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Note

Enantiomeric separation of underivatized α-methyl-α-amino acids by high-performance liquid chromatography

SHULAMITH WEINSTEIN and NELU GRINBERG*
Department of Organic Chemistry, Weizmann Institute of Science, Rehovot (Israel)
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α-Methyl-α-amino acids (α-Me-AA) are present in various biological systems[1–3], amongst others, as constituents of peptide antibiotics. Also, they are of importance in the study of the origin of life and the determination of their enantiomeric composition in meteorites[4,5], in which they have been detected (*e.g.*, the Murchison), is of particular interest.

High-performance liquid chromatographic (HPLC) methods for the resolution of amino acids into enantiomers by a chiral copper complex, as an additive to the mobile phase, have been known for several years[6–9]. Copper complexes of N,N-dialkyl-α-amino acids were introduced by Weinstein and co-workers and found to resolve several classes of compounds[10–12]. This paper describes the HPLC resolution of α-Me-AA into enantiomers with the copper complexes of N,N-dimethyl-L-valine (DMV) and N,N-dipropyl-L-alanine (DPA) using reversed-phase columns. The enantiomers were detected by fluorescence after post-column derivatization with *o*-phthalaldehyde–mercaptoethanol[13].

EXPERIMENTAL

α-Me-AA were purchased from Sigma (St. Louis, MO, U.S.A.) or prepared in our laboratory, by reaction of ketones with ammonium carbonate and sodium cyanide, followed by hydrolysis of the hydantoins formed[14]. DMV and DPA were prepared according to the method of Bowman and Stroud[15]. The compounds were further purified as follows. A solution containing 32 m*M* DMV (or DPA), dissolved in about 20 ml of water, was passed through a glass column (2 × 1 cm I.D.), containing C_{18} reversed-phase particles (43–63 μm), prepared according to the method of Hemetsberger *et al.*[16]. After discarding the first 1 ml, the eluate was collected, the column was washed with 20 ml of water and the solution was diluted to 100 ml to give a final concentration of 0.32 *M*. This stock solution can be preserved for a long period without bacterial growth by adding a few drops of dilute copper(II) acetate solution and keeping it in a refrigerator.

The *o*-phthalaldehyde (OPA) reagent (Merck, Darmstadt, F.R.G.) was prepared by dissolving 500 mg of OPA in 10 ml of ethanol, to which was added 0.5 ml of 2-mercaptoethanol (Fluka, Buchs, Switzerland). This solution was added to 1 l of a 3% aqueous solution of boric acid containing 2.5 g of EDTA sodium salt, which

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had been adjusted to pH 10.5 with potassium hydroxide, filtered through a Millipore filter (0.22 μm pore size) and degassed. The reagent solution was stored in a brown bottle.

All the solvents and reagents were of HPLC grade. A high-pressure liquid chromatograph with a Waters fluorescence detector (Model 420 E), using standard filters (excitation at 340 nm and emission at 425 nm), an Eldex pump (Model A-30-S) and a Milton Roy pump (Model LA-001A) for the mobile phase and the post-column reagent, respectively, was used. A reversed-phase column (24 × 4.6 mm I.D.) packed with Nucleosil C_{18} (Mackerey, Nagel & Co., Düren, F.R.G.) was equilibrated with an aqueous solution prepared from 2.5 ml of 0.32 *M* DMV or DPA solution plus 2.5 ml of 0.16 *M* copper(II) acetate solution diluted to 100 ml. For some of the amino acids a certain amount of acetonitrile was added in order to decrease the retention time. The flow-rate of the mobile phase was 0.3 ml/min and that of the OPA reagent 0.9 ml/min; the dead volume of the column was 2.5 ml.

For post-column derivatization we used a 10-m PTFE loop (0.3 mm I.D.), which was immersed in a water-bath at 50°C.

RESULTS AND DISCUSSION

The results are shown in Tables I–III and Figs. 1 and 2.

The generally most useful and efficient chiral additive to the mobile phase was found to be the copper complex of DMV. The alkyl substitution pattern of this ligand at the nitrogen and α-carbon atoms turned out to be more stereoselective for interaction with α-Me-AA than DPA, leading to good resolution throughout (Tables II and III). The retention times for some of the amino acids were too high when using pure aqueous solutions as the mobile phase, and addition of acetonitrile was necessary to give convenient analysis times.

At pH = 5.5, with $Cu(DPA)_2$ as a chiral additive, no separation was achieved

TABLE I

RETENTION TIMES (t_R) OF α-Me-AA USING THE COPPER COMPLEX OF DPA AS A CHIRAL ADDITIVE

Composition of mobile phase: DPA 8 m*M*, copper(II) acetate 16 m*M*.

Acetonitrile concentration (%)	*α-Me-AA*		*t_R (min)**	*α***
0	Asp	I***	8.7	1.14
		II	9.9	
	Ser	I	4.7	1.21
		II	5.7	
	His	I	9.0	1.00
		II	9.0	
	Glu	I	18.0	1.00
		II	18.0	
2.5	Norval	I	6.0	1.00
		II	6.0	

* t_R = Adjusted retention time.

** $\alpha = t_{RII}/t_{RI}$.

*** Peak assignment of all α-Me-AA has not yet been made.

TABLE II

RETENTION TIMES (t_R) OF α-Me-AA USING THE COPPER COMPLEX OF DMV AS CHIRAL ADDITIVE

[Cu]:[DMV] = 8:16 (m*M*/m*M*); pH = 5.5.

Acetonitrile concentration (%)	*α-Me-AA*		*t_R (min)**	*α***
0	Asp	I***	7.52	1.21
		II	9.10	
	Ser	I	7.7	1.41
		II	10.9	
	Glu	I	11.5	1.19
		II	13.7	
5	Met	I	17.5	1.15
		II	20.1	
	Trp	I	31.5	1.03
		II	32.5	
10	Leu	I	15.9	1.35
		II	21.5	
	Norleu	I	19.1	1.33
		II	25.5	
15	Phe	I	18.1	1.21
		II	21.9	

* t_R = Adjusted retention time.

** $\alpha = t_{RII}/t_{RI}$.

*** Peak assignment of all α-Me-AA has not yet been made.

for some of the compounds studied (Table I), whereas for $Cu(DMV)_2$ baseline separations resulted for almost all of the α-Me-AA compounds examined (Table II). Only α-Me-Trp, under the experimental conditions used, had an α value of 1.03, and was only partially resolved (Table II).

The pH has a large influence on resolution and, in order to improve the results further, we performed a series of experiments at pH 7 with 0.1 *M* sodium acetate in the mobile phase. As shown in Table III, both the retention times and the α values increased, yielding baseline separations for all the amino acids studied.

A problem to which we have given considerable attention is the detection of α-Me-AA. When post-column derivatization is performed with OPA–mercaptoethanol under the same conditions as for α-H-amino acids, using a 1-m loop and room temperature, the fluorescence response is only a few percent of that observed for the latter. Steric hindrance of the methyl group in the α-position probably reduces the reactivity of the compounds. In order to circumvent this problem in the post-column system, we introduced a 10-m loop and immersed it in a water-bath at 50°C[17]. Under these conditions, the detector response for α-Me-AA increased to half of that of the corresponding α-H-amino acids.

The method presented here is a convenient means for the separation of α-Me-AA into enantiomers. The ligand of choice, DMV, is not only more efficient than DPA, but also easier to prepare.

The procedure would appear to be useful, amongst other applications, for the analysis of α-Me-AA in meteorites. The enantiomeric composition of these constitu-

TABLE III

RETENTION TIMES (t_R) OF α-Me-AA USING THE COPPER COMPLEX OF DMV DISSOLVED IN 0.1 *M* SODIUM ACETATE AS A CHIRAL ADDITIVE

[Cu]:[DMV] = 8:16 (m*M*/m*M*); pH = 7.

Acetonitrile concentration (%)	*α-Me-AA*		*t_R (min)**	*α***
0	Asp	I***	10.5	1.25
		II	13.1	
	Ser	I	11.3	1.41
		II	15.9	
	Glu	I	12.9	1.24
		II	16.1	
5	Met	I	23.9	1.21
		II	28.9	
10	Leu	I	16.3	1.37
		II	22.3	
	Norleu	I	20.7	1.47
		II	30.5	
	Trp	I	41.3	1.23
		II	50.7	
	Phe	I	25.5	1.24
		II	31.7	

* t_R = Adjusted retention time.
** $\alpha = t_{RII}/t_{RI}$.
*** Peak assignment of all α-Me-AA has not yet been made.

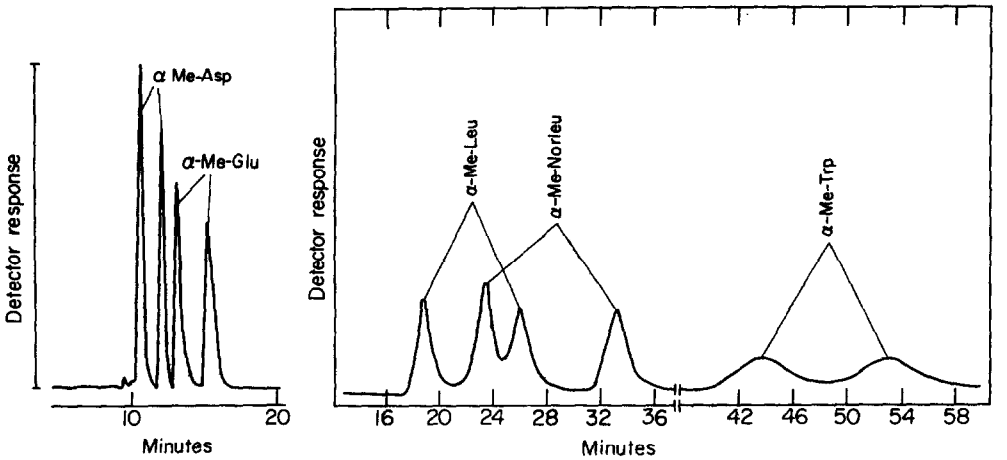

Fig. 1. Separation of α-Me-AA using as a chiral additive the copper complex of DMV [Cu]:[DMV] = 8:16 (m*M*/m*M*), pH = 5.5.

Fig. 2. Separation of α-Me-AA using as a chiral additive the copper complex of DMV dissolved in 0.1 *M* sodium acetate [Cu]:[DMV] = 8:16 (m*M*/m*M*), pH = 7.

ents is of particular interest, as their presence can hardly be ascribed to terrestial contamination[18].

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