Solution Kinetics of a Water-Soluble Hydrocortisone Prodrug: Hydrocortisone-21-lysinate

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Received November 7, 1983, from the *College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109-1065 and the ‡INTERx Research Corporation, Subsidiary of Merck Sharp & Dohme, Lawrence, KS 66044. Accepted for publication April 20, 1984.

Abstract □ Hydrocortisone-21-lysinate was synthesized as an amino acid prodrug of hydrocortisone to serve as a substrate for brush border aminopeptidases. This strategy was developed to demonstrate that an improvement in oral absorption could be obtained through reconversion in vivo. The aqueous stability of hydrocortisone-21-lysinate was studied over the pH range 3–8 at 25°C. Reversible acyl migration of the lysine group between the 21- and 17-position hydroxyl groups was observed as well as hydrolysis. The observed half-life for direct hydrolysis of hydrocortisone-21-lysinate is 40 d at pH 3 and 30 min at pH 7. The relative instability at pH 7 is probably due to electrostatic stabilization of the negatively charged tetrahedral intermediate by the protonated amino groups.

The design of prodrugs through consideration of enzyme–substrate specifities[1] led to the synthesis of estrone lysinate[2] and hydrocortisone-21-lysinate as substrates for brush border aminopeptidases. This strategy was conceived as a method for improving the intestinal absorption of water-insoluble drugs by forming a soluble amino acid derivative which would be enzymatically reconverted into the more membrane-permeable parent drug in the vicinity of the intestinal wall.[3] Hydrocortisone-21-lysinate was chosen as a model compound since its permeability and bioavailability can be compared with that of hydrocortisone-21-phosphate, hydrocortisone-21-hemisuccinate, and free hydrocortisone.[4]

In order to show that brush border reconversion can be utilized to improve oral absorption, the substrate prodrug must be of sufficient stability so that it reaches the site of reconversion intact. Estrone lysinate was quite labile at the pH found in the intestine;[2] however, its rate of absorption was up to five orders of magnitude greater than that for estrone.[3]

To confirm that brush border reconversion was responsible for the improved absorption of estrone lysinate, hydrocortisone-21-lysinate was synthesized to be a more chemically stable substrate for brush border aminopeptidases.

Experimental Section

Melting points were determined on a Mel-Temp melting point apparatus. IR spectra were recorded on a Perkin-Elmer 281 IR spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker WM-360 MHz spectrometer using Me_4Si as a standard. Notation used in the ^{1}H NMR description are as follows: (s) singlet; (d) doublet; (t) triplet; (q) quartet. Microanalyses for the intermediate and the dihydrochloride salt of hydrocortisone-21-lysinate were performed at the University of Kansas, Lawrence, KS and at Schwarzkopf Microanalytical Laboratory, Woodside, NY, respectively.

Synthesis of Hydrocortisone-21-lysinate—Five grams (0.009 mol) of $N_{\alpha,\epsilon}$-di-*t*-BOC-L-lysine monodicyclohexylammonium salt (Sigma Chemical Co.), dissolved in 100 mL of methylene chloride, was neutralized by shaking with 100 mL of 3 M HCl in a separatory funnel. The layers were separated, and then fresh methylene chloride was added. This procedure was repeated until the emulsion slowly disappeared. The solvent was removed from the combined organic phase to give the free acid of $N_{\alpha,\epsilon}$-di-*t*-BOC-L-lysine.

Esterification of $N_{\alpha,\epsilon}$-di-*t*-BOC-L-lysine with hydrocortisone was accomplished by a modification of the method of Neises and Steglich.[5] A mixture of 3.49 g (0.01 mol) of $N_{\alpha,\epsilon}$-di-*t*-BOC-L-lysine, 3.65 g (0.01 mol) of hydrocortisone (Sigma Chemical Co.), 2.15 g (0.01 mol) of *N,N'*-dicyclohexylcarbodiimide, and 0.25 g (0.002 mol) of 4-dimethylaminopyridine in 100 mL of dry methylene chloride was stirred at room temperature for 2 h. Precipitated dicyclohexylurea was removed by filtration, the solvent was removed under reduced pressure, and the residual material was extracted with ether. The organic phase was washed with 5% HCl and saturated sodium bicarbonate and then was dried over anhydrous magnesium sulfate. The solvent was removed under reduced pressure to give material which was purified by chromatography on silica gel (hexane:ethyl acetate, 1:1 and ethyl acetate). The presence of the product was monitored by TLC and reaction with ninhydrin. This afforded 5.73 g (83%) of a white crystalline powder, mp 110–115°C, (R_f 0.28, hexane:ethyl acetate, 1:1). ^{1}H NMR (methanol-d_4): δ 0.87 (s, 3, C-18 CH_3), 1.43 [s, 18, 2 $C(CH_3)_3$], 1.47 (s, 3, C-19 CH_3), 5.00 (d, 1, J = 17.4 Hz, C-21 H), 5.04 (d, 1, J = 17.4 Hz, C-21 H), and 5.65 ppm (s, 1, C-4 H).

Anal.—Calc. for $C_{37}H_{57}N_2O_{10}$: C, 64.3; H, 8.4; N, 4.0. Found: C, 64.41; H, 8.78; N, 3.89.

The *t*-BOC protecting groups were removed by dissolving 5.73 g (0.0083 mol) of the intermediate in methylene chloride and bubbling anhydrous HCl gas through the solution. The dihydrochloride salt of hydrocortisone-21-lysinate precipitated from solution and was removed by filtration. The solution was subjected to this process repeatedly until no more blocked compound was observed in solution (monitored by TLC). The solid material was dissolved in ethanol and filtered through activated charcoal. Ether was added to the filtrate and a precipitate formed. Continued washings with ether produced 0.74 g (16% yield) of a white crystalline hygroscopic powder. The compound decomposed upon heating above 200°C. ^{1}H NMR (methanol-d_4): δ 0.88 (s, 3, C-18 CH_3), 1.46 (s, 3, C-19 CH_3), 5.15 (d, 1, J = 18.5 Hz, C-21 H), 5.20 (d, 1, J = 18.5 Hz, C-21 H), 5.65 (s, 1, C-4 H). HPLC showed one peak (see *Analytical Method*). Elemental analysis for one batch was consistent with the dihydrate and the other for the monohydrate.

Anal.—Calc. for $C_{27}H_{44}Cl_2N_2O_6 \cdot 2\ H_2O$: C, 54.07; H, 8.00; N, 4.66. Found: C, 54.07; H, 7.41; N, 4.47.

Anal.—Calc. for $C_{27}H_{44}Cl_2N_2O_6 \cdot H_2O$: C, 55.75; H, 7.9; N, 4.8. Found: C, 55.33; H, 7.87; N, 4.99.

Kinetic Studies—All chemicals were reagent grade. Acetate and phosphate buffers, consisting of acetic acid, sodium acetate, and monobasic and dibasic potassium phosphate, were used at pH 3, 4, and 5, and 6, 7, and 8, respectively. The ionic strength was held constant at 0.5 M by the addition of potassium chloride. The buffers varied in concentration between 0.05 and 0.15 M to test for buffer catalysis. The buffer pH was measured,

0022-3549/85/0100-0087$01.00/0

added to the prodrug in a reaction vial, mixed, and placed in a temperature-controlled water bath (25.0 ± 0.5°C) to give a reaction solution concentration of ~1×10^{-4} M. Time zero was recorded at the time of mixing, and the pH was not observed to change significantly during the course of the reaction. Water used for buffers was distilled and passed through a water purification system.

Analytical Method—Initial rate methods were used at pH 3 and 4 by monitoring the appearance of hydrocortisone due to the slow rate of reaction. At pH 5, 6, 7, and 8, disappearance of hydrocortisone-21-lysinate was monitored. Disappearance of a second product believed to be hydrocortisone-17-lysinate was also followed at pH 7.

The reaction was monitored using an HPLC system consisting of a high-pressure pump (flow rate, 1 mL/min), a sample injector fitted with a 20-μL sample loop, a reversed-phase column (Hibar II, Lichrosorb RP-18, 10 μm, 250 × 4.6, E Merck, Darmstadt, FRG), an absorbance detector (254 nm, 0.05–0.5 AUFS), and a recorder (chart speed, 20 cm/h). The mobile phase consisted of 25–30% isopropyl alcohol, water, and one vial (PIC B6, Waters Associates) of hexanesulfonic acid added per liter. At a mobile phase concentration of 25% isopropyl alcohol, the retention times for hydrocortisone, hydrocortisone-17-lysinate, and hydrocortisone-21-lysinate were 9, 12.5, and 27.5 min, respectively. As the percentage of isopropyl alcohol was increased to 30%, the hydrocortisone and hydrocortisone-17-lysinate peaks merged at a retention time of 6 min and the retention time for hydrocortisone-21-lysinate decreased to 10.5 min.

Results and Discussion

The ^{1}H NMR spectra of the C-21 methylene groups of hydrocortisone and hydrocortisone-21-lysinate show a simple spin–spin splitting pattern of an AB system.[6] For hydrocortisone in deuterated methanol, the C-21 protons give rise to two doublets, δ_A 4.62 and δ_B 4.26 ppm, both with a geminal coupling constant of 19.2 Hz. For hydrocortisone-21-lysinate, the C-21 proton doublets are shifted downfield by δ 0.58 and 0.89 ppm, respectively (see *Experimental Section*). As a reference point, the C-4 protons of both hydrocortisone and hydrocortisone-21-lysinate occur at δ 5.65 ppm. This evidence indicates that esterification occurred at the 21-position. Chemical shifts and geminal coupling constants for similar C-21 protons have been reported.[6]

At pH 3 and 4, hydrocortisone was observed as the only degradation product. At pH 5, 6, and 7, however, a second product was observed to form. Evidence in the literature suggested that this product was the result of an intramolecular acyl migration of the lysine from the 21- to the 17-hydroxyl group.[7-9] In two of these studies, the starting compounds were the 17-butyrate of hydrocortisone[7] and the 17-valerate of betamethasone.[8] Reverse acyl migration of the 17- to 21-ester was negligible in the case of betamethasone valerate as well as direct hydrolysis of the 17-ester to betamethasone. For hydrocortisone butyrate, reversible isomerization to and from the 21-position occurred. In this case, however, only a marginally better fit was obtained from the data by taking into account direct hydrolysis of the 17-ester.

In another study methylprednisolone 21-hemisuccinate was also observed to undergo acyl migration from the 21- to the 17-hydroxyl group.[9] The 17-ester was isolated and was shown to undergo rearrangement to the 21-ester much faster than direct hydrolysis under alkaline conditions. The above evidence coupled with the observed kinetic behavior of hydrocortisone-21-lysinate suggests Scheme I as the appropriate kinetic model. Although the 17-lysinate ester of hydrocortisone was not isolated, kinetic behavior and literature evidence suggest that the third observed chromatographic peak is the 17-ester. Values for the acyl migration rate constants, k_{12} and k_{21}, and the hydrolysis rate constant, k_{10}, were obtained by fitting the residual chromatographic peak height of hydrocortisone-21-lysinate to a biexponential equation of the form:[10,11] peak height $= Ae^{-\alpha t} + Be^{-\beta t}$; where, $k_{21} = A\beta + B\alpha/A + B$, $k_{10} = \alpha B/k_{21}$, and $k_{12} = \alpha + \beta - k_{21} - k_{10}$.

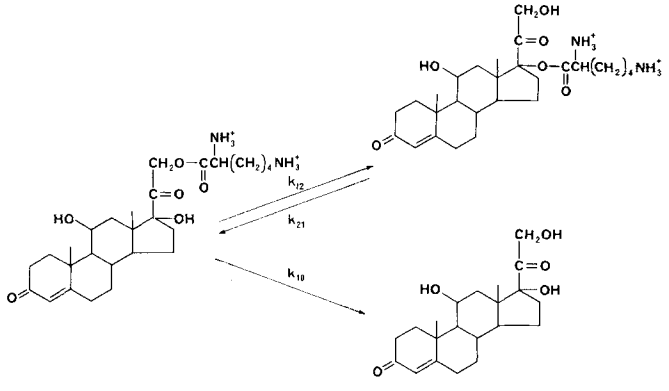
The parameters A, B, α, and β were estimated from the experimental data using a nonlinear regression computer program.[12] No significant buffer effects or hydrocortisone-21-lysinate concentration dependence for k_{12}, k_{10}, and k_{21} were observed. The reported values represent the average obtained at various buffer and hydrocortisone-21-lysinate concentrations. Table I lists the values for k_{12}, k_{10}, and k_{21}.

Using this method, values for k_{12} and k_{21} could only be obtained with accuracy at pH 6 and 7 where all three rate constants, k_{12}, k_{10}, and k_{21}, were similar in magnitude. Below pH 6, k_{21} becomes greater than k_{12} so that the 17-ester was not observed to form at pH 3 and 4. Above pH 7, k_{10} becomes very large in relation to k_{12} so that accurate estimates for the rearrangement rate constants cannot be obtained. At pH 7, after the 21-ester was almost completely hydrolyzed, disappearance of the 17-ester was monitored to give an independent evaluation of k_{21} obtained from the previous method. The two methods gave similar results and the reported value represents an average.

Figure 1 shows the pH–rate profile for hydrocortisone-21-lysinate and estrone lysinate.[2] The curves drawn in the figure represent the theoretical relationship:[2]

$$k_{10} = \frac{[H^+][H_2O]k^{++}_{H_2O} + K_w k^{++}_{OH^-}}{K_a + [H^+]} + \frac{k^{+}_{OH^-}K_w K_a}{[H^+](K_a + [H^+])}$$

where $k^{++}_{H_2O}$ is the water-catalyzed rate constant for the doubly protonated species, $k^{++}_{OH^-}$ is the hydroxide-ion catalyzed rate constant for the doubly protonated species, $k^{+}_{OH^-}$ is the hydroxide-ion catalyzed rate constant of the singly protonated species, K_w is the ionization constant for water, and K_a is the first dissociation constant for the steroid lysinates. Values for the intrinsic rate constants and K_a (1.64×10^{-8}) were found by a least-squares computer fit[12] and are reported in Table II.



Scheme I—Degradation pathways of hydrocortisone-21-lysinate.

Table I—Pseudo-First-Order Rate Constants for the Hydrolysis and Acyl Migration for Hydrocortisone-21-lysinate in Aqueous Solution[a]

pH	$k_{10} \times 10^3$, min^{-1}	$k_{12} \times 10^3$, min^{-1}	$k_{21} \times 10^3$, min^{-1}
3	0.012 (0.0017)	—	—
4	0.036 (0.0096)	—	—
5	0.311 (0.079)	—	—
6	3.730 (0.47)	7.2 (1.2)	6.20 (1.20)
7	23.300 (2.60)	9.2 (1.9)	2.26 (0.49)
8	130.000 (12.00)	—	—

[a] At 25°C, μ = 0.5 M; 95% confidence limits in parentheses.

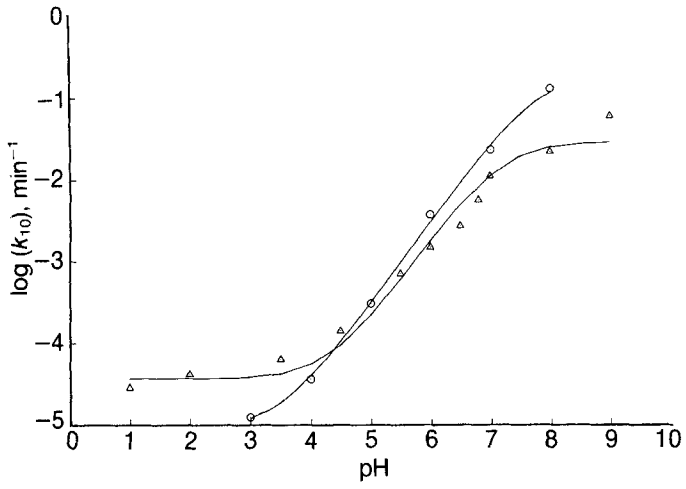


Figure 1—*Plots of the logarithms of the pseudo-first-order rate constants, k_{10}, as a function of pH for hydrocortisone-21-lysinate (○) and estrone lysinate (△). The lines represent calculated data from the theoretical equation described in the text.*

Table II—Intrinsic Rate Constants

Compound	$k^{++}_{H_2O} \times 10^7$, $M^{-1} \cdot min^{-1}$	$k^{++}_{OH^-} \times 10^{-5}$, $M^{-1} \cdot min^{-1}$	$k^{+}_{OH^-} \times 10^{-3}$, $M^{-1} \cdot min^{-1}$
Hydrocortisone-21-lysinate	1.60 (0.4)[a]	3.05 (0.28)[a]	7
Estrone lysinate[b]	6.50 (1.2)	1.80 (0.30)	5.70 (1.8)
α-Carbobenzoxy-L-lysine *p*-nitrophenyl ester[c]	—	6.0	—
p-Acetamidophenol glycinate[d]	61.0 (5.2)	1.870 (0.110)	1.440 (0.050)
p-Acetamidophenol β-aspartate[d]	77.5 (6.5)	0.397 (0.033)	0.100 (0.008)

[a] Standard deviation obtained from computer fit. [b] Ref 2. [c] Ref 14. [d] Ref. 13.

Hydrocortisone-21-lysinate was originally synthesized as a model prodrug for brush border reconversion; it was expected to offer increased pharmaceutical stability over estrone lysinate on the basis that the more basic alkoxy group of hydrocortisone would be a poorer leaving group from the tetrahedral intermediate formed in ester hydrolysis. This was observed to be the case in the lower pH range. At higher pH, attack by hydroxide ion to form the tetrahedral intermediate appears to be rate determining, as both esters hydrolyze at approximately the same rate. Both esters are quite labile in this pH range which may be explained by intramolecular electrostatic stabilization of the negative charge formed in the tetrahedral intermediate by the α and ε amino groups of lysine. This explanation is supported by the related kinetic studies. For example, introduction of a protonated amino group in the *O*-acyl moiety of *p*-acetamidophenol and ethyl acetate resulted in a 500- and 150-fold increase in the hydroxide-ion catalysis constant, respectively.[13] The difference between the two rate enhancement factors was explained as a change in the rate-determining step. The rate of breakdown of the tetrahedral intermediate becomes increasingly important as the leaving group becomes more basic, whereas the rate of formation of the tetrahedral intermediate is rate determining in the hydroxide-ion catalyzed hydrolysis of phenolic esters.[13] Intramolecular electrostatic stabilization of the negative charge of a oxygen in the tetrahedral intermediate by the positive charge of the ε-amino group was also given as an explanation for the high rate constants for the hydrolysis of α-carbobenzoxy-L-lysine *p*-nitrophenyl ester compared with other *p*-nitrophenyl esters studied.[14] In the pH range 5–7, hydrocortisone-21-lysinate, estrone lysinate, α-carbobenzoxy-L-lysine *p*-nitrophenyl ester, *p*-acetamidophenol-β-aspartate,[13] and *p*-acetamidophenol glycinate[13] all hydrolyze at approximately the same rate, indicating that the rate-determining step is the formation of the tetrahedral intermediate stabilized by both α, β, and ε $^+NH_3$ groups. In the lower pH range, however, the rate-determining step appears to be the breakdown of the tetrahedral intermediate as observed in the greater stability of hydrocortisone-21-lysinate. Intrinsic rate constants for the above esters are reported in Table II, where $k^{+}_{H_2O}$ is the water-catalyzed rate constant for the singly protonated species and k_{OH^-} is the hydroxide-ion catalyzed rate constant for the neutral species.

Previous work has shown that the peptidases found in the brush border require substrates to have a free α-amino group for hydrolysis.[15] As shown in this study however, this amino group leads to instability of the prodrug. Hydrocortisone-21-lysinate has a higher solubility than the parent drug, resulting in a faster rate of dissolution and a larger concentration gradient across the wall of the intestine. However, this prodrug is also quite labile at the pH found in the small intestine and is a likely substrate for pancreatic enzymes found in the bulk of the lumen. Consequently, significant hydrolysis of the prodrug in the gut lumen is likely to occur, resulting in a smaller increase in intestinal absorption rate than is theoretically possible. However, the lysinate derivative of hydrocortisone would still have the advantage of an increased dissolution rate compared with that of the parent compound.

References and Notes

1. Amidon, G. L.; Pearlman, R. S.; Leesman, G. D. in "Design of Biopharmaceutical Properties through Prodrugs and Analogs"; Roche, E. B., Ed.; APhA/APS: Washington, DC, 1977; pp 281–315.
2. Leesman, G., Ph.D. Thesis, University of Wisconsin, Madison, WI, 1980.
3. Amidon, G. L.; Leesman, G. D.; Elliott, R. L. *J. Pharm. Sci.* **1980,** *69,* 1363.
4. Fleisher, D., Ph.D. Thesis, University of Wisconsin, Madison, WI, 1983.
5. Neisis, B.; Steglich, W. *Angew. Chem. Int. Ed.* **1978,** *17,* 522.
6. Bhacca, N. S.; Williams, D. H. "Applications of NMR Spectroscopy in Organic Chemistry, Illustrations from the Steroid Field"; Holden-Day Inc.: San Francisco, 1964; pp 42, 90–92.
7. Yip, Y. W.; Li Wan Po, A.; Irwin, W. J. *J. Pharm. Sci.* **1983,** *72,* 776.
8. Bungaard H.; Hansen, J. *Int. J. Pharm.* **1981,** *7,* 197.
9. Anderson, B. D.; Tapouse, V. *J. Pharm. Sci.* **1981,** *70,* 181.
10. Gibaldi, M.; Perrier, D. "Pharmacokinetics"; Marcel Dekker: New York, 1975; p 95.
11. Moore, J. W.; Pearson, R. G. "Kinetics and Mechanism"; Wiley: New York, 1981.
12. Yamaoka, K.; Tanigawara, Y.; Nakagawa, T.; Uno, T. *J. Pharm. Dyn.* **1981,** *4,* 879.
13. Kovach, I. M.; Pitman, I. H.; Higuchi, T. *J. Pharm. Sci.* **1981,** *70,* 881.
14. Ascenzi, P.; Sleiter, G.; Antonini, E. *Gazz. Chim. Ital.* **1982,** 112, 307.
15. Amidon, G. L.; Chang, M.; Fleisher, D.; Allen, R. *J. Pharm. Sci.* **1982,** *71,* 1138.