HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF FLUORESCENT DERIVATIVES OF ADENINE AND ADENOSINE AND ITS NUCLEOTIDES

OPTIMIZATION OF DERIVATIZATION WITH CHLOROACETALDEHYDE AND CHROMATOGRAPHIC PROCEDURES

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SUMMARY

The use of chloroacetaldehyde (CAA) as a potential precolumn fluorimetric labelling reagent for adenine compounds was examined in detail. The reaction kinetics was greatly influenced by parameters such as the pH, temperature and CAA concentration. These parameters were optimized with regard to the reaction yield. The resulting procedure for CAA derivatization of adenine compounds was found to be excellent for quantitative analysis. Because the CAA derivatives of the adenine compounds studied were markedly stable, precise quantitative results were easily obtained using high-performance liquid chromatography. CAA derivatives of the five adenine compounds, *i.e.*, $1,N^6$-etheno-adenine, -adenosine, -adenosine 5'-monophosphate, -adenosine 5'-diphosphate and -adenosine 5'-triphosphate were separated. The detection limits for the $1,N^6$-etheno derivatives were 0.5–1.7 pmol per 10-μl injection. Due to its simplicity, speed, sensitivity and selectivity, this procedure is recommended for use in studies on the metabolism and biology of adenine compounds.

INTRODUCTION

Purine and pyrimidine bases, nucleosides and nucleotides are not only components of nucleic acids and coenzymes, but also play a rôle as mediators of hormone action. The analysis of purine and pyrimidine compounds in biological materials is essential for understanding nucleic acid metabolism. With the advent of high-performance liquid chromatography (HPLC), precise determination and high resolution of these compounds was made feasible[1–12]. Notwithstanding this radical development, very little attempt has been made to develop detection procedures with high sensitivity and selectivity. Nevertheless, some results on the specific detection of adenine compounds and cytidine as fluorescent derivatives have been reported, wherein chloroacetaldehyde (CAA) was used as the fluorimetric labelling reagent[13–19]. The essential features of the reaction of CAA and the application of its derivatized nucleo-

tides and coenzymes to biological functions have been independently reviewed by Kost and Ivanov[20] and by Leonard[21].

In this paper, data on the optimization of the CAA derivatization of adenine compounds and their chromatographic separation are presented. In addition, the first successful application of a method developed to measure adenine compounds in rat serum is described.

EXPERIMENTAL

Apparatus

A Japan Spectroscopic (JASCO) Model 800-MP-15 high-performance liquid chromatograph with a JASCO FP-210 spectrofluoro monitor was used. Chromatograms were recorded on a JASCO Model 805-GI graphic integrator, while fluorescence spectra were obtained on a JASCO FP-770 spectrofluorometer.

HPLC columns

The properties of the following columns were examined: silica gel Finepak Sil (250 mm × 4.6 mm) and octadecylsilane-bonded silica gel Finepak Sil C_{18}T-5 (reversed phase, 250 mm × 4.6 mm) (both from JASCO); vinyl alcohol copolymers Asahipak GS-320H (dual mode of adsorption and gel permeation chromatography, 250 mm × 7.6 mm) (Asahi, Tokyo, Japan).

Chemicals

Adenine (Ade), adenosine (Ado), adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP) were obtained from Sigma (St. Louis, MO, U.S.A.), chloroacetaldehyde solution (40%, practical grade) from Wako (Osaka, Japan). All other chemicals used were of analytical grade from commercial sources. Standard solutions were freshly prepared by dissolving the carefully weighed adenine compounds in distilled water to a concentration of 1 mg/ml. These standard solutions were diluted to the target concentrations in distilled water and stored at 4°C.

Chromatographic conditions

Separations were performed at a flow-rate of 1.0 ml/min at 40°C. The eluting solvents were: A, 0.2 *M* phosphate buffer (pH 5.0); B, 0.2 *M* phosphate buffer (pH 5.0)–methanol (70:30, v/v). Elution was carried out for 5 min with solvent A, followed by solvent B for 25 min. The column effluent was monitored fluorometrically at an excitation wavelength of 290 nm and at an emission wavelength of 415 nm.

Preparation of CAA derivatives

A 0.1 *M* CAA solution was prepared by diluting a commercial chloroacetaldehyde solution (40% CAA) in distilled water. Unless specified otherwise, the following procedure was used. To 100 μl of serum sample or 100 μl of diluted standard solutions in a reaction tube, 100 μl each of 0.2 *M* phosphate buffer (pH 5.0) and 0.1 *M* CAA were added. The reaction tube was stoppered hermetically and heated at 100°C for 30 min. The pH of the reaction mixture after derivatization was 4.5. Thereafter the mixture was diluted to 2 ml by adding 1.7 ml of 0.1 *M* Tris–HCl buffer (pH 7.4)

resulting in a pH of 7.0. Immediately after dilution, an equal volume of water-saturated diethyl ether was added to the mixture to remove the excess of CAA by extraction. A 5–10 μl aliquot of the aqueous phase was injected into the HPLC system.

Preparation of the sample fluid from rat serum

Blood samples were obtained individually from eight male rats (age, 3 months; strain, Sprague-Dawley). After 2 h at room temperature, erythrocytes were removed from the collected blood by centrifugation at 1500 g for 10 min. The resulting serum was deproteinized using an equal volume of cold 12% trichloroacetic acid solution. After a 5-min incubation in an ice-bath, the mixture was centrifuged at 1500 g for 10 min and the supernatant was collected. Trichloroacetic acid was removed using water-saturated diethyl ether. The supernatant was adjusted to pH 4.5 by titrating with 0.1 M KOH. The protein-free supernatants were stored at $-20°C$ until analysis. Sample volumes of 100 μl of the protein-free supernatants were sufficient for analysis by the above-mentioned procedure.

RESULTS

Fluorescence spectra of $1,N^6$-etheno derivatives

Standard samples containing 1 μg each of the $1,N^6$-etheno derivatives of the adenine compounds were analyzed. The fluorescence spectra are given in Fig. 1. As can be deduced, all $1,N^6$-etheno derivatives exhibited similar fluorescence emission spectra patterns, however the fluorescence excitation intensities at 223 nm increased in the order of $1,N^6$-ethenoadenine, $1,N^6$-ethenoadenosine, $1,N^6$-etheno-AMP, $1,N^6$-etheno-ADP and $1,N^6$-etheno-ATP. For HPLC analysis, the fluorescence intensity was measured using excitation at 290 nm and emission at 415 nm for simultaneous analysis of the $1,N^6$-etheno derivatives of the five adenine compounds.
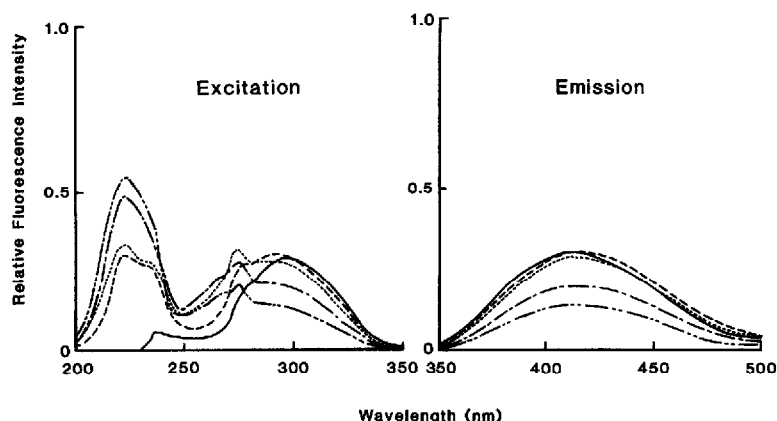

Fig. 1. Fluorescence spectra of $1,N^6$-ethenoadenine (———), $1,N^6$-ethenoadenosine (– – –), $1,N^6$-etheno-AMP (------), $1,N^6$-etheno-ADP (– · –) and $1,N^6$-etheno-ATP (– · · –). Emission was measured at an excitation wavelength of 290 nm, while excitation was measured at an emission wavelength of 415 nm.

Separation of 1,N⁶-etheno derivatives by HPLC

Three columns were examined for their ability to separate 1,N⁶-etheno derivatives: Finepak Sil, Sil C_{18}T-5 and Asahipak GS-320H. A significantly better separation was obtained using the Asahipak GS-320H column. Hence, the latter was used throughout the present study and all results presented were obtained on this column.

The separation of the 1,N⁶-etheno derivatives of the adenine compounds is shown in Fig. 2A, while Fig. 2B shows the separation of non-derivatized adenine compounds as monitored by UV absorbance at 260 nm. The 1,N⁶-etheno derivatives were more sharply separated from each other *vis-à-vis* the separation of the non-derivatized adenine compounds.

The effect of the pH of the eluting solvents (see Experimental) on the separation of 1,N⁶-etheno derivatives was examined. Following an increase from pH 5 to 7, a worsening in the separation of each derivative was noted. A value of 5.0 was selected as the pH of the eluting solvents A and B, because of the good separation of the 1,N⁶-etheno derivatives of five adenine compounds.

Assay linearity and detection limit

Calibration graphs were constructed by analyzing a series of serum samples containing adenine compounds of know concentrations. Each concentration was studied in triplicate. The fluorescence intensities, measured as peak areas, were linear over a range of detection limits up to 40 pmol per 10-μl injection. The detection limits for 1,N⁶-ethenoadenine, 1,N⁶-ethenoadenosine, 1,N⁶-etheno-AMP, 1,N⁶-etheno-ADP and 1,N⁶-etheno-ATP were 1.7, 0.5, 0.6, 0.9 and 1.3 pmol per 10-μl injection respectively, at a signal-to-noise ratio of about five. Conversely, the detection limits for non-derivatized adenine compounds monitored by UV absorbance at 260 nm were 10–30 pmol per 10-μl injection. This is taken to indicate that the fluorimetric analyses were approximately 10- to 30-times more sensitive than the UV-monitoring method.
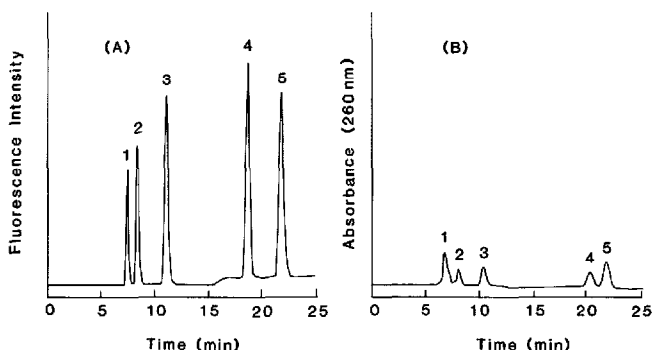

Fig. 2. Chromatograms of 1,N⁶-etheno derivatives of adenine compounds (11.4 ng of each per 10-μl injection) (A) and of non-derivatized adenine compounds (49.4 ng of each per 10-μl injection) monitored by UV absorbance at 260 nm (B). Peaks: (A) 1 = 1,N⁶-etheno-ATP; 2 = 1,N⁶-etheno-ADP; 3 = 1,N⁶-etheno-AMP; 4 = 1,N⁶-ethenoadenosine; 5 = 1,N⁶-ethenoadenine; (B) 1 = ATP; 2 = ADP; 3 = AMP; 4 = adenosine; 5 = adenine.

Assay precision

The intra-assay precision was determined by analyzing, on the same day, a set of five replicate serum samples containing various amounts of adenine compounds. The inter-assay precision was determined by analyzing serum samples containing various amounts of adenine compounds for 5 days over a period of 2 weeks. As regards the intra-assay precision, all coefficients of variation were below 4%; higher coefficients of variation were found for the inter-assay precision (2.7–8.1%).

Recovery

The extraction recoveries of the adenine compounds were determined by comparing the fluorescence intensities obtained by direct injection of 1,N^6-etheno derivatives with those obtained after extraction of the spiked adenine compounds (10 μg/ml) from rat serum. The extraction recoveries of Ade, Ado, AMP, ADP and ATP were 93.8 $\pm$ 5.1, 92.2 $\pm$ 6.1, 87.6 $\pm$ 5.8, 91.4 $\pm$ 5.6 and 93.8 $\pm$ 5.8% (mean $\pm$ S.D., $n = 5$), respectively.

Effect of pH on the derivatization reaction

To examine the effect of the pH in the reaction mixture on the reaction yield, standard samples containing 1 μg of each adenine compound in the reaction mixture were derivatized using 0.2 M phosphate buffers (pH 3.2–6.0) for pH adjustments. As shown in Fig. 3, the effect on the derivatization of pH values below 4.5 was small, however the fluorescence intensities decreased markedly above pH 4.5.

Effect of reaction temperature and reaction time on the derivatization yield

The derivatization with CAA was carried out using the procedure described in the Experimental, except that the reaction temperature was varied from 60 to 120°C. A reaction temperature of 100°C was found to be the most effective. The effect of the
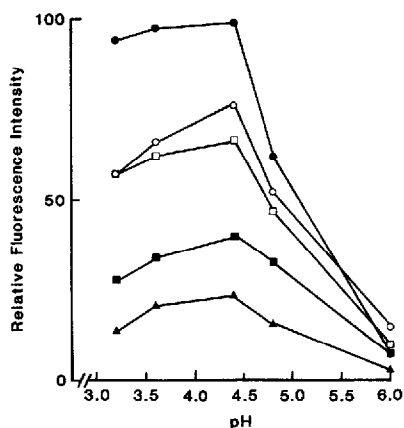

Fig. 3. Effect of the pH in the reaction mixture on chloroacetaldehyde derivatization of adenine compounds. ○, 1,N^6-Ethenoadenine; ●, 1,N^6-ethenoadenosine; □, 1,N^6-etheno-AMP; ■, 1,N^6-etheno-ADP; ▲, 1,N^6-etheno-ATP. Each point represents the mean of triplicate determinations of the fluorescence intensity of each peak of the CAA derivative separated on the Asahipak GS-320H column. Chromatographic conditions similar to those in Fig. 2.
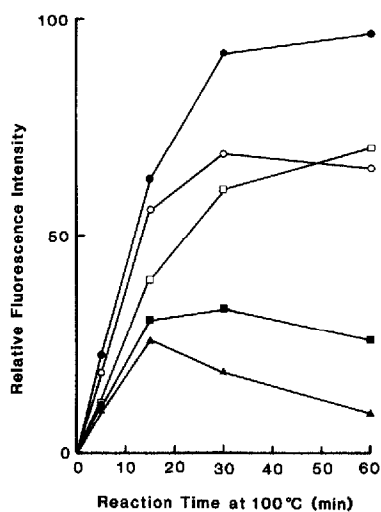
Fig. 4. Effect of reaction time at 100°C on chloroacetaldehyde derivatization of adenine compounds. For details see Fig. 3.

reaction time at 100°C was examined over a period of 60 min. The results, Fig. 4, clearly indicate that the fluorescence intensities of 1,N^6-ethenoadenosine and 1,N^6-etheno-AMP increased with reaction time, whereas those of 1,N^6-ethenoadenine, 1,N^6-etheno-ADP and 1,N^6-etheno-ATP gradually decreased. Hence, for the simultaneous analysis of the five adenine compounds, the derivatization was performed at 100°C for 30 min.

Effect of CAA concentration on quantitative analysis

The molar ratio of CAA to total adenine compounds was varied from $0.7 \cdot 10^2$ to $70 \cdot 10^2$. The total amount of adenine compounds present was always 0.018 μmol. A minimum molar ratio of $7 \cdot 10^2$ of CAA to adenine compounds is required for quantitative analysis. When the ratio fell below this minimum the assay response always became non-linear. Therefore, samples containing high concentrations of adenine compounds had to be diluted prior to derivatization in order to ensure a linear response.

Stability of CAA derivatives

The stability of the derivatives as a function of pH was examined at pH 4.5 and 7.0. The pH of the reaction mixture was adjusted by adding the appropriate buffer after the CAA derivatization reaction. The results obtained are shown in Fig. 5. At pH 4.5, 1,N^6-etheno-AMP, 1,N^6-etheno-ADP and 1,N^6-etheno-ATP were stable throughout the investigation period, although 1,N^6-ethenoadenosine underwent up to 40% decomposition within 7 days. During this time, the fluorescence response of 1,N^6-ethenoadenine increased by approximately the same percentages. At pH 7.0 (Fig. 5B), the fluorescence intensity of 1,N^6-ethenoadenosine increased slightly. Due to the rapid decomposition of 1,N^6-ethenoadenosine at pH 4.5, the reaction mixture was adjusted to pH 7.0 immediately after the reaction.
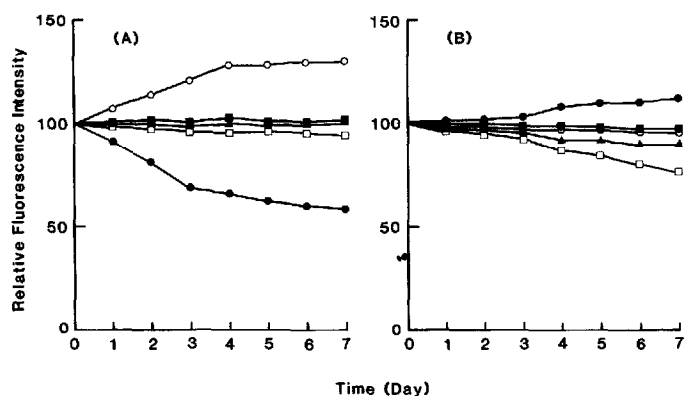
Fig. 5. Stability of 1,N^6-etheno derivatives of adenine compounds at pH 4.5 (A) and 7.0 (B). Details as in Fig. 3.

Specificity

The reaction specificity was examined not only with the major bases, but also with their nucleosides and nucleotides. Under conditions similar to those used in this study, no fluorescent CAA derivatives were detected with guanine, cytosine, thymine and uracil, as well as with their respective nucleosides and nucleotides.

Application to biological fluid

Typical chromatograms showing analyses of CAA derivatives in serum samples with and without addition of standards prior to extraction and CAA derivatization are shown in Fig. 6A and B, respectively. Chromatograms showing analyses of non-derivatized serum samples with monitoring of the absorbance at 260 nm are given in
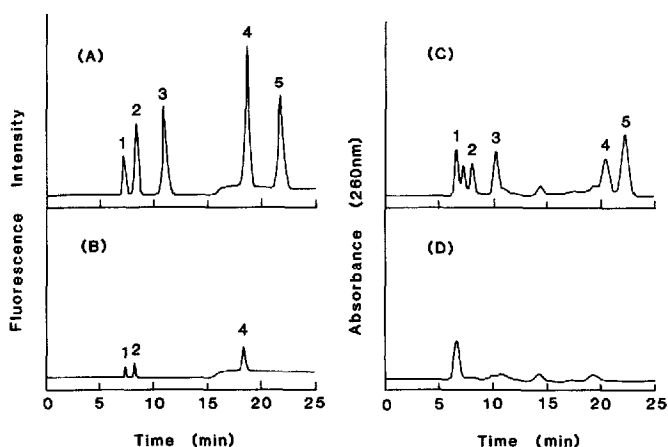

Fig. 6. Typical chromatograms of 1,N^6-etheno derivatives (A) in a serum sample spiked with adenine compounds (10 μl of 1 mg/ml of each) prior to extraction and derivatization and (B) in a blank serum sample. Chromatograms obtained from analyses of non-derivatized serum samples with (C) and without (D) addition of adenine compounds by monitoring the UV absorbance at 260 nm are shown for comparison. Peak notations are similar to those in Fig. 2.

TABLE I

CONCENTRATIONS OF ADENINE COMPOUNDS IN RAT SERUM

Compound	Concentration (nmol/ml serum)
Adenosine	2.21 ± 0.79
ADP	0.47 ± 0.14
ATP	0.41 ± 0.14

Fig. 6C and D. There was no interference by the matrix in the separation in chromatogram A, although some interference was evident in the separation in chromatogram C. Ado, ADP and ATP were detected as $1,N^6$-etheno derivatives in blank serum samples, as shown in Fig. 6B. Except for interference, no adenine compounds were detected by monitoring the absorbance at 260 nm, as shown in Fig. 6D. The concentrations of adenine compounds detected in rat serum are summarized in Table I. The results are given as mean values ± standard deviation for eight male rats. Ade and AMP were not detected by the method described.

DISCUSSION

In studies of nucleic acid metabolism, the microanalysis of nucleic acid components with high sensitivity and selectivity is required.

To cite a few references, Secrist *et al.*[14,15] found that fluorescent $1,N^6$-etheno derivatives of adenosine and its nucleotide were obtained by the reaction with CAA. Similarly, Barrio *et al.*[15] reported that cytidine reacted more rapidly with CAA when near pH 3.5, while 4.5 was the optimum pH required for reaction with adenosine. Also, Kochetkov *et al.*[13] have revealed a dependence of the selectivity of the CAA reaction on pH. Moreover, Sattsangi *et al.*[22] have reported that the synthesis of the fluorescent $N^2,3$-ethenoguanine by sterically hindering the reaction of CAA at N-1 of the guanine ring. Yoshioka and Tamura[17] reported the determination of Ade, Ado, AMP and cyclic AMP as their $1,N^6$-etheno derivatives by HPLC, using a porous polystyrene column.

In the present study, we attempted to optimize the CAA derivatization and chromatographic separation of adenine compounds. Likewise, we have proposed a procedure having some advantages for the analysis of adenine and related compounds, as summarized below. (1) The $1,N^6$-etheno derivatives of the adenine compounds were distinctly separated from each other using an Asahipak GS-320H column, as compared with the previous method[17–19]. Hence, simultaneous quantitative analysis of Ade, Ado, AMP, ADP and ATP was possible. The solid phase of the Asahipak GS-320H column is a hydrophilic hard gel composed of copolymers of vinyl alcohol. (2) The $1,N^6$-etheno derivatives were deemed to be more stable at pH 7.0, hence precise analysis was possible. Upon degradation of $1,N^6$-ethenoadenosine at pH 4.5, a gradual increase in the fluorescence intensity of $1,N^6$-ethenoadenine was observed. This seems to be caused by hydrolysis of $1,N^6$-ethenoadenosine by an acid, thereby yielding $1,N^6$-ethenoadenine. At pH 7.0, the fluorescence intensity of $1,N^6$-ethenoadenosine increased slightly. In this case, gradual dephosphorylation of $1,N^6$-

etheno-AMP yielded 1,N^6-ethenoadenosine. (3) The CAA derivatization was found to be specific for the adenine compounds. Although CAA derivatization of cytidine and guanine had been reported[15,22], under the conditions used in this study, however, no fluorescent derivatives were detected with cytosine and guanine, as well as with their nucleosides and nucleotides. This discrepancy is apparently due to differences in the reaction conditions such as pH, temperature and CAA concentration.

This study was primarily undertaken to develop an analytical method for measuring adenine compounds in biological materials. The significance of the concentrations of adenine compounds found in rat serum remains to be elucidated. Suffice it to say that the developed methodology should enhance not only the possibility of measuring other adenine compounds, but also the study of the metabolism and biological roles of adenine compounds.

REFERENCES

1 M. Zakaria and P. R. Brown, *J. Chromatogr.*, 226 (1981) 267.
2 P. R. Brown (Editor), *HPLC in Nucleic Acid Research*, Marcel Dekker, New York, 1984, p. 247.
3 R. A. Hartwick, A. M. Krstulovic and P. R. Brown, *J. Chromatogr.*, 186 (1979) 659.
4 G. A. Taylor, P. J. Dady and K. R. Harrap, *J. Chromatogr.*, 183 (1980) 421.
5 A. McBurney and T. Gibson, *Clin. Chim. Acta*, 102 (1980) 19.
6 R. J. Simmonds and R. A. Harkness, *J. Chromatogr.*, 226 (1981) 369.
7 R. Boulieu, C. Bory, P. Baltassat and C. Gonnet, *Anal. Biochem.*, 129 (1983) 398.
8 D. M. Tidd and D. S. Dedhar, *J. Chromatogr.*, 145 (1978) 237.
9 R. Meyer and K. G. Wagner, *Anal. Biochem.*, 148 (1985) 269.
10 R. Boulieu and C. Bory, *J. Chromatogr.*, 339 (1985) 380.
11 K. Nakano, K. Shindo, T. Yasaka and H. Yamamoto, *J. Chromatogr.*, 343 (1985) 21.
12 P. Rotllan, A. Liras and P. Llorente, *Anal. Biochem.*, 159 (1986) 377.
13 N. K. Kochetkov, V. N. Shibaev and A. A. Kost, *Tetrahedron Lett.*, (1971) 1993.
14 J. A. Secrist, III, J. R. Barrio, N. J. Leonard and G. Weber, *Biochemistry*, 11 (1972) 3499.
15 J. R. Barrio, J. A. Secrist, III and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 46 (1972) 597.
16 G. Avigad and S. Damle, *Anal. Biochem.*, 50 (1972) 321.
17 M. Yoshioka and Z. Tamura, *J. Chromatogr.*, 123 (1976) 220.
18 J. F. Kuttesch, F. C. Schmalstieg and J. A. Nelson, *J. Liq. Chromatogr.*, 1 (1978) 97.
19 G. C. Mills, F. C. Schmalstieg, K. B. Trimmer, A. S. Goldman and R. M. Goldblum, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 2867.
20 A. A. Kost and M. V. Ivanov, *Chem. Heterocycl. Compd.*, 16 (1980) 209.
21 N. J. Leonard, *CRC Crit. Rev. Biochem.*, 15 (1984) 125.
22 P. D. Sattsangi, N. J. Leonard and C. R. Frihart, *J. Org. Chem.*, 42 (1977) 3292.