Journal of Chromatography, 386 (1987) 243–249
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1023

COMPARISON OF AUTOMATED PRE-COLUMN AND POST-COLUMN ANALYSIS OF AMINO ACID OLIGOMERS

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SUMMARY

It has been shown that various amino acids will polymerize under plausible prebiotic conditions on mineral surfaces, such as clays and soluble salts, to form varying amounts of oligomers (n = 2–6). The investigation of these surface reactions required a quantitative method for the separation and detection of these amino acid oligomers at the picomole level in the presence of nanomole levels of the parent amino acid. In initial high-performance liquid chromatography (HPLC) studies using a classical postcolumn *o*-phthalaldehyde (OPA) derivatization ion-exchange HPLC procedure with fluorescence detection, problems encountered included lengthy analysis time, inadequate separation and large relative differences in sensitivity for the separated species, expressed as a variable fluorescent yield, which contributed to poor quantitation. We have compared a simple, automated, pre-column OPA derivatization and reversed-phase HPLC method with the classical post-column OPA derivatization and ion-exchange HPLC procedure. A comparison of UV and fluorescent detection of the amino acid oligomers is also presented. The conclusion reached is that pre-column OPA derivatization, reversed-phase HPLC and UV detection produces enhanced separation, improved sensitivity and faster analysis than post-column OPA derivatization, ion-exchange HPLC and fluorescence detection.

INTRODUCTION

The involvement of minerals in adsorption and catalytic reactions of biologically important molecules in chemical evolution was first suggested by Bernal[1] in 1951. Since that time, many students of chemical evolution have utilized minerals in their investigations and consider them an integral and necessary component of the geochemical environment that has been postulated for the primordial reactions of biologically important monomers.

In the course of studying the oligomerization of amino acids, it was found that their analysis by postcolumn derivatization with *o*-phthalaldehyde (OPA) was time-

0021-9673/87/$03.50

consuming (90 min between injections). Moreover, serious problems were encountered in separation and reproducibility due to a large difference in the relative fluorescent response to the OPA derivatives of glycine and its oligomers. The sensitivity of the postcolumn OPA analysis of glycine by fluorescence detection is at least one order of magnitude greater than for the *n*-mers (n = 2–6) of glycine. Since these samples contain high levels of cations, sample cleanup by cation-exchange chromatography, followed by lyophilization was required. It was this series of difficulties that prompted us to investigate pre-column techniques for optimizing the quantitative analysis of picomole levels of glycine oligomers in the presence of nanomole levels of the parent amino acid.

We sought to develop an automated precolumn method of OPA derivatization using reversed-phase HPLC and UV detection to circumvent the problems of postcolumn OPA derivatization and fluorescent detection in the analysis of amino acid oligomers in the presence of their parent amino acids. Following the work of Jones and Gilligan[2], we developed a reversed-phase isocratic elution method for glycine and its oligomers. The IBM LC/9505-SE automatic sample handler allowed us to inject both OPA and samples, to mix them on-line, and to control timing precisely. Elimination of the variations in manual pre-column OPA derivatization resulted in a very accurate and precise method. Ultraviolet detection has the added advantage of providing a simple relationship between concentration of the amino acid derivative and the detector signal, whereas fluorescence detection showed significantly lower quantum yields for oligomers which made quantitation at the required levels difficult.

EXPERIMENTAL

Materials

Glycine and its oligomers (di-, tri-, tetra-, penta- and hexamers) were obtained from Sigma (St. Louis, MO, U.S.A.). The OPA solution (1.0 mg/ml) was prepared as follows: 100 mg of OPA (Pierce, Rockford, IL, U.S.A.) was dissolved in 1.0 ml methanol; 0.2 ml of a 30% (w/w) Brij 35 solution (Pierce) and 0.3 ml of mercaptoethanol (Pierce) were added; this mixture was diluted to 100 ml with 1.0 *M* potassium borate buffer (pH 10.4). Spectral grade methanol (EM Science, Cherry Hill, NJ, U.S.A.) was used in the reversed-phase mobile phase. Sodium acetate was HPLC grade (J. T. Baker, Phillipsburgh, NJ, U.S.A.). The buffer solutions used for ion exchange (Li-220, Li-275 and Li-750) were obtained from Pickering Labs. (Mountain View, CA, U.S.A.). All other reagents used were of the highest purity available; all aqueous solutions were prepared in doubly-distilled, de-ionized water.

Pre-column instrumentation

The HPLC system consisted of an IBM Instruments (Danbury, CT, U.S.A.) LC/9505-SE automatic sample handler for sample injections and an IBM LC/9560 ternary gradient liquid chromatograph for separations. Detection was accomplished using an IBM LC/9563 variable-wavelength UV detector (340 nm) and an IBM LC/9524H fluorescence detector (excitation 360 nm, emission 418–700 nm). System control, quantitation and data analysis were done using the IBM System 9000 laboratory computer. All reversed-phase HPLC separations were done using two IBM octadecyl (C_{18}) 3-μm columns (4.6 × 100 mm) coupled in series. An IBM C_{18} guard

column (2.0 × 50 mm) was used to protect the analytical columns and a SSI 2-μm in-line high-pressure filter (Scientific Systems, State College, PA, U.S.A.) was installed between the injector and pre-column to accomplish on-line mixing of the samples and OPA reagent. The columns and high-pressure filter were maintained at 35°C using the built-in oven on the IBM liquid chromatograph.

Pre-column methology

A solution 0.020 m*M* in glycine and each of the oligomers was prepared in a 100 m*M* sodium acetate buffer (pH = 7.2). Using the automation capabilities of the IBM HPLC system described above, a 10-μl aliquot of sample sandwiched between two 10-μl aliquots of OPA reagent was directly injected into the high-pressure filter for on-line pre-column mixing and pre-column derivatization of the samples. The flow-rate of the mobile phase [24% methanol in 100 m*M* sodium acetate (pH = 7.2)] was set at 0.8 ml/min for isocratic elution.

Post-column instrumentation

The HPLC system was identical to that described earlier for the precolumn method with the addition of a Pickering Labs. post-column reaction system between the column outlet and the detector inlet. All ion-exchange separations were done using the Pickering Fast-Li cation exchange 7-μm column (150 × 4.6 mm). A Pickering Li guard column (20 × 2.0 mm) was used to protect the analytical column. The columns were maintained at 45°C using the built-in oven on the IBM liquid chromatograph.

Post-column methodology

A solution 0.200 m*M* in glycine and each of the oligomers was prepared in the Li-220 buffer solution. Using the automatic sample handler, 20-μl sample aliquots were injected directly onto the columns. A 40-min linear gradient from 100% Li 275 to 80% Li 275 in Li 750 was used at a flow-rate of 0.3 ml/min. The OPA solution (0.3 ml/min) was continuously mixed with the column effluent, reacted at 60°C in the Pickering post-column reactor, and the derivatized products were consecutively detected by UV and fluorescence.

RESULTS AND DISCUSSION

Post-column separation and detection

The separation of glycine from its oligomers is more complex than an ordinary amino acid separation because of the similar chemical nature of the species. The ion-exchange conditions outlined in the Experimental section were obtained after an extensive search for optimum conditions. Although the use of a sodium buffer eluent would have given a shorter run time, it was impossible to obtain adequate resolution of the oligomers in a sodium system.

Fig. 1 shows the optimum separation obtained using the ion-exchange HPLC post-column OPA derivatization procedure. The elution order by ion-exchange HPLC was as follows: glycine, hexamer, pentamer, tetramer, dimer and trimer. The individual retention times were verified by separate injections of the pure standards. Although this separation was adequate for quantitation of nanomole levels of these

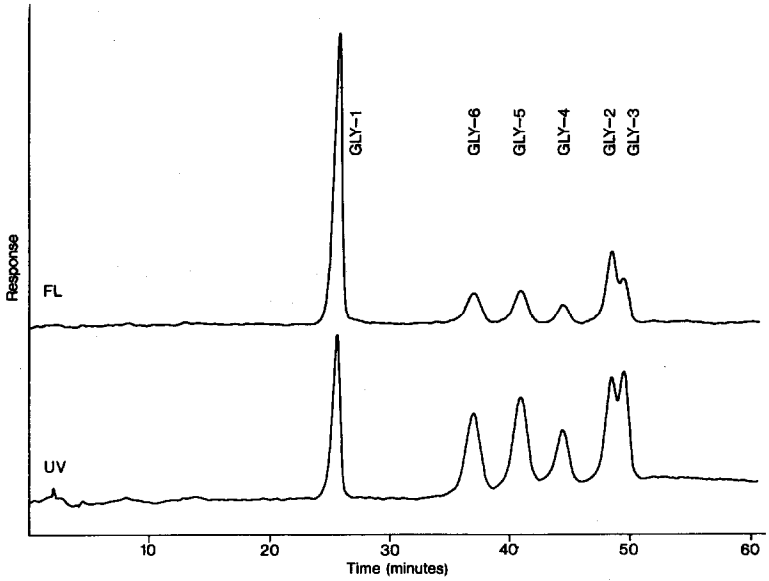

Fig. 1. Chromatogram of glycine and its oligomers obtained by post-column OPA derivatization ion-exchange HPLC and detection by UV (lower curve) and fluorescence (upper curve). The standard contained 4 nmoles of each species.

species, it was not useful for our condensation samples. The main difficulty was the large difference in fluorescence detection sensitivity for the oligomers relative to the parent glycine (Table I). Although the use of a UV detector in this post-column system overcame this problem, the overall sensitivity by UV was limited (minimum detectable quantity = 100 pmoles) due to the high background from the continuous addition of the highly absorbing OPA reagent. In our samples, glycine was normally present at the nanomole level while the oligomers were present at the low picomole level. The above detection limitations coupled with problems of excessive sample clean-up (samples contained large amounts of cations), irreproducibility of retention times, excessive analysis times (90 min between injections including column equilibration), excessive consumption of expensive reagent (27 ml per analysis) and more

TABLE I

RESPONSE RATIOS (PEAK HEIGHT OF OLIGOMER:PEAK HEIGHT OF GLYCINE)

	Post-column		*Pre-column*	
	UV	*Fluorescence*	*UV*	*Fluorescence*
Glycine	1.00	1.00	1.00	1.00
Dimer	0.77	0.24	0.67	0.18
Trimer	0.77	0.18	0.91	0.10
Tetramer	0.43	0.08	0.50	0.06
Pentamer	0.62	0.14	0.91	0.13
Hexamer	0.53	0.11	0.83	0.11

costly hardware prompted us to look at a pre-column OPA derivatization, reversed-phase HPLC system.

Pre-column separation and detection

Fig. 2 shows the optimum separation of glycine and its oligomers by reversed-phase HPLC using the conditions described earlier. Although the system uses a simple isocratic binary mixture, a wide variety of conditions were explored. Methanol gave better resolution than acetonetrile, tetrahydrofuran or any combination of these three organic modifiers. A variety of buffers, ionic strengths and pH values were also investigated with no improvements noted. Several gradient profiles were run but none were better than the isocratic system.

The elution order of these six compounds by reversed-phase HPLC was as follows: hexamer, pentamer, tetramer, trimer, glycine and dimer. This elution order is quite different than that obtained by ion-exchange HPLC (especially for glycine) but this should be expected since the two modes ofg HPLC have different retention mechanisms. It is curious that the elution order by reversed-phase HPLC is nearly opposite of what one might expect at first glance (*i.e.* increasing elution time with increasing size), but since glycine (H_2N–CH_2–COOH) does not have a non-polar side chain, the polarity increases with increasing size of the oligomers. In the case of glycine and the dimer, the overall effect of the OPA complex must be more significant.

One can also see from Fig. 2 and Table I that the same decreasing response problem exists for fluorescence detection in this pre-column OPA derivatization, reversed-phase HPLC system as in the post-column system. The UV detection in the pre-column system does not suffer from the same limitations as the post-column

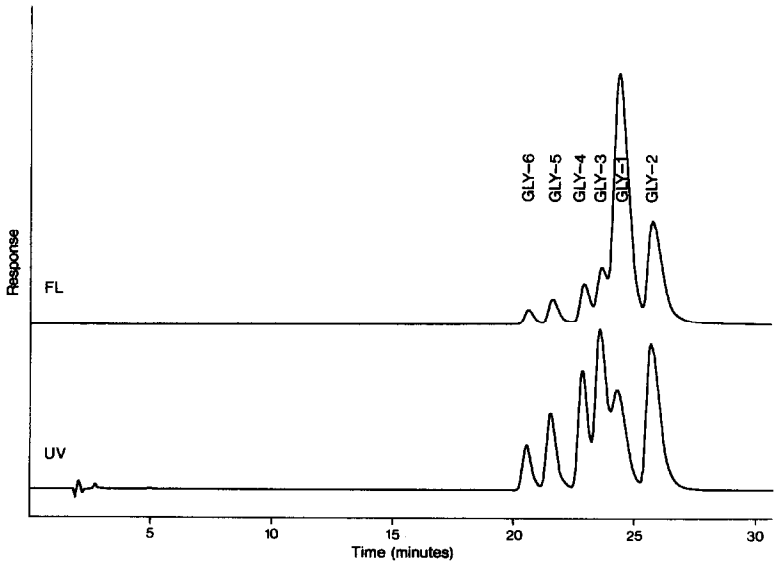

Fig. 2. Chromatogram of glycine and its oligomers obtained by derivatization reversed-phase HPLC and detection by UV (lower curve) and fluorescence (upper curve). The standard contained 0.20 nmoles of each species.

system and, therefore, a detection limit of 10 pmoles for glycine and the oligomers was easily obtained.

Sample analysis

Fig. 3 shows the results of an actual condensation sample analyzed by pre-column OPA derivatization and reversed-phase HPLC. Although the fluorescence trace shows less sample components, one can see that because of the relative response problem, quantitation of the oligomers (especially the dimer) is difficult. The UV trace shows several other sample components early in the elution profile, but the detection and quantitation of glycine and its oligomers was easily obtained. Since this sample was only filtered (0.5 μm) prior to injection, an equivalent chromatogram of this sample on the post-column OPA derivatization, ion-exchange HPLC system could not be obtained (due to the rigorous sample clean-up requirements prior to ion-exchange separation).

Pre-column method validation

One of the major objections to the use of OPA for pre-column derivatization is that the reaction is extremely time dependent which leads to large variations in response for manual reactions[3,4]. The HPLC system used in this study eliminates this problem because the system precisely controls the sampling, mixing and timing of the reaction. This automated control leads to extremely good reproducibility of both retention times (R.S.D. 0.5%) and peak area quantitations (R.S.D. 1–3%) as shown in Table II. This system has been successfully applied to a large number of

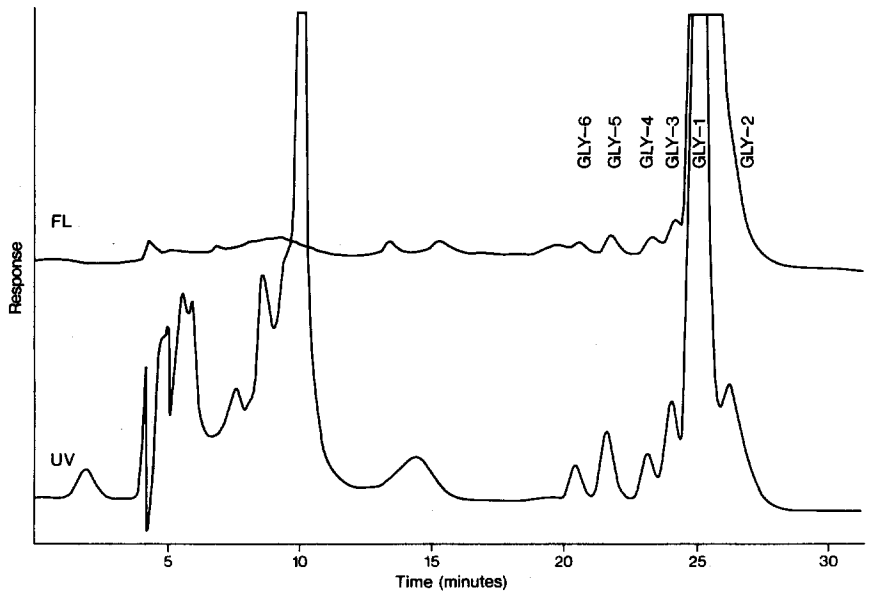

Fig. 3. Chromatogram of glycine and its oligomers using pre-column OPA derivatization reversed-phase HPLC and detection by UV (lower curve) and fluorescence (upper curve). The sample was from a condensation reaction. Glycine is at the 20 nmole level while the oligomers are at the 100–200 pmole level.

TABLE II

REPRODUCIBILITY OF RETENTION TIMES AND PEAK AREAS BY PRE-COLUMN OPA DERIVATIZATION AND UV DETECTION

$n = 10$.

Species	*Retention time (min)*		*Area ($\times 10^7$ μV-s)*	
	Mean	*R.S.D. (%)*	*Mean*	*R.S.D. (%)*
Glycine	24.3	0.5	1.62	1.0
Dimer	25.5	0.6	1.98	2.0
Trimer	23.7	0.5	1.64	3.4
Tetramer	23.0	0.5	1.04	2.9
Pentamer	21.7	0.5	0.72	3.0
Hexamer	20.7	0.4	0.37	2.9

condensation samples as well as a variety of various primary amino acid analysis samples.

CONCLUSIONS

The automated pre-column OPA derivatization, reversed-phase HPLC, UV detection method developed and described herein is ideally suited to the quantitation of amino acid oligomers from condensation experiments. This methodology offers a variety of advantages over the classical post-column OPA derivatization, ion-exchange HPLC fluorescence detection method including: simplicity, lower cost, shorter analysis time, less sample preparation and improved quantitation.

Although this pre-column OPA system meets the needs and requirements of the analysis described in this work, it may not be competitive with post-column derivatization using fluorescence detection for the analysis of more complex solutions such as physiological fluid amino acid analysis. Complex solutions may demand selective separation first to eliminate interferences and thus the use of post-column derivatization. We will be evaluating this procedure on more complex systems in the future.

ACKNOWLEDGEMENT

This research was supported in part by grants to J.B.O. from the National Science Foundation (DMB-8507726) and the National Aeronautics and Space Administration (NAGW-324).

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