only in the position of a double bond can be so well separated demonstrates that excellent efficiency and selectivity are provided by the chromatographic system. For all steps in the procedure other than HPLC, the two compounds behave identically. In addition, it is advantageous that the internal standard is easily prepared from the sample. The only problem in this situation is that the internal standard may also be a metabolite of the drug; but, this is not the case here. The plasma sample (Fig. 2B) which was analyzed with no internal standard was taken from an animal 3 days after dosing and 2 days after the highest drug concentration had been observed. No peak corresponding to III is present. Similarly, plasma samples taken at later times also show nothing eluting at this retention time.

CONCLUSION

The analytical procedure presented here is a substantial simplification of the original fluorescence derivatization HPLC method. At a typical therapeutic plasma concentration of 40 ng/ml, the observed mean deviation of 1.7 ng/ml corresponds to an accuracy of 4% mean relative error. Similarly, the standard deviation of 2 ng/ml typically observed corresponds to a precision of 5% relative standard deviation. These values are essentially the same as those observed for the fluorescence method. As one would expect, the fluorescence method has a definite advantage with a detection limit of one-tenth that of the direct method. However, the direct method is more rapid and reliable, and with a detection limit of 2 ng/ml, it is entirely suitable for quantitative determination of avermectins in plasma at normally effective dosage levels. Numerous bioavailability studies with both cattle and swine in which peak drug concentrations have ranged from 50 to 100 ng/ml have been successfully conducted in these laboratories using this analytical procedure.

REFERENCES

(1) G. Albers-Schönberg, B. H. Arison, J. C. Chabala, A. W. Douglas, P. Eskola, M. H. Fisher, A. Lusi, H. Mrozik, J. L. Smith, and R. L. Tolman, *J. Am. Chem. Soc.*, **103**, 4216 (1981).

(2) J. P. Springer, B. H. Arison, J. M. Hirshfield, and K. Hoogsteen, *J. Am. Chem. Soc.*, **103**, 4221 (1981).

(3) J. C. Chabala *et al.*, *J. Med. Chem.*, **23**, 1134 (1980).

(4) J. R. Egerton, J. Birnbaum, L. S. Blair, J. C. Chabala, J. Conroy, M. H. Fisher, H. Mrozik, D. A. Ostlind, C. A. Wilkins, and W. C. Campbell, *Br. Vet. J.*, **136**, 88 (1980).

(5) J. R. Egerton, D. A. Ostlind, L. S. Blair, C. H. Eary, D. Suhayda, S. Cifelli, R. F. Riek, and W. C. Campbell, *Antimicrob. Agents Chemother.*, **15**, 372 (1979).

(6) J. W. Tolan, P. Eskola, D. W. Fink, H. Mrozik, and L. A. Zimmerman, *J. Chromatogr.*, **190**, 367 (1980).

(7) P. C. Tway, J. S. Wood, and G. V. Downing, *J. Agric. Food Chem.*, **29**, 1059 (1981).

(8) R. Wachowiak and K. A. Connors, *Anal. Chem.*, **51**, 27 (1979).

(9) K. A. Connors and N. K. Pandit, *Anal. Chem.*, **50**, 1542 (1978).

(10) R. W. Burg *et al.*, *Antimicrob. Agents Chemother.*, **15**, 361 (1979).

(11) T. W. Miller *et al.*, *Antimicrob. Agents Chemother.*, **15**, 368 (1979).

ACKNOWLEDGMENTS

The authors thank J. B. Williams, F. P. Baylis, and their staffs for dosing the animals and providing plasma samples for the bioavailability experiment.

Coil → Helix Transition in Polyadenylic Acid Induced by the Binding of Epinephrine, Norepinephrine, and Isoproterenol: Circular Dichroism Study

HANNA N. BORAZAN ˣ and SONA N. KOUMRIQIAN

Received October 21, 1981, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, University of Baghdad, Baghdad, Republic of Iraq.* Accepted for publication October 19, 1982.

Abstract □ A circular dichroism spectropolarimetric study on the conformation of polyadenylic acid (poly A) in neutral solutions demonstrated a coil → helix transition induced by intercalative binding of critical amounts of epinephrine, norepinephrine, and isoproterenol relative to poly A. Theoretical treatment of the experimental data indicated a first-order kinetic transition in poly A. It was possible to measure transition rate constants of the epinephrine–poly A and norepinephrine–poly A systems and to calculate the activation energies. The results indicate a high level of temperature dependence of the rate constants. The effects can be reversed by increasing ionic strength, indicating the significance of the electrostatic interactions. The importance of the results is discussed in terms of the possible role of the catecholamines as control mechanisms for the poly A-regulated translation of the genetic code on mRNA.

Keyphrases □ Polyadenylic acid—coil → helix transition, intercalative binding of epinephrine, norepinephrine, and isoproterenol, circular dichroism □ Catecholamines—epinephrine, norepinephrine, isoproterenol, intercalative binding to polyadenylic acid, coil → helix transition, circular dichroism □ Coil → helix transition—of polyadenylic acid, induced by catecholamines, intercalative binding of epinephrine, norepinephrine, and isoproterenol, circular dichroism

Most mRNA molecules contain stretches of polyadenylic acid (poly A) at the 3′-end (1), with length of the poly A depending on the evolutionary level of the organism; the larger segments of poly A exist in highly differentiated cells (2). The exact function of this poly A segment is not yet known. Some researchers have proposed that the segments are responsible for increasing the stability of mRNA by inducing a circular structure, while others found that the translation of the genetic code becomes far more efficient in the presence of poly A, attributed to the greater stability of mRNA afforded by poly A (3).

In a continuing effort to study the diversity of the biological effects of adrenergic compounds at the molecular level, the investigation of the possibility that nucleic acids are the target molecules of these drugs was conducted in this laboratory. The present study deals with circular di-

0022-3549/83/1200-1450$01.00/0

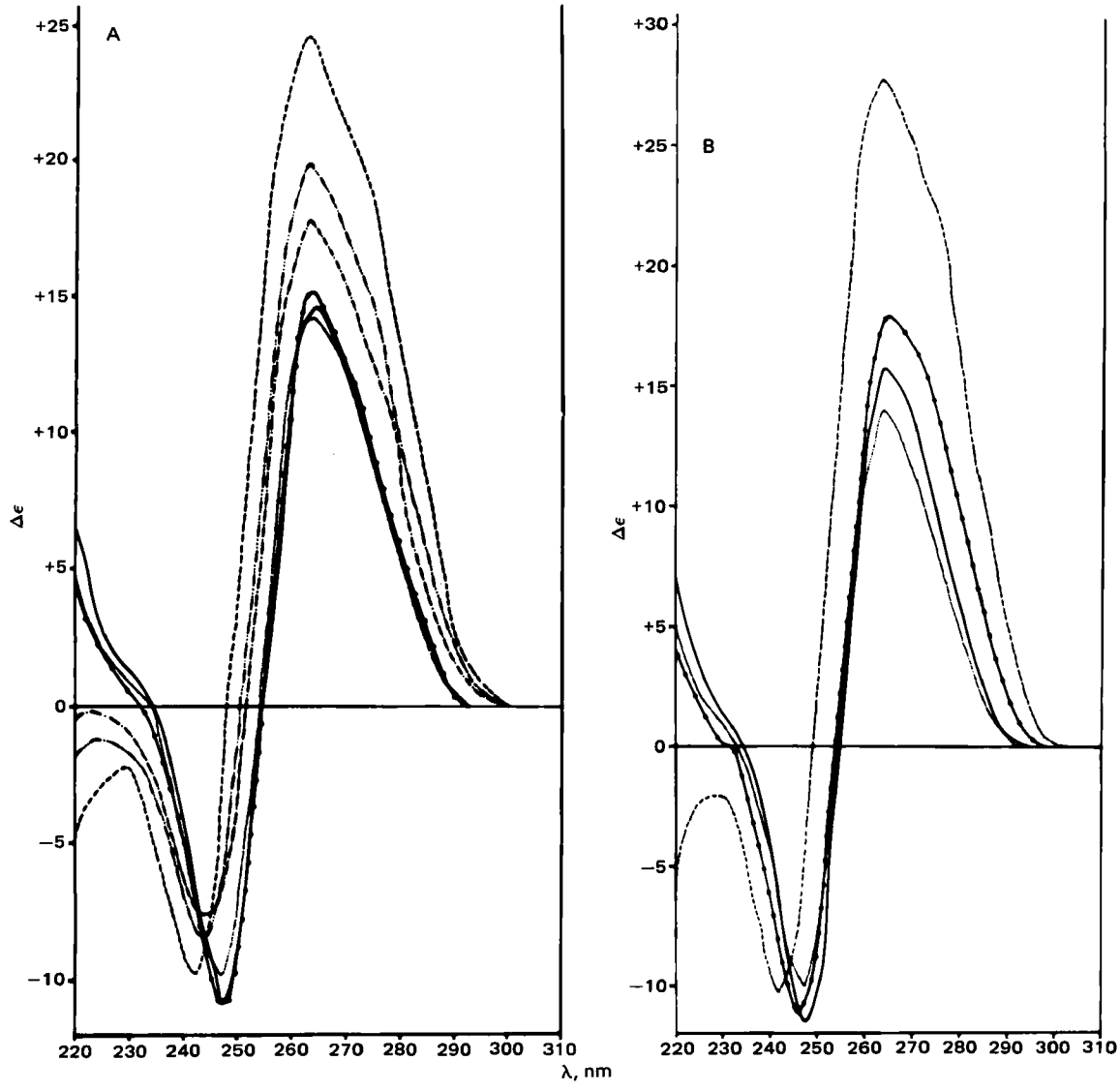

Figure 1—*CD spectra. (A) 5 × 10^{-4} M poly A in the absence (—) and presence of epinephrine hydrogen tartrate at pH 7 (1 mM sodium cacodylate, 1 mM NaCl, and 0.2 mM EDTA), temperature 25°. Key: (. . . . . .) 0.5:1 epinephrine–poly A; (–·–·), (–·· –·· ··), (- - - - - - -) 1:1 epinephrine–poly A at 9, 18, and 126 min, respectively; (–●–●–●–●–●–) effect of increasing ionic strength (0.2 M NaCl) after completion of the transition. The time indicated is at the middle of the scan, speed = 20 nm/min. (B) 5.11 × 10^{-4} M poly A in the absence (—) and presence of 1.022 × 10^{-3} M isoproterenol sulfate at pH 7 (same buffer system as A), temperature 25°. Key: (. . . . . .), (- - - - - - - -) after 7 min and 2 days, respectively; (–●–●–●–) effect of sodium chloride (0.125 M) after the transition is completed.*

chroism (CD) spectropolarimetric investigation of conformational changes in poly A, at pH 7, induced by epinephrine, norepinephrine, and isoproterenol.

EXPERIMENTAL

Polyadenylic acid[1], epinephrine hydrogen tartrate[2], EDTA (disodium ethylenediaminetetraacetate)[2], (+)-tartaric acid[2], norepinephrine[3], sodium cacodylate[3], isoproterenol sulfate dihydrate[4], and sodium chloride[5] were obtained from commercial sources. All chemicals were used without further purification, since they were of the highest commercially available purity.

The prepared solutions contained a fixed concentration of poly A (~5 × 10^{-5} *M*), with varying concentrations of the catecholamines (one-half to twice the concentration of poly A). A value of 10^4 was assumed for the molar absorptivity (ϵ) per nucleotide residue of poly A at 257 nm when preparing standard solutions. The norepinephrine hydrogen tartrate solution was prepared from an equimolar mixture of the acid and the base. The buffer system used was 1 m*M* sodium cacodylate, pH 7, containing 1 m*M* NaCl and 0.2 m*M* EDTA.

Spectroscopic measurements were performed on a spectrophotometer[6] and a circular dichroism spectropolarimeter[7]. All equations used were written on programs and executed on a programmable calculator[8].

The temperature of solutions in the cuvettes (quartz, with a 0.1-cm path length) was maintained constant throughout measurements by using a thermostated cell holder. When working at low temperatures, the spectra were recorded under dry conditions. The experiments were done in a room with subdued lighting to avoid undesired photooxidation of the catecholamines. Each experiment was replicated at least three times.

RESULTS AND DISCUSSION

It is known that poly A in neutral solutions exist in a single-stranded coil conformation (4). This conformation of poly A exhibits a charac-

[1] Boehringer Ingelheim, West Germany.
[2] BDH Chemical Co., Poole, England.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Evans Medical Co., Ltd., Liverpool, England.

[6] Pye Unicam UV-Visible Spectrophotometer, Model SP8-200.
[7] JASCO CD Spectropolarimeter, Model J-40C.
[8] Hewlett-Packard, Model 9810A.

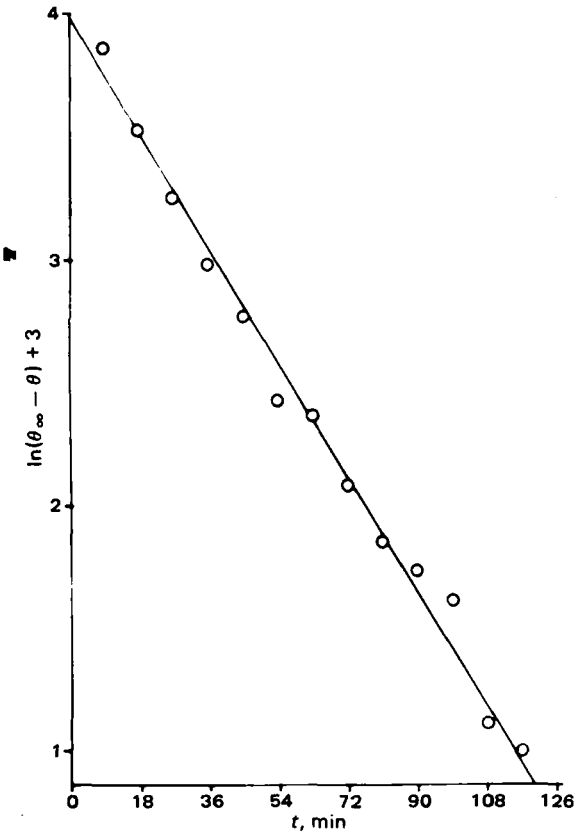

Figure 2—*Ln* $(\theta_\infty - \theta) + 3$ versus t *corresponding to changes in the intensity of the positive band at* λ_{max} *of poly A* (5×10^{-4} M) *in the presence of an equimolar mixture of norepinephrine hydrogen tartrate at pH 7. The conditions are as indicated in Fig. 1.* θ *is expressed in centimeters.*

teristic CD spectrum that is due to ordered base-stacked structures composed of two bands of nearly equal intensities: a positive band with a maximum located at 264 nm and a negative band at 247 nm, with a crossover at 254 nm (5–8).

In the present study, it was found that addition of epinephrine hydrogen tartrate or norepinephrine hydrogen tartrate to poly A at a concentration ratio (amine/poly A) of 0.5 caused a slight reduction of the intensities of the positive and negative bands of the CD spectrum of poly A without altering its shape. This pattern of change could indicate an intercalative binding of the aromatic nucleus between the stacked adenine bases (9, 10). When the concentration ratio was increased to 1.0, an increase in the positive band and a decrease in the negative band of the CD spectrum, as well as shifts in the λ_{max} and λ_{min} toward shorter wavelengths, were observed with time (Fig. 1). After the change was completed, the CD spectrum was composed of two bands, a positive band with a maximum at 263 nm and a negative band with a minimum at 242 nm; at 248 nm the CD signal had a zero value. This spectrum is considered to be identical to that of the double-stranded intertwined helical conformation of poly A existing in acidic solutions, pH 4.8, (5–7). These results indicate a coil → helix transition catalyzed by the binding of epinephrine and norepinephrine.

In order to have a basic understanding of the mechanism of this coil → helix transition, several kinetic models were tested. The best model that seems to be consistent with our experimental data is a first-order transition process in poly A. This led us to the following equation:

$$\ln (\theta_\infty - \theta) = \ln (\theta_\infty - \theta_0) - kt \qquad \text{(Eq. 1)}$$

where θ_0, θ, and θ_∞ represent the ellipticity of the CD signal at times 0, t, and ∞, respectively, and k is the rate constant of the transition. Thus, a plot of ln $(\theta_\infty - \theta)$ *versus* t should lead to a slope of $-k$.

Naturally, the activation energy of the coil → helix transition can be measured in principle according to the Arrhenius equation:

$$\ln k = -\frac{E_a}{RT} + \text{constant} \qquad \text{(Eq. 2)}$$

by plotting ln k *versus* $1/T$. In Eq. 2, E_a is the activation energy, R is the gas constant, and T is the absolute temperature in °K. Representative plots of Eqs. 1 and 2 are shown in Figs. 2 and 3, respectively; the data are summarized in Table I. To minimize errors, the slopes of the curves in Figs. 2 and 3 and those used to calculate the kinetic parameters presented in Table I were calculated by the method of least squares.

Isoproterenol can also induce the coil → helix transition, but this process is very slow. A 2:1 isoproterenol–poly A mixture can bring about the transition completion in >2 days at 25° (Fig. 1B). However, this transition is too slow to enable measurement of k or of the corresponding E_a.

It is evident from Fig. 2 that the transition is a first-order process in poly A when based on measurements of the positive band (264 → 263 nm) and the total peak-to-peak $\lambda_{max} - \lambda_{min}$ amplitude (λ_{min} shifts from 248 to 242 nm). The transition at 4° is a very slow process; it was therefore necessary to leave the solution overnight to bring the transition to completion and to measure θ_∞. Even though the transition occurring at 15° is faster than that at 4°, it was necessary to leave the solution overnight to achieve the most stable double-helical structure. θ_∞ was attained within 2 hr at 25° for both systems.

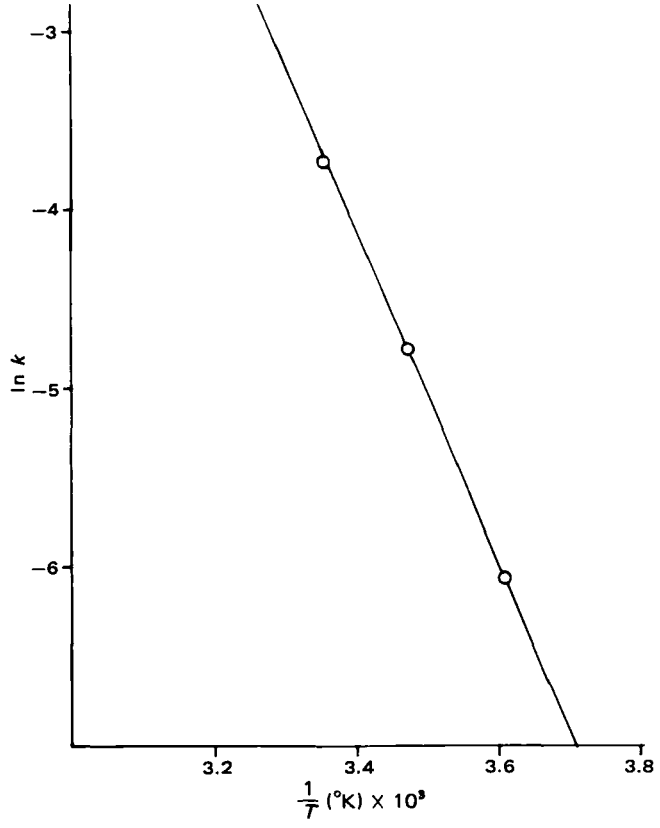

Figure 3—*Ln* k versus *1/*T *for the transition of poly A in the norepinephrine-poly A system based on measurements of the total peak-peak* $(\lambda_{max} - \lambda_{min})$ *amplitude.*

Table I—Rate Constants (k) and Coil → Helix Transition Activation Energies (E_a) of Poly A Based on CD Signals

Temperature ± 1°	Total Peak–Peak Amplitude k, min^{-1} × 10^3	Total Peak–Peak Amplitude E_a, kcal/mole	Positive Amplitude k, min^{-1} × 10^3	Positive Amplitude E_a, kcal/mole
	Epinephrine–Poly A			
4°	3.16		4.02	
15°	9.40	16.408	9.42	16.480
25°	25.50		33.22	
	Norepinephrine–Poly A			
4°	2.31		2.89	
15°	8.45	18.478	8.51	17.104
25°	24.16		25.59	

The positive CD band assumes the stable structure at a faster rate than that of the negative band (as reflected from measurements of the total amplitude) in the case of the epinephrine–poly A system (Table I). In the norepinephrine–poly A system, however, both bands of poly A attain their stable features at approximately the same rate. The activation energy calculated from the changes in the total amplitude and the positive band is very similar in the epinephrine–poly A system, while a slight difference appears to exist in the norepinephrine–poly A (Table I). In comparing both systems, one may observe the high temperature dependence of the rate constants; a change from 4° to 25° can bring about an 8- to 10-fold increase in the rate constant.

The significance of the contributions of electrostatic interactions to the total binding energy was verified by changing the ionic strength of the solutions. It was found that increasing the ionic strength to 0.2 *M* by the addition of sodium chloride had the effect of restoring almost entirely the initial CD spectra of poly A in the three systems (Fig. 1). This can be explained on the basis of competition occurring between sodium ions and the positively charged amino groups existing at pH 7 in the negatively charged phosphate residues. The contribution of other weaker forces to the intercalative binding cannot be neglected, however, since it has been demonstrated (11–13) that adenine can form charge transfer complexes with catechol, epinephrine, and isoproterenol.

The exact geometry of the complexes formed between the catecholamines and poly A in its single- and double-stranded conformations cannot be determined from the CD investigation alone. On the other hand, it should be emphasized that the conformational changes occurring in the poly A molecule cannot be attributed solely to the electrostatic interactions, due to the fact that isopropylamine does not change the CD spectrum of poly A (10). Finally, catecholamines may exert a control mechanism through induction of the coil → helix transition on the regulatory role in genetic code translation which has been hypothesized for the poly A segments present in most mRNA molecules.

REFERENCES

(1) J. D. Watson, "Molecular Biology of the Gene," 3rd ed., Benjamin, Menlo Park, Calif., 1976, pp. 482–483.

(2) R. K. Carlin, *J. Theor. Biol.*, **71**, 323 (1978).

(3) G. Brawerman, *Prog. Nucleic Acid Res. Mol. Biol.*, **17**, 117 (1976).

(4) J. Fresco and P. Doty, *J. Am. Chem. Soc.*, **79**, 3928 (1957).

(5) J. Brahms, *Nature (London)*, **202**, 797 (1964).

(6) J. Brahms, A. M. Michelson, and K. E. Van Holde, *J. Mol. Biol.*, **15**, 467 (1966).

(7) F. H. Wolfe, K. Oikawa, and C. M. Kay, *Can. J. Biochem.*, **47**, 637 (1969).

(8) H. Hashizume and K. Imahori, *J. Biochem.*, **61**, 738 (1967).

(9) M. Durand, J. C. Maurizot, H. N. Borazan, and C. Helene, *Biochemistry*, **14**, 563 (1975).

(10) M. Durand, H. N. Borazan, J. C. Maurizot, J. L. Dimicoli, and C. Helene, *Biochimie*, **58**, 395 (1976).

(11) F. A. Al-Obeidi and H. N. Borazan, *J. Pharm. Sci.*, **65**, 892 (1976).

(12) F. A. Al-Obeidi and H. N. Borazan, *J. Pharm. Sci.*, **65**, 982 (1976).

(13) H. M. Taha, F. A. Al-Obeidi, and H. N. Borazan, *J. Pharm. Sci.*, **68**, 631 (1979).

ACKNOWLEDGMENTS

Abstracted in part from a dissertation submitted by S. N. Koumriqian to the University of Baghdad in partial fulfillment of the Master of Science degree requirements.

The authors thank Dr. Waleed R. Sulaiman, Dean, for his encouragement. They also thank the University of Baghdad for the financial support of this work.

Stability of Triamcinolone Acetonide Solutions as Determined by High-Performance Liquid Chromatography

V. DAS GUPTA

Received July 6, 1982, from the *Department of Pharmaceutics, University of Houston, Houston, TX 77030.* Accepted for publication October 14, 1982.

Abstract □ A stability-indicating assay method based on reverse-phase high-performance liquid chromatography has been developed for the quantitation of triamcinolone acetonide. The method was used to study the stability of triamcinolone acetonide in water–ethanol solutions of varying pH, buffer concentration, and ionic strength. The decomposition of triamcinolone followed pseudo-first-order law and was minimal at pH ~ 3.4. Above pH 5.5, the decomposition increased rapidly and was directly related to phosphate buffer concentration. The decomposition decreased with increasing ionic strength when the pH of the solution was >7. Two new peaks corresponding to decomposition products were noted in the chromatogram; their ratio varied significantly with the composition of the vehicle.

Keyphrases □ Triamcinolone acetonide—decomposition in solution, effect of pH, buffer concentration, and ionic strength, high-performance liquid chromatography □ High-performance liquid chromatography—stability indicating, triamcinolone acetonide and its decomposition products, effect of pH, buffer concentration, and ionic strength □ Stability—triamcinolone in solutions, effect of pH, buffer concentration, and ionic strength, high-performance liquid chromatography

Triamcinolone acetonide (I) is available in different dosage forms such as creams, ointments, and suspensions. Despite its extensive use, little is known about the stability of I in aqueous systems and water-washable ointment bases such as polyethylene glycol ointment base USP (1). In general, corticosteroid decomposition is first order (2) with two parallel routes of decomposition. One route (attack on ring A) produces neutral product(s) and the other (attack on the C-17 side chain) produces acidic product(s).

This study evaluates the stability of triamcinolone acetonide (*a*) in water–ethanol solutions of varying pH with different buffer concentrations and ionic strengths and (*b*) in polyethylene glycol ointment base USP (1) using a stability-indicating reverse-phase high-performance liquid chromatographic (HPLC) assay method developed in our laboratory.

EXPERIMENTAL

Reagents and Chemicals—All reagents and chemicals were either ACS, USP, or NF grade and were used without further purification. Triamcinolone acetonide[1] was used as received.

Chromatographic Conditions—Two columns (30 cm × 4-mm i.d.) were used. One contained a semipolar material[2], the other a nonpolar

[1] E. R. Squibb & Sons, Princeton, N.J.

[2] μBondapak CN; Waters Associates, Milford, Mass.

0022-3549/83/1200-1453$01.00/0