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HPLC Analysis of Methylated Amino Acids: Methylated Amino Acids on HPLC

Kwang Sook Park, Sung-Youl Hong,* Hyang Woo Lee,* Sangduk Kim and Woon Ki Paik

Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A.
**Department of Biochemistry, School of Pharmacy, Sung Kyun Kwan University, Suwon 170, Kyunggi-Do, Korea*
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Abstract□Various naturally occuring methylated amino acid derivatives were resolved on high performance liquid chromatography (HPLC), using *o*-phthaldialdehyde as a fluorogenic reagent.

We separated ε-N-monomethyllysine, ε-N-dimethyllysine, and ε-N-acetyllysine from lysine derivatives. N^G-Monomethylarginine and N^G-dimethylarginine were separated from arginine derivatives. However, ε-N-monomethyllysine and ε-N-trimethyllysine, N^G, N^G-dimethylarginine and N^G, N'^G-dimethylarginine were not resolved under the conditions employed. S-Methylmethionine, S-methylcysteine, and 1-N-methylhistidine or 3-N-methylhistidine were clearly separated from their reference amino acids, even though 1-N-methyl- and 3-N-methylhistidine could not be separated.

Keywords□Methylated amino acids, HPLC

Various methylated amino acids are widely distributed in nature as either free or protein-bound. Lysine and arginine derivatives in particular are present ubiquitously in every organism examined.[1-3]. Most of the analytical techniques currently available have been applied to the analysis of methylated amino acids; paper chromatography[4] and electrophoresis[5], thin-layer chromatography and electrophoresis[6], and ion-exchange chromato-graphy.[7-10] In spite of its extremely high sensitivity, however, high performance liquid chromatography (HPLC) has not been commonly utilized for quantitative or qualitative analysis of methylated amino acids. To the best of our knowledge, only a few reports are available so far in the literature. David *et al.*[11] and Kohse *et al.*[12] determined the amount of free ε-N-trimethyllysine in plasma on HPLC using 0-phthaldialdehyde (OPA), and Lischwe *et al.*[13] recently resolved various N^G-methylated arginine derivatives on HPLC, using phenylthiohydantoin (PTH). Thus, as part of an on-going research project on protein methylation, we felt it worthwhile to characterized the naturally occurring various methylated amino acids on HPLC, employing OPA. The results obtained are described herein.

EXPERIMENTAL METHODS

Materials:

All of the methylated amino acid derivatives were obtained either from Sigma Chemical Co., St. Louis, MO. or from Calbiochem Brand Biochemicals, San Diego, CA. All the other reagents used for HPLC were from J. T. Baker Chemical Co., Phillisburg, NJ. *o*-Phthaldialdehyde (OPA) was purchased from Sigma Chemical Co. The remaining reagents were obtained from various commercial sources and were of the highest grade available.

High performance liquid chromatography (HPLC):

Analysis on HPLC was carried out using a Waters Associates Liquid Chromatograph equipped with a Model 721 programmable system controller, two Model 510 pumps, Model 730 Data module, and an automatic sampler WISP Model 710B. OPA adducts of methylated amino acids and internal standard amino acids produced by pre-column mixing were monitored using a Model 420-AC fluorescence detector set at $\lambda_{ex} = 338$ nm and $\lambda_{em} = 425$ nm. The analysis was performed on a Resolve C_{18} column (3.9 mm i.d. × 15 cm) from Waters, which

was maintained at 45°C using a water bath. The fluorogenic reagent was prepared by dissolving 10 mg of OPA in 0.200 ml of methanol, to which was added 0.100 ml of 2-mercaptoethanol. The mixture was then added to 4 ml of 0.5 N sodium borate buffer at pH 10.4. Samples were eluted from Resolve C_{18} column using gradients containing solvent A (methanol: tetrahydrofuran:water = 2:2:96 with 50 mM sodium acetate, 50 mM Na_2HPO_4 to pH 7.5) and solvent B (methanol:water = 65:35). The elution program is shown in the following table.

Time (min)	Flow (ml/min)	%A	%B
Initial	0	100	0
2.00	0.10	100	0
2.50	1.50	100	0
14.00	1.50	50	50
23.00	1.50	0	100
28.00	1.50	100	0
35.00	0	100	0

Amino acids were quantitated by measuring their peak area with the data module.

RESULTS AND DISCUSSION

Separation of various methylated amino acids with OPA on HPLC:

Fig. 1 illustrates the separation of various standard protein-derived amino acids on HPLC, using OPA. Here, glutamine, asparagine, and tryptophan are not included, because they are sensitive to HCl-hydrolysis which is routinely employed for hydrolysis of protein. Proline and hydroxyproline are not reactive with OPA, since this reagent is specific towards amino acids containing primary amines[14]. Furthermore, it should be mentioned that the fluorogenic yield of cysteine with OPA is extremely low, so that the amount of this amino acid used (50 picomoles) does not give rise to any measurable fluorogenic intensity.

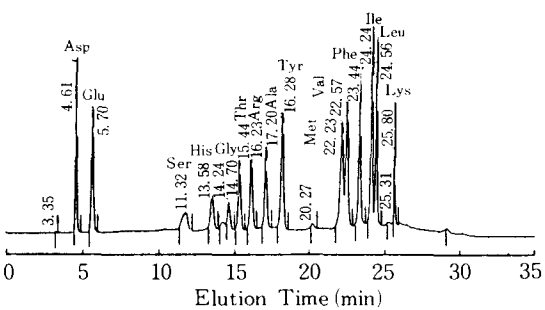

Fig. 1. Separation of standard amino acids on HPLC

Fifty picomoles each of standard amino acids were separated on HPLC using OPA as a fluorogenic reagent. The numbers on each peak indicates its retention time. Detailed chromatographic conditions are described under Methods.

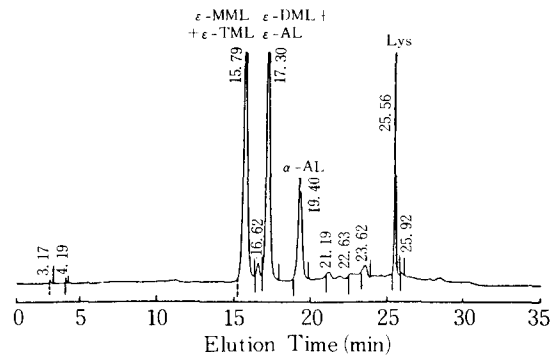

Fig. 2. Separation of various methyl-and acetyl derivatives of lysine on HPLC

One hundred picomoles each of various lysine derivatives were processed. MML, DML, TML, ε-AL, and α-AL represent ε-N-monomethyllysine, ε-N-dimethyllysine, ε-N-trimethyllysine, ε-N-acetyllysine and α-N-acetyllysine, respectively. Detailed experimental conditions are described under Methods.

Fig. 2 shows the elution pattern of 5 variously modified naturally occurring lysine derivatives together with lysine. Unfortunately, however, ε-N-monomethyllysine (MML) and ε-N-trimethyllysine (TML), and ε-N-dimethyllysine (DML) and ε-N-acetyllysine (ε-AL) coelute. α-N-Acetyllysine (α-AL) was clearly resolved from the rest of the lysine derivatives.

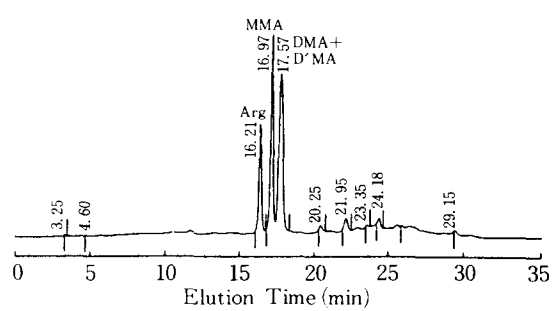

Fig. 3. HPLC analysis of various naturally occurring methylated arginines

On hundred picomoles of each amino acid derivative were analyzed. MMA, DMA, and D'MA represent N^G-monomethylarginine, N^G, N^G-dimethylarginine (asymmetric) and $N^G\ N'^G$-dimethylarginine (symmetric), respectively.

Separation of various naturally occurring methylated arginine derivatives is illustrated in Fig. 3. Two N^G-dimethylarginine [N^G, N^G-dimethylarginine (asymmetric, DMA) and N^G, N'^G-dimethylarginine (symmetric, D'MA)] are not resolved under the conditions employed. These two compounds, however, have been resolved on HPLC, employing PTH as a fluorogenic reagent.[13]

Two N-methylhistidine derivatives [1-N-methyl- (1-MH) and 3-N-methyl- (3-MH)], two S-methyl substituted amino acids [S-methylmethionine (S-MM) and S-methylcysteine (S-MC)], and their reference compounds were examined on HPLC (Fig. 4). Here, 1-N-methyl- and 3-N-methylhistidine could not be separated. Incidently, S-methylmethionine results from methylation of cytochrome *c* catalyzed by an enzyme isolated from *Euglena gracilis*.[15] S-Methylcysteine is formed during DNA repair process.[16]

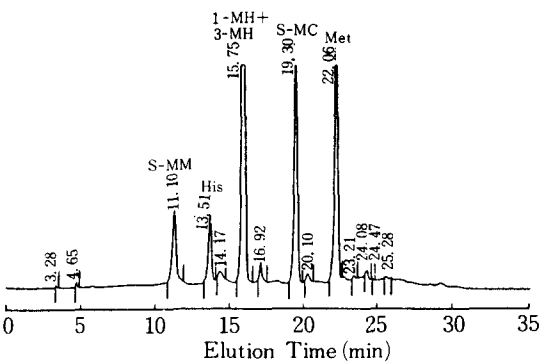

Fig. 4. HPLC analysis of methylated histidines and S-methyl substituted amino acids

One hundred picomoles of each amino acid were analyzed. 1-MH, 3-MH, S-MM and S-MC represent 1-N-methylhistidine, 3-N-methylhistidine, S-methylmethionine and S-methylcysteine, respectively.

Attempts have been made to resolve the above coeluting compounds by either changing the gradients or by changing the initial concentration of the mobile phase without any notable improvement.

Table I lists the results on the fluorescent yield of various amino acid derivatives examined. It is noteworthy that, even though cysteine has an extremely weak fluorogenicity[14], the OPA-adduct of S-methylcysteine exhibits a strong fluorescence. It has been reported that conversion of cysteine to cysteic acid by performic acid increased the fluorescence intensity by 50-fold.[14,17] It is also noted that the relative fluorescence intensities observed in the present experiment do not necessarily coincide with those reported by Roth.[14] However, this is most likely due to the differences in wavelengths used for fluoroscopy (see the Table I).

Table I. Fluorescence intensities(relative units) observed with variously modified amino acid derivatives.

Amino acids and their derivatives	Relative fluorescence intensities	
	Observed	Literature(14)
Arginine	100	100*
N^G-Monomethyl-L-arginine	181	
N^G, N^G-Dimethyl-L-arginine (asymmetric)	113	
N^G, N'^G-Dimethyl-L-arginine (symmetric)	107	
Lysine	89	6
ε-N-Monomethyl-L-lysine	82	
ε-N-Dimethyl-L-lysine	136	
ε-N-Trimethyl-L-lysine	84	
α-N-Acetyl-L-lysine	212	
ε-N-Acetyl-L-lysine	434	
Methionine	218	229
S-Methyl-L-methionine	115	
Cysteine		4
S-Methyl-L-cysteine	338	
Histidine	82	167
1-N-Methyl-L-histidine	319	
3-N-Methyl-L-histidine	196	
Tyrosine	161	169
O-Methyl-L-tyrosine	167	
Serine	48	208
O-Methyl-L-serine	24	
O-Methyl-L-threonine	130	

*Reported values in reference(14) were recalculated based on 100 of arginine. These were determined at λ_{ex}=395nm and λ_{em}=475nm, while present observation was made at λ_{ex}=338nm and λ_{em}=425nm.

The fluorogenic reaction of OPA with primary amines in the presence of a thiol (here, 2-mercaptoethanol) employed in the present experiments yields intensely fluorescent 1,2-disubstituted isoindoles,[14,18] and the reaction has been considered to be specific towards amino acids with primary amines.[14] Thus, even proline and hydroxyproline are completely unreactive with OPA. The side chain of the amino acid has been suggested to influence the stability of the OPA-adduct.[19]

Finally, practical applicability of the present HPLC method for analysis of various methylated amino acid derivatives appears to be rather limited. A few compounds whose structures are extremely similar such as

N^G, N^G- and N^G, N'^G-dimethylarginine, and 1-N-methylhistidine and 3-N-methylhistidine could not be resolved under the conditions employed. Furthermore, when examined for the presence of minute amounts of methylated derivatives in protein hydrolyzate or physiological fluids, the chromatogram would be very much "congested". It is, therefore, advisable to remove acidic and neutral amino acids through Dowex 50 H^+ resin prior to the analysis on HPLC.[11] However, the present HPLC method might be most useful when examined for the identity as well as purity of the compound synthesized.

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