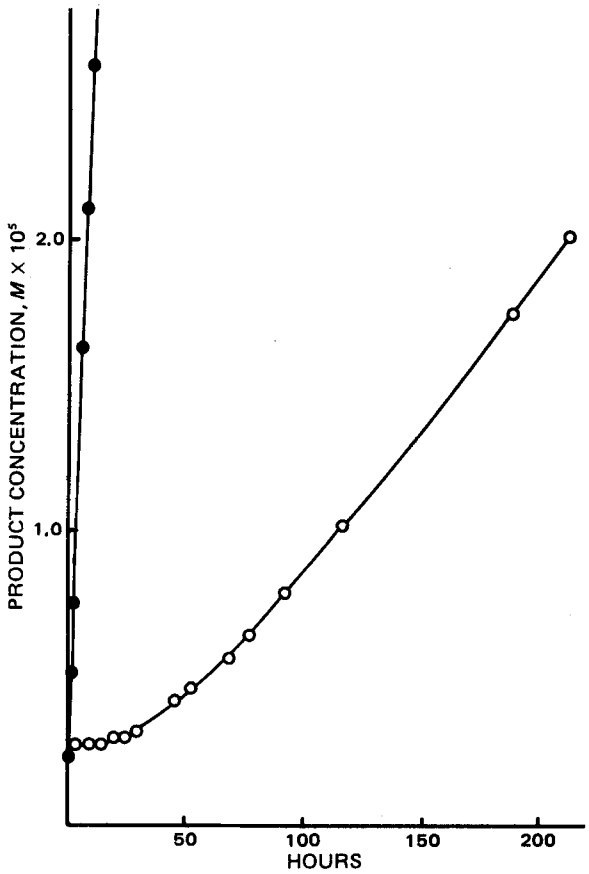


Figure 4—*Appearance of free methylprednisolone (○) and 21-ester (●) versus time in a solution containing 4 × 10⁻⁴ M methylprednisolone 17-hemisuccinate buffered at pH 7.2 at 25°.*

21-hydroxyl group. Except for their vertical position, the curves are virtually superimposable. An equation identical to Eq. 2 but with the pseudo-first-order rate constant k_{-2} and second-order rate constants $k_{OH^-}^{17\rightarrow 21}$ and $k_{OH^-}^{\prime 17\rightarrow 21}$ can be used to describe the 17 → 21 rearrangement. Table III lists the computer-determined best values for the second-order rate constants and the kinetically determined pKa for the 17-succinate.

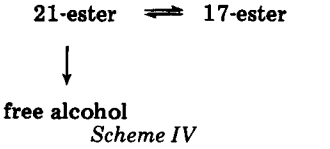
Relative Stabilities of Methylprednisolone 17- and 21-Succinates—A true equilibrium between the 17 and 21-succinates never is attained in aqueous solution due to rapid hydrolysis. However, from a comparison of the migration rate constants in Table III, the relative thermodynamic stability of the two isomers can be estimated.

In the rearrangement reaction, the transition state is the same for both the forward and reverse acyl migration by the principle of microscopic reversibility (17), so that the ratio of the migration rates depends only on the relative reactant stabilities. From Table III, this ratio ($k_{OH^-}^{17\rightarrow 21}/k_{OH^-}^{21\rightarrow 17}$ or $k_{OH^-}^{\prime 17\rightarrow 21}/k_{OH^-}^{\prime 21\rightarrow 17}$) is ~3–5, indicating that the 21-succinate is thermodynamically more stable by 650–950 cal/mole.

The 21-succinate is an ester of a primary hydroxyl group, while the 17-succinate, being an ester of a tertiary hydroxyl group, is more hindered. If the difference in ester stability observed is due to a steric effect, the 21-ester is expected to be favored. The relatively small difference seen suggests that steric hindrance affects the ground-state stability of these compounds to only a minor extent.

Even though the 21-ester is thermodynamically more stable, it hydrolyzes much faster than the 17-ester. Direct hydrolysis of the 17-succinate does not appear to be an important reaction, at least under alkaline conditions. Figure 4 shows the concentrations of free methylprednisolone and its 21-hemisuccinate formed as a function of time in a solution of the 17-hemisuccinate buffered at pH 7.2. A lag time is clearly apparent in the formation of free methylprednisolone, suggesting that hydrolysis occurs after 21-ester formation. This evidence indicates that the reaction shown in Scheme IV applies to these systems.

It is apparent that steric effects on the relative rates of hydrolysis of these esters are pronounced. Steric effects are known to be important in hydrolysis reactions, because the transition state involving the formation of a tetrahedral intermediate is more crowded than the initial state and



Scheme IV

is more highly ordered since two molecules have combined to form one (18). From a consideration of steric parameters of alkyl substituents at the oxygen in acetate esters (19), base-catalyzed hydrolysis rates of primary alkyl acetates are expected to be faster than hydrolysis of tertiary alkyl acetates by a factor of >100. Thus, the hydrolysis of the 17-hemisuccinate is expected to be quite slow, as was observed in this study.

CONCLUSIONS

Because current analytical techniques are superior in sensitivity and specificity to those used in the past for drug degradation studies, reevaluation of earlier data may be useful. A reexamination of the aqueous degradation of methylprednisolone 21-hemisuccinate serves as an example.

A specific HPLC method for determining low levels of methylprednisolone and methylprednisolone 17-hemisuccinate in the presence of the 21-hemisuccinate was developed. Initial rate kinetic studies of the degradation of methylprednisolone 21-hemisuccinate in aqueous solution at 25° showed that degradation proceeds by parallel first-order reactions in the initial phase. Although hydrolysis is a significant reaction at all pH values, reversible acyl migration dominates at pH 3.6–7.4. Therefore, acyl migration is more important in such systems than previously was recognized.

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ACKNOWLEDGMENTS

The authors acknowledge the work of many people at The Upjohn Co., Kalamazoo, Mich., who played a role in the demonstration of acyl migration discussed in this report, including H. J. Rodriguez, L. E. Fox, W.

F. Beyer, and S. Nail. They also thank J. R. Boal for assistance in interpretation of the mass spectral data and S. Mizsak for interpretation of the ^{13}C-NMR data.

V. Taphouse participated in this study through a senior independent research program sponsored jointly by The Upjohn Co. and Kalamazoo College, Kalamazoo, Mich.

Fluorometric Determination of Tetracyclines in Small Blood and Tissue Samples

C. van den BOGERT and A. M. KROON ˣ

Received May 27, 1980, from the *Laboratory of Physiological Chemistry, State University School of Medicine, Bloemsingel 10, 9712 KZ Groningen, The Netherlands.* Accepted for publication July 25, 1980.

Abstract □ A reported fluorometric method to determine tetracyclines in biological material was modified to determine low levels of the antibiotics when using only small amounts of starting material. The method is applicable to tissue distribution studies in small experimental animals and also can be used for serial determinations in blood samples of these animals and for biopsy material. The tetracyclines are extracted in the presence of ethylenediaminetetraacetic acid, and the phosphate precipitation steps are omitted. This omission makes the procedure considerably less time consuming. The recoveries of the tetracyclines, oxytetracycline and doxycycline, are ~80–100%. The values obtained by this method were consistent with those from microbiological assays, and its reliability was shown further in a comparison with the fate of radioactive tetracycline.

Keyphrases □ Tetracyclines—fluorometric determination in small blood and tissue samples □ Oxytetracycline—fluorometric determination in small blood and tissue samples □ Doxycycline—fluorometric determination in small blood and tissue samples □ Fluorometry—analysis, tetracyclines in small blood and tissue samples

Several methods are available to determine the amount of tetracycline in biological material. Microbiological assays often are preferred even though their precision is low and data can be obtained only after prolonged incubation (1).

Spectrophotometric methods (2) are insensitive, and interference from other material cannot always be excluded. High-pressure liquid chromatography (HPLC) (3) and polarographic analysis (4) of tetracyclines also have been described, but they require special equipment. Fluorometric analysis is relatively simple and specific for the tetracyclines (5, 6).

BACKGROUND

Studies have been made of the inhibition of mitochondrial protein synthesis by tetracyclines and the consequences of this inhibition on the functional activity of cells, tissues, and whole animals (7–10). Because of the many other possible effects of tetracyclines, which occur mainly at drug concentrations much higher than those at which mitochondrial protein synthesis is impaired, careful control of tetracycline levels and amounts is necessary in such studies.

For these reasons, the fluorometric method described by Poiger and Schlatter (6) was selected for measurement of tetracycline concentrations in tissues and serum samples of rats after prolonged drug administration. This method is based on buffer extraction of tetracyclines from blood and tissue samples. After deproteinization and elimination of phosphates, the tetracyclines are extracted into an organic solvent as ion-pairs with calcium and trichloroacetate ions. Fluorescence of the tetracyclines is induced by the addition of a base and magnesium ions. The method gives reproducible results, although the recoveries are low—on the order of 35–40% for tetracycline and chlortetracycline. Only for oxytetracycline was 60% recovery from serum recorded (6).

A major disadvantage of the method is the large quantity of material required (3 ml of serum or 2 g of tissue). Therefore, the amounts of tetracyclines in blood and tissue samples of experimental animals such as mice and rats are difficult to determine with this method. By critical evaluation of the various steps of the method, it was possible to modify it in a way that allows the detection of small amounts of the tetracyclines in as little as 0.2 ml of serum or 50 mg (wet weight) of tissue.

EXPERIMENTAL

Chemicals and Antibiotics—All chemicals were analytical grade. Oxytetracycline[1] (as hydrochloride), doxycycline[2] (as hydrochloride), and [7-^{3}H-(N)]tetracycline[3] (0.94 Ci/mmole) were used as obtained.

Experimental Animals and Preparation of Homogenates—In all recovery studies, after the addition of tetracyclines (oxytetracycline, doxycycline, or tetracycline), rat tissue homogenates or serum samples were incubated for 20 min at 37° to obtain maximal binding. Tissue homogenates were prepared in saline using a glass–polytef homogenizer.

Extraction of Tetracyclines—Two milliliters of 0.1 *M* glycine–hydrochloric acid (pH 2) was added per milliliter of a 20% homogenate or dilutions thereof or per milliliter of serum with a known tetracycline content. Proteins were precipitated by the addition of 2 ml of 1 *M* trichloroacetic acid. The mixture was shaken vigorously and then centrifuged. Four milliliters of the supernate was mixed with 1.2 ml of a solution containing sodium acetate (2 *M*) and lead nitrate (0.06 *M*) and allowed to stand for 45 min at room temperature before centrifugation. Then 0.85 ml of saturated potassium iodide solution was added to 5 ml of the supernate, and the mixture was left for 15 min. After centrifugation, 5 ml of the supernate was mixed with 50 μl of 1 *M* calcium chloride and saturated with sodium chloride.

The pH was adjusted to 9.5 with concentrated ammonia. After addition of 3 ml of ethyl acetate, the tetracycline was extracted by shaking the mixture for 1.5 min. Deviations from this general procedure will be indicated.

Fluorescence Measurements—Fluorescence of tetracyclines extracted into the organic layer was induced by adding 0.5 ml of a solution containing 200 mg of barbital sodium and 600 mg of magnesium acetate in 100 ml of methanol to 3 ml of the ethyl acetate extract just prior to the measurement of fluorescence. When small amounts of tissue or serum were used, 1 ml of water, treated as for a sample containing tetracycline, was a suitable blank. In other cases, a sample of tissue or serum without tetracycline was used as a blank because of the background fluorescence.

After reading the fluorescence of a sample, a known amount of tetracycline was added to the cell to calculate the recovery of tetracycline from the sample.

Fluorescence measurements were carried out on a fluorescence spectrophotometer[4] equipped with a xenon arc lamp. The spectral bandwidth was 10 nm for both excitation and emission. The instrument settings were 400 nm for excitation and 505 nm for emission.

[1] Vendarcin, Gist-Brocades, Delft, The Netherlands.
[2] Pfizer Inc., Brussels, Belgium.
[3] New England Nuclear, Dreiech, West Germany.
[4] Perkin-Elmer MPF-43, Charles Goffin, Maastricht-de Bilt, The Netherlands.

0022-3549/81/0200-0186$01.00/0