# Direct Determination of Avermectins in Plasma at Nanogram Levels by High-Performance Liquid Chromatography

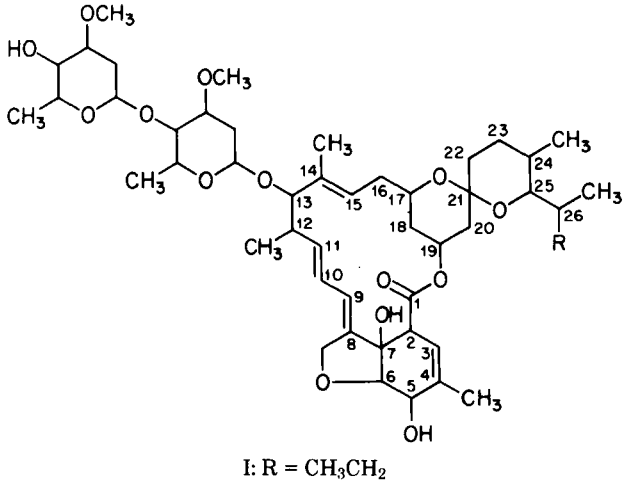
**JAMES V. PIVNICHNY ˣ, JUNG-SOOK K. SHIM, and LAURIE A. ZIMMERMAN**

Received June 16, 1982, from the *Merck Sharp and Dohme Research Laboratories, Rahway, NJ 07065.* Accepted for publication October 21, 1982.

**Abstract** □ 22,23-Dihydroavermectin $B_{1a}$ (I) is determined in animal plasma over the concentration range 5–60 ng/ml by reverse-phase high-performance liquid chromatography (HPLC) with UV photometric detection. Prior to HPLC the sample is isolated by gravity-fed adsorption column chromatography on Florisil. The $\Delta^2$ isomer of I (designated as compound III) is used as an internal standard, and the conversion of I to this isomer by base hydrolysis is described. An accuracy of 2 ng/ml (mean deviation) and a precision in the range of 1–3 ng/ml (standard deviation) were observed for the method. The limit of detection is 2 ng/ml based on the background observed for normal cattle plasma. The method is applicable to bioavailability studies of I at usual therapeutic concentrations.

**Keyphrases** □ Avermectins—direct determination in plasma at nanogram levels, high-performance liquid chromatography, isomerization by hydrolysis □ High-performance liquid chromatography—direct determination of avermectins in plasma at nanogram levels, isomerization by hydrolysis □ Isomerization—avermectins in plasma, hydrolysis, direct determination at nanogram levels, high-performance liquid chromatography

The avermectins are disaccharide derivatives of a structurally similar group of pentacyclic 16-membered lactones (1, 2) which function as broad-spectrum antiparasitic agents (3, 4). Studies in a variety of animals (5) have indicated that these compounds are exceptionally potent. Because of this, effective dosage levels are unusually low (typically 0.2 mg/kg), and the concentrations of this drug found in plasma samples from animals treated with such doses are correspondingly low (<100 ng/ml). The analytical procedure originally reported for determining the avermectins in animal plasma (6) is based on conversion of these compounds to fluorescent derivatives followed by high-performance liquid chromatography (HPLC) with fluorometric detection. The derivatization reaction, which involves dehydration using acetic anhydride–pyridine to form a six-membered aromatic ring in conjugation with a butadiene unit, is time consuming and sensitive to minor experimental variations. A recently reported modification of this method applied to cattle and sheep tissue (7) makes use of the catalyst 1-methylimidazole, as developed by Connors and coworkers for the acetylation of hydroxy compounds (8, 9), to decrease both the reaction time and variability.



I: R = $CH_3CH_2$
II: R = $CH_3$

This report describes a direct HPLC procedure with UV photometric detection for determining the avermectins in animal plasma. The particular avermectin component used, 22,23-dihydroavermectin $B_{1a}$ (I), is one of major pharmaceutical interest. The method requires no derivatization and yet has a detection limit approaching that of the original fluorescence procedure.

## EXPERIMENTAL

**Instrumentation**—A high-performance liquid chromatograph was used which consisted of a pump[1] operated at a constant flow rate of 0.8 ml/min, an automatic sample injector with the capability of handling 0.25-ml sample volumes[2], a prepacked microparticulate octadecylsilyl column[3], and a variable-wavelength photometric detector[4] set at 245 nm. The detector output (0.02 AUFS) was displayed on a strip chart recorder[5], and the column was completely enclosed in a water jacket maintained at 30° with a circulating constant-temperature bath[6].

**Reagents**—The production and isolation of the avermectins has been previously described (10, 11). The purity of the various lots of I used in this study, each of which contained <10% 22,23-dihydroavermectin $B_{1b}$ (II), was established by HPLC. The conversion of I to its $\Delta^2$ isomer (III) for use as an internal standard is described below. All solvents were either HPLC grade[7] or distilled in glass[8]. Distilled water was further purified by passing through an ultrapure water system[9]. The chromatographic eluant was acetonitrile–methanol–water (53:35:7).

**22,23-Dihydroavermectin $B_{1a}$ $\Delta^2$ Isomer (III)**—A solution of 20 mg/ml of I in methanol–water (9:1) was mixed with an equal volume of 0.1 *M* KOH in the same solvent. After 4 hr at room temperature, portions of the reaction mixture were injected into the HPLC system described above, and III was collected as a solution in the eluant as it emerged from the detector. A total amount of III in excess of 5 mg was collected from 10 separate 0.1-ml injections. After dilution with methanol, the concentration of III in this solution was determined by comparison of its HPLC peak area to that of I using the assumption that the molar absorptivities for the two compounds are equal at the detector wavelength.

**Florisil Columns**—Florisil[10], 100–200 mesh, was washed thoroughly with chloroform–ethyl acetate (3:1), dried, and then washed with water

[1] Model 740B; Spectra-Physics, Santa Clara, Calif.
[2] Model 710B WISP; Waters Associates, Milford, Mass.
[3] ZORBAX ODS, 4.6 mm i.d. × 250 mm; DuPont, Wilmington, Del.
[4] Spectromonitor III; Laboratory Data Control, Riviera Beach, Fla.
[5] Model 561; Linear Instruments, Irvine, Calif.
[6] Model FS, Haake Instruments, Saddle Brook, N.J.
[7] Fisher Scientific, Pittsburgh, Pa.
[8] Burdick and Jackson, Muskegon, Mich.
[9] Super-Q; Millipore Corp., Bedford, Mass.
[10] Floridin, Pittsburgh, Pa.

0022-3549/83/1200-1447$01.00/0

and dried overnight at 120°. Glass columns (0.7-cm i.d. × 10 cm) were fabricated with conical funnel tops (6.5-cm diameter) and constricted bottoms. A small plug of silanized glass wool was inserted into each column followed by 0.40 g of Florisil. Each prepared column was rinsed with 10 ml of chloroform immediately before use.

**Procedure**—A plasma sample was combined with the internal standard by first evaporating 1.00 ml of a 140-ng/ml solution of III in an extraction vessel (50-ml centrifuge tube) at 50° under a stream of dry nitrogen and then adding 5.00 ml of the plasma. The sample was then extracted with three 15-ml portions of ethyl acetate, and the combined extracts were evaporated to dryness as above. The residue was dissolved in 1 ml of chloroform and placed on the Florisil column. Two chloroform rinses (1 ml each) and one additional 10-ml portion of chloroform were added to the column. Elution with 25 ml of chloroform–ethyl acetate (3:1) which had been saturated with water afforded the desired fraction. After evaporating to dryness at 50° with dry nitrogen, this was dissolved in 0.25 ml of methanol–water (19:1).

A 0.10-ml aliquot was injected into the chromatographic system. An analytical reference standard was processed as 1.0 ml of a chloroform solution containing 100 ng/ml of I and 140 ng/ml of III, starting with the Florisil chromatography. Quantitation was by peak height measurement with normalization using the internal standard.

## RESULTS AND DISCUSSION

**Characterization of the Analytical Method**—The suitability of ethyl acetate for extracting the avermectins from animal plasma has previously been demonstrated using radiolabeled I in the original fluorescence HPLC procedure (6). The only modification employed here was the elimination of the initial treatment with ethanol. The Florisil column described in the original method was used here, but a total of 13 ml of chloroform was used to load the column instead of the 6 ml originally used. The additional chloroform removed more potential interfering material from the Florisil column before the desired material was eluted with water-saturated ethyl acetate–chloroform.

Typical chromatograms for a sample of normal cattle plasma supplemented with 40 ng/ml of I and the analytical reference standard are shown in Fig. 1. The sample (I) and the internal standard (III) elute with capacity factors ($k'$) of 8.8 and 11.1, respectively, and are completely resolved from each other as well as from interfering endogenous substances. A small amount of the avermectin component II is apparent in the chromatogram of the analytical reference standard, but any of this which might be in the sample is obscured by extraneous plasma components.

No plasma components, however, interfere significantly with either the sample or the internal standard. This is shown in Fig. 2. Chromatogram A, which was obtained for a sample of drug-free cattle plasma, shows only a small peak eluting at the retention time of I. For this particular sample, the peak corresponds to an apparent drug concentration of 1.5 ng/ml. Chromatogram B was obtained from a plasma sample from an animal which had been dosed with I as described below. The sample was processed through the analytical procedure in the absence of internal standard; nothing can be seen eluting at the retention time of this compound. The peak and shoulder that are seen in this chromatogram eluting with retention times slightly less than that of III are due to extraneous plasma components which have been observed in varying amounts in the plasma of different animals. This case represents the maximum amount of these components encountered for any animal examined in this study, and, in fact, the components are completely absent in the other chromatograms illustrated here.

This analytical method is accurate and precise when applied to samples of cattle plasma containing I in the concentration range of 5–60 ng/ml. When normal cattle plasma was supplemented with I over this range, a mean deviation of 1.7 ng/ml from the expected concentration was observed. Linear least-squares analysis of these found *versus* expected results, which are listed in Table I, yield a coefficient of determination ($r^2$) of 0.979 with a slope of 0.936 and an intercept of 1.6 ng/ml. Replicate determinations at supplemented levels of 20, 30, 40, and 60 ng/ml indicate within-run precision values in the range of 1–3 ng/ml (standard deviation). Since the data of Table I are the combined results of five individual runs, an indication of between-run precision is given by the standard error of estimate for the least-squares fit. This value is 2.2 ng/ml. Analyses of samples from eight control animals showed an average apparent concentration of 1.1 ng/ml of I with a standard deviation of 0.9 ng/ml, demonstrating that the method is free of interferences at concentrations >5 ng/ml.

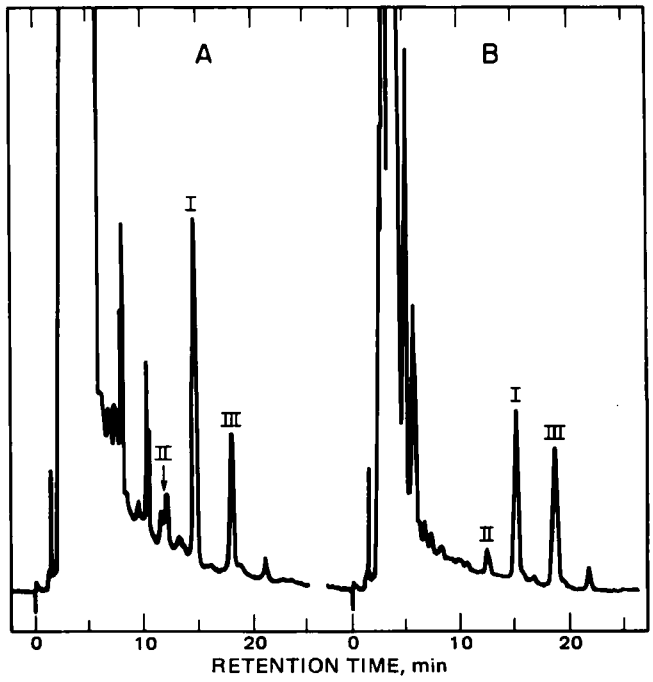



**Figure 1**—*Chromatograms obtained with this analytical procedure. Key: (A) normal cattle plasma supplemented with 40 ng/ml of I; (B) analytical reference standard corresponding to a sample containing 20 ng/ml of I carried through the procedure.*

The overall recoveries of both sample and internal standard carried through the analytical procedure were ~80%. This was determined by supplementing several 5-ml samples of normal cattle plasma with 100 ng of I and 140 ng of III, processing them through the entire procedure, and comparing the resultant chromatographic signals to those obtained for the same amounts of I and III chromatographed directly. These recovery values are essentially the same as that previously determined for I in the portion of the fluorescence procedure preceding the derivatization reaction (6).

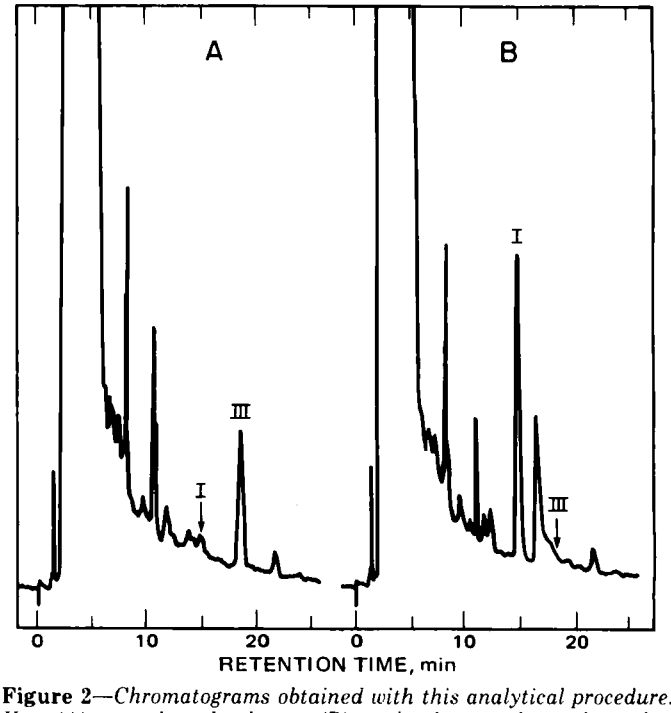



**Figure 2**—*Chromatograms obtained with this analytical procedure. Key: (A) normal cattle plasma; (B) cattle plasma taken 3 days after dosing with 0.2 mg/kg of I. The internal standard was omitted from the analytical procedure.*

**Table I—Accuracy and Precision of the Method Applied to Normal Cattle Plasma Supplemented with Various Amounts of I**

| Concentration Added, ng/ml | Mean Concentration Determined, ng/ml | Number of Determinations | Standard Deviation ng/ml |
|---|---|---|---|
| 5.0 | 6.0 | 1 | — |
| 15.0 | 14.4 | 1 | — |
| 20.0 | 21.5 | 5 | 1.0 |
| 25.0 | 25.9 | 1 | — |
| 30.0 | 30.1 | 8 | 0.8 |
| 35.0 | 31.6 | 1 | — |
| 40.0 | 37.4 | 8 | 2.5 |
| 45.0 | 45.8 | 1 | — |
| 55.0 | 54.9 | 1 | — |
| 60.0 | 58.1 | 7 | 2.8 |

**Application**—To further test this analytical procedure, it was applied to plasma samples obtained from an *in vivo* study involving an Angus steer. The animal was dosed subcutaneously with a solution of I at a dosage level of 0.2 mg/kg, and samples were collected over the period of 2 weeks. The observed concentration–time profile is shown in Fig. 3. A maximum concentration of ~70 ng/ml was reached in 1 day, and the elimination phase closely follows first-order decay kinetics as shown by the semilogarithmic insert in the figure. Least-squares fitting of the results covering 1–14 days gives the expression:

$$C = 80.6e^{-0.236t}$$

where $C$ is the concentration in ng/ml and $t$ is time in days. The coefficient of determination ($r^2$) is 0.993, and the half-life for elimination is 2.9 days.

**The $\Delta^2$ Isomer (III)**—The avermectins react with hydroxide in methanol–water solutions to give a number of products as shown by the chromatograms of Fig. 4. Starting with I under the conditions chosen here, the predominant initial reaction is an epimerization of the hydrogen at C-2 to form IV. This compound has a slightly greater retention time than I in the chromatographic system, and the amount of IV increases as the amount of I decreases. The eventual result might be complete conversion of I to IV or the establishment of an equilibrium mixture of I and IV were it not for the fact that a third product forms by the shifting of a double bond from the C-3 position to C-2 to produce III.

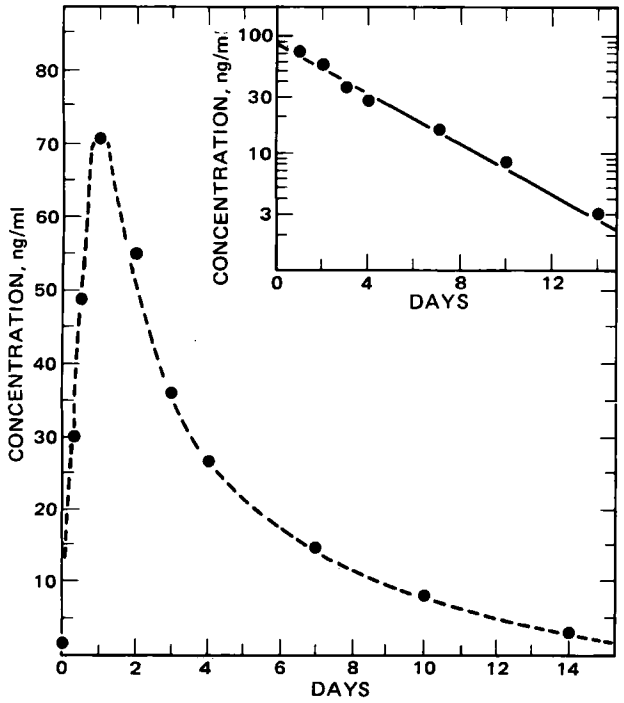



**Figure 3**—*Plasma concentration of I observed for an Angus steer dosed subcutaneously with I at a level of 0.2 mg/kg. The insert presents the elimination phase data on a logarithmic scale.*

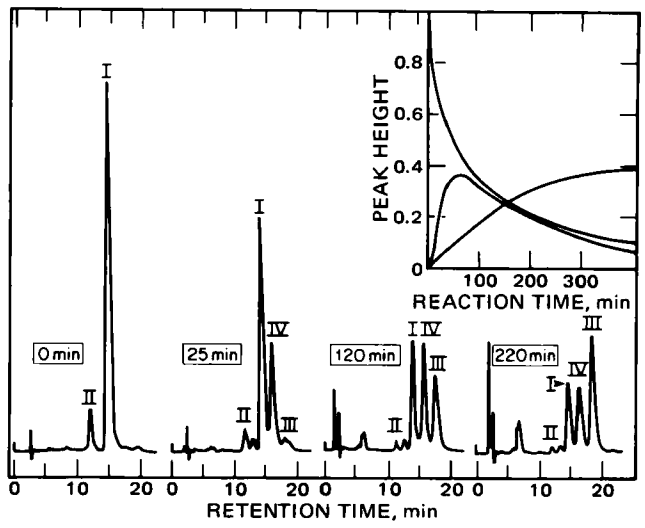
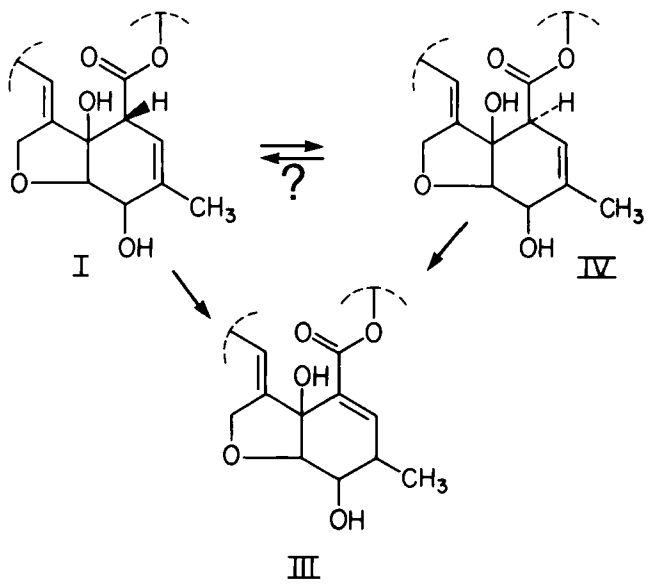



**Figure 4**—*Chromatograms showing the conversion of I to its 2-epimer and $\Delta^2$ isomer by reaction with 0.05 M KOH in methanol–water (9:1). The inset shows the variation in chromatographic peak heights over the course of the reaction.*




*Scheme I*

In the chromatographic system, III elutes after IV, and although its concentration increases more slowly than IV early in the reaction, it continues to increase even after IV begins to decrease. Eventually it becomes the predominant product, and at this point it is isolated by semipreparative HPLC. It is not known how stable III is in the basic reaction mixture, but after isolation it is completely stable in methanolic solution at 5° for at least several months. Also, it is not known whether the mechanism is strictly stepwise, that is, I goes to IV which then goes to III, or whether I and IV simultaneously are converted to III as indicated in Scheme I. Compound IV was identified by comparison of its HPLC retention time to that of an authentic sample which had been independently synthesized[11], and III was identified on the basis of its 300-MHz $^1$H-NMR spectrum[12].

Using III as an internal standard is appropriate since its structure is very similar to that of the sample. That two such large molecules differing

[11] H. Mrozik, Merck Sharp and Dohme Research Laboratories, June 1979, personal communication.

[12] B. H. Arison, Merck Sharp and Dohme Research Laboratories, June 1979, personal communication.

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*