

1.00000
11-22-94
078000

ELSEVIER

Journal of Chromatography A, 690 (1995) 55-63

JOURNAL OF
CHROMATOGRAPHY A

Determination of α -dialkylamino acids and their enantiomers in geological samples by high-performance liquid chromatography after derivatization with a chiral adduct of *o*-phthaldialdehyde

Meixun Zhao¹, Jeffrey L. Bada*Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, USA*

First received 13 June 1994; revised manuscript received 23 September 1994

Abstract

Derivatization with *o*-phthaldialdehyde (OPA) and the chiral thiol N-acetyl-L-cysteine (NAC) is a convenient and sensitive technique for the HPLC detection and resolution of protein amino acid enantiomers. The kinetics of the reaction of OPA-NAC with α -dialkylamino acids was investigated. The fluorescence yield of α -dialkylamino acids was only about 10% of that of protein amino acids when the derivatization was carried out at room temperature for 1-2 min, which is the procedure generally used for protein amino acid analyses. The fluorescence yield of α -dialkylamino acids can be enhanced by up to ten-fold when the derivatization reaction time is increased to 15 min at room temperature. The OPA-NAC technique was optimized for the detection and enantiomeric resolution of α -dialkylamino acids in geological samples which contain a large excess of protein amino acids. The estimated detection limit for α -dialkylamino acids is 1-2 pmol, comparable to that for protein amino acids.

1. Introduction

Developments in two areas of research have resulted in efforts to detect α -dialkylamino acids in nature. For example, α -dialkylamino acids have been identified in abiotic synthesis experiments simulating primitive Earth conditions [1], in carbonaceous chondritic meteorites [2] and in sediments (the Cretaceous/Tertiary boundary) associated with the impact of a large asteroid or comet with the Earth [3]. In addition, α -

dialkylamino acids have also been shown to be present in several polypeptidic antibiotics [4] and have been incorporated into enzymes without greatly affecting their activity [5]. The observation that these amino acids may serve as specific inhibitors of enzymes that use protein amino acids as substrates has raised interest in their potential as pharmacologically active agents [6].

Interest in the α -dialkylamino acids has led to the utilization of several chromatographic methods for their detection and optical isomer separation. The separation of isovaline enantiomers by gas chromatography (GC) after derivatization with N-trifluoroacetyl anhydride (TFA) and a

¹ Present address: Environmental Sciences Program, University of Massachusetts, Boston, MA 02125-3393, USA.

chiral alcohol has been successfully achieved [7]. However, attempts to separate optical isomers of α -dialkylamino acids using commercial chiral GC columns has been largely unsuccessful: either poor isomer separation or on-column degradation was observed [8, 9]. Separations by high-performance liquid chromatography (HPLC) either utilizing a chiral mobile complex or employing pre- or postcolumn chiral derivatization have been more successful [10].

Almost all of the reports of the attempted resolution of the enantiomers of α -dialkylamino acids either used authentic compounds, or α -dialkylamino acids were the major components in the mixtures [11]. In efforts to detect α -dialkylamino acids in marine sediments and other geological samples where α -dialkylamino acids are minor components, we have found that most of these methods do not give satisfactory results. Interfering peaks often hindered the identification of the α -dialkylamino acids. In other instances higher sensitivity was required.

The purpose of this study was to develop a sensitive, reliable and routine HPLC method for the detection and simultaneous resolution of optical isomers of α -dialkylamino acids in geological samples in the presence of a large excess of the protein amino acids. A widely used HPLC method for the determination of amino acids is based on the reaction of amino acids with OPA and a thiol [12]. The derivatives formed can be separated on reversed-phase HPLC columns and detected using a fluorescence detector. If the thiol also has a chiral centre, diastereomers will be formed and they often can be separated by HPLC. Aswad [13] used NAC as the chiral thiol and successfully separated the enantiomers of aspartic acid. Nimura and Kinoshita [14] extended the OPA–NAC method to resolve most of the protein amino acids enantiomers. No attempt was made to separate α -dialkylamino acid enantiomers using OPA–NAC in these studies. We demonstrate here that the OPA–NAC method can be used for the detection and resolution of several α -dialkylamino acid enantiomers. Derivatization conditions for the OPA–NAC technique were

optimized to enhance the detection of α -dialkylamino acids. The geochemical applications of this method are demonstrated by the identification of α -aminoisobutyric acid (AIB) and racemic isovaline in Cretaceous/Tertiary (K/T) boundary sediments.

2. Experimental

2.1. Reagents and standards

α -aminoisobutyric acid (AIB), α -methylaspartic acid (α -Me-Asp) and α -methylglutamic acid (α -Me-Glu) were obtained from Sigma. Isovaline (Isoval), α -methylvaline (α -Me-Val), α -methylnorvaline (α -Me-n-Val) and α -ethyl- α -aminobutyric acid (α -Et-ABA) were kindly synthesized by Professor Stanley Miller (Department of Chemistry, University of California, San Diego, CA, USA).

HPLC-grade methanol and sodium acetate were obtained from Fisher and OPA and NAC from Sigma. Doubly distilled water was prepared in the laboratory by two consecutive distillations of deionized water in all-glass stills.

NAC solution (1M) was prepared by dissolving NAC in doubly distilled water and adjusting the pH to 5–6 with 2 M sodium hydroxide. The 1 M NAC solution was stored frozen. OPA–NAC derivatization solution was prepared by (1) dissolving 4.00 mg of OPA in 300 μ l of methanol, (2) adding 250 μ l of 0.4 M sodium borate buffer (pH 9.4) to ensure that the solution would be basic, (3) adding 15 μ l of 1 M NAC and (4) adding 435 μ l of water to give a total volume of 1 ml. OPA–NAC solution was prepared fresh weekly and kept frozen in the dark.

2.2. HPLC analysis

Derivatization of amino acids with OPA–NAC was carried out by thoroughly mixing 20 μ l of an amino acid solution (normally 1–100 pmol/ μ l) with 5 μ l of OPA–NAC reagent in a poly-

ethylene "microfuge" tube. After 1–15 min, 475 μ l of 50 mM sodium acetate buffer (pH 5.2) were added to stop the reaction. A 50- μ l volume of this solution was injected into an Altex Model 332 HPLC system. The highly fluorogenic derivatives were separated on an Alltech Econosphere C₁₈ column (250 \times 4.6 mm I.D.). The mobile phases were (A) methanol and (B) 50 mM sodium acetate (pH 5.4)–methanol (92 : 8). The column was equilibrated with 100% B. Four minutes after injection, a gradient was started to change the mobile phase to 63% B in 10 min, then to 58% B in another 10 min and to 40% B in 5 min, and this concentration of B then maintained to the end of the analysis. Finally, the mobile phase was changed back to 100% B in 15 min and the system was ready for the next injection. The flow-rate was 1 ml/min. The column effluent was monitored with a Shimadzu RF-530 fluorescence HPLC monitor at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The results were recorded with a Hitachi D-2500 Chromato-Integrator. The amino acid concentrations in an actual sample were calculated from peak-area integration using the peak area determined from a known amount of authentic standard for comparison.

2.3. HCl cation-exchange chromatography

A preparative-scale acid cation-exchange chromatographic method was utilized to separate AIB and isovaline from interfering protein amino acids [15]. A column of cation-exchange resin (AG 50W-X8, 100–250 μ m, hydrogen form) was equilibrated with 1.0 M HCl. A mixture of amino acids was placed on the top of the column. Increasing concentrations of HCl were used to elute the amino acids from the column; the eluate was collected with a Gilson fraction collector at a flow-rate of 0.2 ml/min. Each fraction consisted of 2–4 ml of solution. Evaporation of the HCl fractions in a desiccator yielded the purified amino acids. The fractions were then dissolved in 1 ml of water and analyzed by the technique described above.

3. Results and discussion

3.1. Kinetics of the derivatization reaction

Under the derivatization conditions given by Aswad [13] and Nimura and Kinoshita [14], protein amino acids react with OPA–NAC rapidly at room temperature. However, it has been noted that α -dialkylamino acids react much more slowly with OPA under similar conditions [16]. We therefore decided to carry out a systematic investigation of the reaction kinetics of α -dialkylamino acids with OPA–NAC to test whether this technique can be modified for their sensitive detection.

Initially the same reaction conditions as recommended by Aswad [13] were used, with OPA at 6 mM and NAC at 12 mM in the derivatization solution (OPA to NAC ratio = 0.5). The amino acid concentration is normally adjusted to about 10–100 μ M. Alanine was chosen as an example to observe the reaction of OPA–NAC with protein amino acids. Under these conditions the reaction of alanine with OPA–NAC reaches its maximum yield in less than 2 min, as observed by Aswad [13]. In the next step, the concentrations of OPA and NAC were varied and the reaction kinetics monitored. The results indicated that as long as the concentration of NAC is within the range 1–20 mM and the concentration of OPA is more than 10–20 times that of alanine, the reaction is completed within 1–2 min. The rate of reaction and the fluorescence yield are not very sensitive to the ratio of OPA to NAC. If the concentration of OPA is comparable to that of alanine, the derivatization slows considerably. Svedas et al. [17] observed a similar behavior for the reaction of protein amino acids with OPA and mercaptoethanol. They concluded that the OPA concentration should be 2–3 times higher than that of the amino acid. Based on our study and earlier reports, it is recommended that the OPA concentration be at least ten times higher than that of the amino acid for protein amino acid analysis using OPA–NAC.

The same approach was utilized to investigate

the reaction of α -dialkylamino acids with OPA-NAC using AIB as an example. Under the same conditions as used by Aswad [13], this reaction proceeds much more slowly than that with alanine. A detailed study of the kinetics of the AIB reaction was carried out by using various combinations of OPA, NAC and AIB concentrations to determine the best reaction parameters (Fig. 1). These experiments demonstrated that as long as the OPA and NAC concentrations are roughly 10–20 times higher than that of AIB, both the rate of the derivatization reaction and the maximum fluorescence yield are a function of the ratio of OPA to NAC. Fig. 1 shows the fluorescence response of OPA-NAC with AIB as a function of derivatization time at room temperature using various OPA to NAC ratios from 0.25 to 4. It is clear that, at room temperature, this reaction needs at least 15 min to reach equilibrium. When the OPA to NAC ratio is low (e.g., 0.25 and 0.5), equilibrium is not reached even after 25 min. When the OPA to NAC ratio is increased to 1, the reaction is accelerated and equilibrium is achieved within 15 min. At an OPA to NAC ratio of 2, the rate of the reaction is the fastest and the maximum yield is the

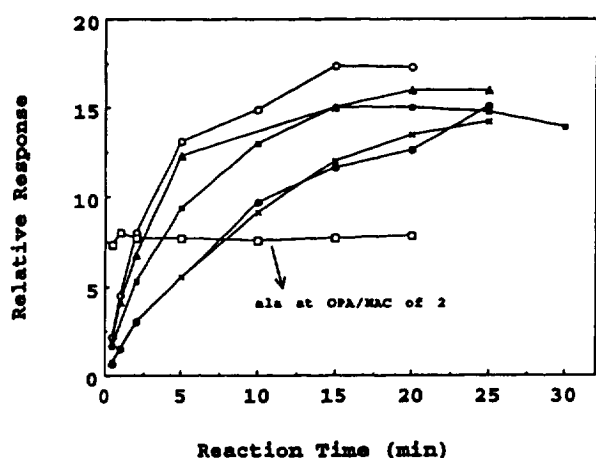


Fig. 1. Kinetics of the reaction of AIB with OPA-NAC with different ratios of OPA to NAC: ○ = 2; ▲ = 4; ■ = 1; ● = 0.5; × = 0.25. □ = Alanine. Also shown are the reaction kinetics of alanine with OPA-NAC for OPA/NAC = 2. Concentrations in the derivatization reaction: OPA = 6 mM, AIB = 12 μ M and alanine = 6 μ M. The amount of AIB injected into the column was 30 pmol and that of alanine was 15 pmol.

highest. When the OPA to NAC ratio is increased to 4, the reaction proceeds at roughly the same rate as with a ratio of 2, but the maximum fluorescence yield is about 15% lower. Fig. 1 clearly demonstrates that for the OPA-NAC reaction with AIB, an OPA to NAC ratio of 2 is the optimum to maximize both the reaction rate and the final fluorescence yield.

Our results are in agreement with the report by Cronin et al. [16] that the fluorescent yield of the OPA- β -mercaptoethanol derivative of α -dialkyl-substituted amino acids is much less than that for amino acids with an α -hydrogen when derivatization is carried out for 1–2 min at room temperature. Cronin et al. [16] were able to increase the yield of α -dialkylamino acids by carrying out the reaction at 100°C. Based on our results and those of Cronin et al. [16], it seems that there are two approaches to improve the detection of α -dialkylamino acids: (1) carry out the derivatization at higher temperature (100°C) for 1–2 min, or (2) carry out the derivatization at room temperature for 15–20 min. With the OPA-NAC reaction, derivatization at 100°C increased the fluorescence yield of α -dialkylamino acids but the response of the α -hydrogen amino acids was also substantially affected (e.g., the yield for glycine was decreased by a factor of 10–15). Also, when amino acids extracted from sediments were derivatized at 100°C, several side-reactions occurred that gave rise to unknown peaks which interfered with the detection of several amino acids.

Our kinetic studies of the OPA-NAC reaction with AIB imply that the fluorescent yield for the α -dialkylamino acids can also be increased up to eight-fold if derivatization is carried out for 15 min compared with 1–2 min at room temperature while the yield for the α -hydrogen amino acids is more or less unaffected (see Table 1). This not only improves the detection for α -dialkylamino acids, but also provides another criterion for the identification of the α -dialkylamino acids. By carrying out the derivatization for both 1 and 15 min, we can easily recognize the α -dialkylamino acids by observing the dramatic increase of 5–10-fold in their fluorescent peak size when the chromatograms

Table 1
Retention time, Y15/Y1 ratio and relative molar fluorescence for protein and α -dialkylamino acids

Amino acid	Retention time (min)	Y15/Y1 ^a	Molar fluorescence ^b
Asp	7.5–8.9	1	1.04
Glu	11.3	1	1.14
α -Me-Asp	12–12.5	4.6	0.40
α -Me-Glu	12.9–13.6	3.9	1.06
Gly	14.7	0.8	1
Ala	16.7–16.9	1	1.47
AIB	18	8	1.19
Isovaline	19.3–19.6	12	0.93
Val	20.4–21.7	6.1–12	0.43
α -Et-ABA	21.7	6.1–12	0.43
α -Me-Val	22.7–23.5	8–10	0.64
α -Me-Nva	22.5–22.9	3.7–6.8	0.40
Leu	26.8	1	0.73

The retention times would be expected to vary depending on the instrument, column and elution scheme used in the analyses. The values of Y15/Y1 and the molar fluorescence depend on the room temperature and the age of the OPA-NAC reagent. The values given here are those which were found to be representative of those routinely obtained with the present analytical set-up.

^a Y15/Y1 is the relative fluorescence yield ratio for the 15- and 1-min derivatization times.

^b Relative to the glycine = 1.0.

for the two reaction times are compared. It is therefore recommended that the OPA-NAC derivatization be carried out for both 1 and 15 min at room temperature for the identification of α -dialkylamino acids.

3.2. OPA-NAC reaction with several α -dialkylamino acids

Our study was extended to include several other α -dialkylamino acids to test whether this method could be generally applied for their identification. The derivatization conditions for all these amino acids were 6 mM OPA and 3 mM NAC (OPA to NAC ratio = 2) in the derivatization solution. Fig. 2 shows chromatograms for several α -dialkylamino acids. The results in Fig. 2 demonstrate that the same reaction condition as used for AIB analysis can also be applied for other α -dialkylamino acids. With all the α -dialkylamino acids that we have investigated, the yield for the 15-min derivatization (Y15) increased compared with those for 1-min derivatization (Y1). However, the yield enhancement was not the same for all α -dialkylamino acids. The Y15/Y1 ratio ranged from 4 to 12,

with isovaline showing the largest increase and α -methylglutamic acid the smallest. Even with the same amino acid, the Y15/Y1 ratio can vary by up to a factor of 2 depending on the age of the OPA-NAC reagent and the room temperature when the analyses are carried out. However, the derivatization yield for all the α -dialkylamino acids is always significantly greater for the 15-min reaction time. The approximate HPLC elution times for several amino acids (both protein and α -dialkyl) are summarized in Table 1 along with the Y15/Y1 ratio.

With the 15-min derivatization, the molar fluorescence yields of α -dialkylamino acids are comparable to those of protein amino acids (see Table 1). In fact, the yields for AIB and isovaline are higher than those for some protein amino acids, such as valine and leucine. The estimated detection limit is 0.3–1 pmol for α -dialkylamino acids based on analyses of authentic compounds. The detection limit for geological samples would be expected to be higher owing to interference from other components present in these complex mixtures.

In studying the OPA-NAC reaction with α -substituted glutamic acid analogues, Maurs et al.

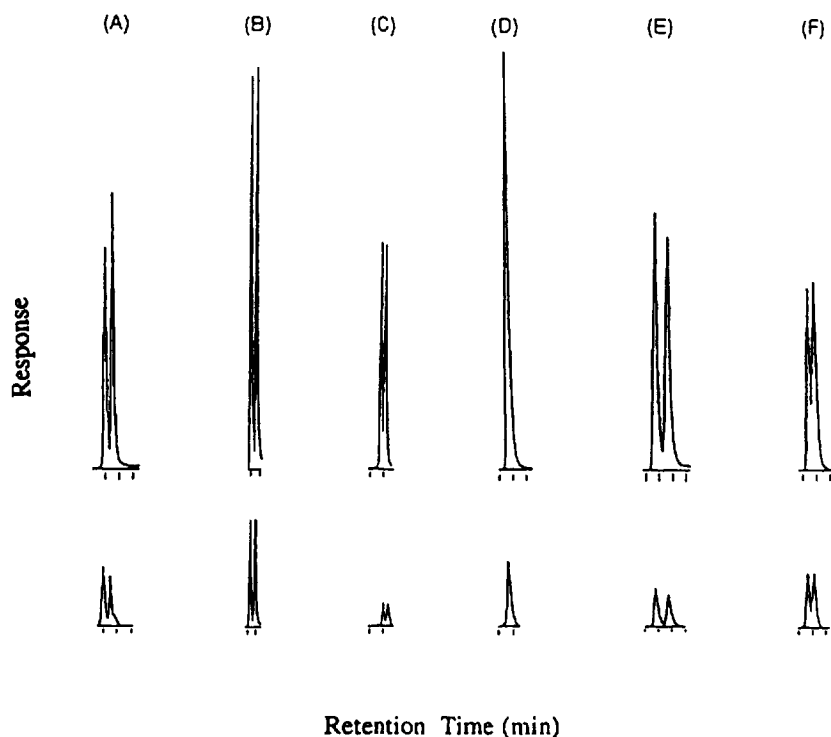


Fig. 2. HPLC of OPA-NAC derivatives of α -dialkylamino acids, obtained with derivatization for (bottom) 1 min and (top) 15 min. (A) D/L- α -Me-Asp; (B) D/L- α -Me-Glu; (C) D/L-Isoval; (D) α -Et-ABA; (E): D/L- α -Me-Val; (F) D/L- α -Me-n-Val. The approximate retention times for these various amino acids are given in Table 1.

[18] gave some conflicting results. They first stated that, despite the steric congestion around the amino group due to α -substitution, α -substituted analogues of glutamic acid react as rapidly as protein amino acids with the OPA-NAC reagent under alkaline conditions. However, they then noticed a significant decrease in the fluorescence response with all the α -substituted amino acids compared with protein amino acids (10–30%). Our results unambiguously demonstrate that α -dialkylamino acids react much more slowly with OPA-NAC at room temperature, but the fluorescence response can be increased with a longer reaction time.

3.3. Resolution of enantiomers

It is critical to evaluate the stereochemistry of α -dialkylamino acids in order to establish their possible biotic or abiotic origin [2]. Although the OPA-NAC technique was originally designed

for the detection of protein amino acids and their enantiomers, the chromatograms shown in Fig. 2 illustrate that this technique can also be used to separate the enantiomers of α -dialkylamino acids. It should be pointed out that we do not know the elution order of the enantiomers for the α -dialkylamino acids as we do not have pure isomers. For protein amino acids, the D-enantiomers elute before the L-enantiomers with hydrophilic amino acids such as aspartic acid, whereas the L-enantiomers elute before the D-enantiomers with hydrophobic amino acids such as valine [11, 14]. We predict that the same elution order would also apply to α -dialkylamino acids.

3.4. Detection of AIB and isovaline in geological samples

To demonstrate the application of the OPA-NAC technique for the detection of α -dialkylamino acids in geological samples, we

selected sediments from the K/T boundary sequence at Stevns Klint, Denmark. The K/T boundary marks one of the largest mass extinction episodes in the Earth's history [19]. Convincing evidence has suggested that this mass extinction was caused by the collision of a large extraterrestrial object with the Earth [20-23]. The collision of an asteroid or comet with the Earth may have added extraterrestrial organic matter to the earth since certain meteorites and comets contain a variety of organic compounds [24]. We decided to investigate AIB and isovaline in K/T boundary sediments as these two amino acids have been found to be among the most abundant non-protein amino acids in the Murchison meteorite [2].

Fig. 3 shows the HPLC traces for the total amino acid extract from a Stevns Klint K/T boundary sediment sample. Regions where AIB and isovaline are expected to elute are indicated by arrows. Examination of the chromatograms for the 1- and 15-min derivatization times indicate that AIB and isovaline are probably present in this sample. However, it is obvious that their concentrations are minute compared with those of the protein amino acids. This complicates the detection and quantitation of the α -dialkylamino acids because many small unknown peaks in the chromatogram of the sediment extract may co-elute with AIB and isovaline. However, careful evaluation of the chromatograms still provides preliminary evidence that AIB and isovaline are present. Comparing the 1- and 15-min results, it is seen that, for the peak in the AIB retention time region, the fluorescence yield for the 15-min derivatization was double that for the 1-min derivatization. However, if we examine this peak carefully in the 15-min chromatogram, we notice that peak 9 appears to be a doublet. One explanation is that there are two peaks with a retention time roughly corresponding to AIB. For the 1-min analysis the AIB peak is much smaller than the unknown peak so AIB is totally hidden beneath this unknown peak. For the 15-min analysis, the AIB peak increases 5-8-fold whereas the unknown peak stays more or less the same. As a result, the AIB peak height is comparable to that of the unknown peak.

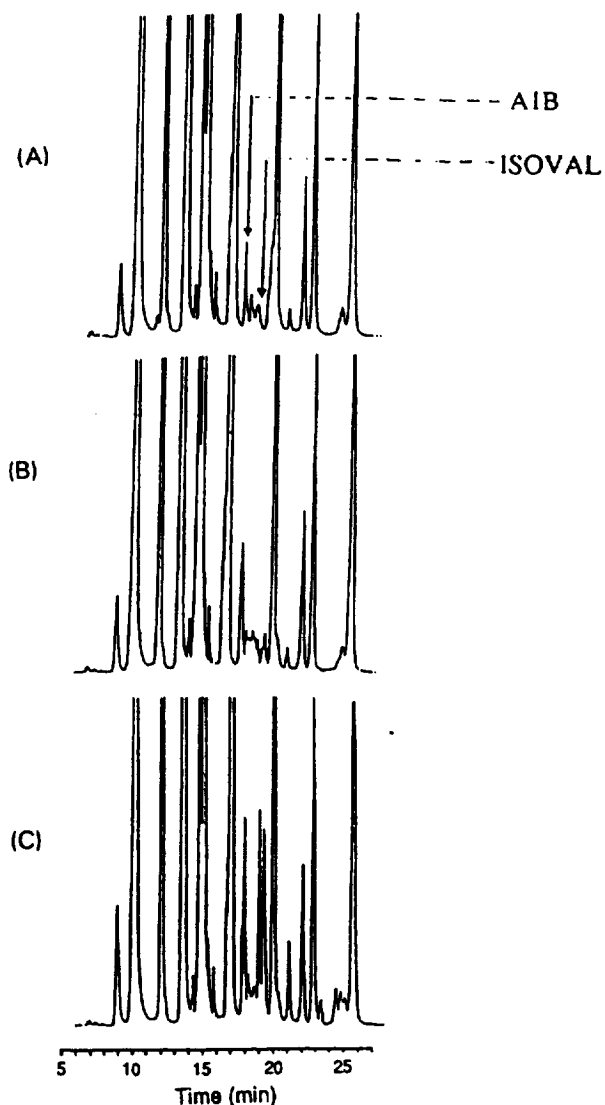


Fig. 3. HPLC of the total amino acid fraction from a sediment sample 0.5 m below the K/T boundary at Stevns Klint, Denmark. HPLC conditions are given in the text. (A) 1-min derivatization; (B) 15-min derivatization; (C) 15-min derivatization after spiking with authentic AIB and racemic isovaline.

Because their retention times are not exactly the same, AIB and the unknown peak appear to be partially separated. The 1-min analysis gives no indication of isovaline in the sample. However, when the sample is derivatized for 15-min the corresponding isovaline peaks apparently emerge. There seems to be two peaks, as would be expected if racemic isovaline was present.

The retention times of both AIB and racemic isovaline in this sample were confirmed by spiking the sediment extract with the authentic amino acids (Fig. 3C).

The analyses of bulk amino acids in K/T boundary sediment extracts gave an indication that K/T boundary sediments probably contain AIB and racemic isovaline, and demonstrated that the OPA-NAC technique could be applied for the detection of these unusual non-protein amino acids in geological samples. However, these analyses are by no means satisfactory. Because the concentrations of AIB and isovaline in K/T boundary sediments are very low compared with those of protein amino acids (the AIB concentration is about 5% of that of alanine in the sample shown), impurities that normally do not interfere with the detection of protein amino acids may interfere with the detection of AIB and isovaline.

To improve this situation, the total amino acid extracts were further separated into fractions using HCl cation-exchange chromatography (see

Experimental). With this separation procedure, AIB and isovaline are partially resolved from protein amino acids. Their relative concentration compared with the background of protein amino acids is increased and co-eluting peaks are usually eliminated. The chromatograms of the AIB and isovaline fractions for the same sample shown in Fig. 3 are given in Fig. 4. Here the presence of AIB and racemic isovaline in this K/T boundary sample is clearly demonstrated. By analyzing known amounts of authentic AIB and racemic isovaline, and comparing the resulting peak areas with those in the K/T boundary sample, we were able to estimate [3] that this sediment contains 350 ng/g of AIB and 120 ng/g of racemic isovaline.

4. Conclusions

Based on these investigations, we recommended the following procedure for the detection of α -dialkylamino acids in geological samples. The total amino acid extract obtained from a sample should be analyzed using the OPA-NAC technique with both 1- and 15-min derivatization times. If a peak, or peaks, corresponding to a specific α -dialkylamino acid increases several-fold, this provides the first indication that an α -dialkylamino acid is present. Further verification can be accomplished by first partly separating the amino acids using either HCl cation exchange chromatography or some other technique, and then repeating the OPA-NAC analyses. The method provides for the highly sensitive and selective detection of α -dialkylamino acids in natural samples which are dominated by the protein amino acids.

Acknowledgments

This research was supported by NSF grant EAR89-15829. We thank Professor Stanley Miller for synthesizing and providing several of the α -dialkylamino acids used in this study.

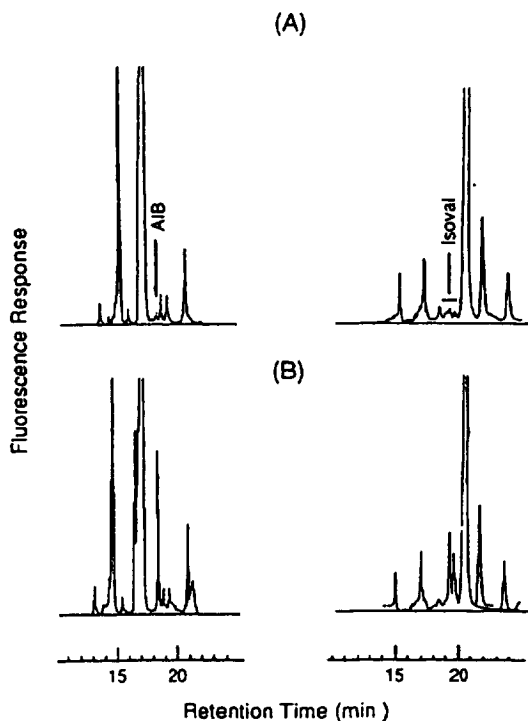


Fig. 4. HPLC of AIB and isovaline HCl cation-exchange chromatographic fractions from the same K/T boundary sample as shown in Fig. 3. HPLC conditions are given in the text. (A) 1-min derivatization; (B) 15-min derivatization.

References

- [1] S.L. Miller, *J. Am. Chem. Soc.*, 77 (1955) 2351.
- [2] K.A. Kvenvolden, J.G. Lawless and C. Ponnamparuma, *Proc. Natl. Acad. Sci. U.S.A.*, 68 (1971) 486.
- [3] M. Zhao and J.L. Bada, *Nature*, 339 (1989) 463.
- [4] M.K. Das, S. Raghothama and P. Balaram, *Biochemistry*, 25 (1986) 7110.
- [5] J.A. Ellman, D. Mendel and P.G. Schultz, *Science*, 255 (1992) 197.
- [6] R.C. Sheppard (Senior Reporter), *Amino Acids, Peptides, and Proteins* (Specialist Periodical Report, Vol. 10), Chemical Society, London, 1979, p. 11.
- [7] G.E. Pollock, C.-N. Cheng, S.E. Cronin and K.A. Kvenvolden, *Geochim. Cosmochim. Acta*, 39 (1975) 1571.
- [8] S.-C. Chang, R. Charles and E. Gil-Av, *J. Chromatogr.*, 238 (1982) 29.
- [9] S. Weinstein, B. Feibush and E. Gil-Av, *J. Chromatogr.*, 126 (1976) 97.
- [10] M.H. Engel and B. Nagy, *Nature*, 296 (1982) 837.
- [11] H. Bruckner, I. Bosch, T. Graser and P. Furst, *J. Chromatogr.*, 395 (1987) 569.
- [12] M. Roth, *Anal. Chem.*, 43 (1971) 880.
- [13] D.W. Aswad, *Anal. Biochem.*, 137 (1984) 405.
- [14] N. Nimura and T. Kinoshita, *J. Chromatogr.*, 352 (1986) 169.
- [15] C.H.W. Hirs, S. Moore and W.H. Stein, *J. Am. Chem. Soc.*, 76 (1954) 6063.
- [16] J.R. Cronin, S. Pizzarello and W.E. Gandy, *Anal. Biochem.*, 93 (1979) 174.
- [17] V.J.K. Svedas, I.J. Galaev, I.L. Borisov and I.V. Berezin, *Anal. Biochem.*, 101 (1980) 188.
- [18] M. Maurs, F. Trigalo and R. Azerad, *J. Chromatogr.*, 440 (1988) 209.
- [19] D.M. Raup, *Science*, 231 (1986) 1528.
- [20] L.W. Alvarez, F. Asaro and H.V. Michel, *Science*, 208 (1980) 1095.
- [21] A. Montanari, R.L. Hay, W. Alvarez, F. Asaro, H.V. Michel, L.W. Alvarez and J. Smit, *Geology*, 11 (1983) 668.
- [22] B.F. Bohor, P.J. Modreski and E.E. Foord, *Science*, 236 (1987) 705.
- [23] J.F. McHone, R.A. Nieman, C.F. Lewis and A.M. Yates, *Science*, 243 (1989) 1182.
- [24] F. Mullie and J. Reisse, *Top. Curr. Chem.*, 139 (1987) 83.