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LIQUID CHROMATOGRAPHIC SEPARATION AND DETECTION OF N-METHYLAMINO ACIDS USING MOBILE PHASES CONTAINING COPPER(II) IONS

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SUMMARY

The chromatographic separation of N-methylamino acids can be accomplished using a reversed-phase column with an aqueous mobile phase containing copper ions. The N-methylamino acid–Cu complex absorbs UV radiation with λ_{max} around 235 nm. Thus copper ions influence the retention times and allows the detection of the above solutes. Unsubstituted amino acids also absorb radiation at 235 nm. The molar absorptivities of the two types of solutes seem to be of the same order of magnitude and the detection limits are therefore also close. The method can be utilized in the simultaneous analysis of amino acids and their N-methyl derivatives. Although the method cannot compete with the sensitivity of amino acid analysers, its simplicity and its detection limits make it useful in such research fields as peptide synthesis.

INTRODUCTION

N-Methylamino acids constitute an important class of biologically active compounds. For example, they have been used in drugs[1] and for the elucidation of active sites in enzymes[2]. The synthesis of the N-methylamino acid derivatives is well documented (*e.g.*, ref. 3), but little has been published on their chromatographic separation and analysis.

Coggins and Benoiton[4] used an amino acid analyser with ninhydrin reagent. As ninhydrin does not react well with secondary amines, the resulting detector signals were low. In addition, low flow-rates had to be used in order for the ninhydrin reaction to take place at all. Audhya and Russell[5] encountered similar difdiculties. In addition, the resolution they reported was insufficient. Felix and Terkelsen[6] used an amino acid analyser with fluorescamine as the labelling reagent. As fluorophore does not react well with secondary amines, they added N-chlorosuccinimide to the

mobile phase in order to obtain primary amines. Two main difficulties were associated with this approach: (a) the separation between N-methylamino acids, after reaction with N-chlorosuccinimide, and the unmethylated acids was poor, as the retention times were very short; and (b) the detector signals due to the parent amino acids were very weak, being less than 5% of their original values without the reagent. To overcome these problems, Felix *et al.*[7] utilized a dual fluorometric–colorimetric detection system. It can be concluded from the above that the separation and detection of N-methylamino acids is difficult. The analysis is particularly complicated if the parent amino acids are also present.

We have recently described a simple high-performance liquid chromatographic (HPLC) system for the separation of amino acids[8]. Copper ions were added to the mobile phase not only to control the analysis time but also to form complexes with the amino acids. These complexes absorb radiation fairly strongly around 235 nm. Although the sensitivity of the method could not rival that of amino acid analysers, it is nonetheless sufficient for many applications such as peptides synthesis.

N-Alkyl-substituted amino acids are also known to form copper(II) complexes (*e.g.*, ref. 9). The absorption spectrum of the complexes has not been reported. As the complex is similar to that of the present amino acids, it is reasonable to assume that it will absorb UV radiation with $\lambda_{max} \approx 235$ nm. It was the aim of the present work to extend the method described previously[8] to the HPLC analysis of N-methylamino acids and their parent compounds.

EXPERIMENTAL

Instrumentation

A Spectra-Physics Model SP 8000 liquid chromatograph with a variable-wavelength detector was used. The column was 250 × 4.6 mm O.D. reversed phase (Perkin-Elmer, Norwalk, CT, U.S.A.). The mobile phase consisted of purified water containing 0.1 M sodium chloride for constant ionic strength and, when needed, $5 \cdot 10^{-4}$ M copper(II) chloride. Hydrophobic solutes were eluted with water–methanol as the mobile phase.

Reagents

All amino acids and most of the N-methylated ones were purchased from Sigma (St. Louis, MO, U.S.A.). Some N-methylated amino acids were synthesized in our laboratory using the procedure of McDermott and Benoiton[3].

Procedure

The flow-rate of the mobile phase was kept at 2.0 ml/min. Stock solutions of amino acids were freshly prepared and diluted before each set of experiments. The sample size injected was 10 μl. As mentioned above, we could not find any literature references concerning the spectral properties of the N-methylamino acid–copper complexes. A UV study carried out in our laboratory has shown that the spectra of these complexes are very similar to those of the unmethylated compounds, with $\lambda_{max} \approx$ 235 nm. Hence the chromatographic detection was carried out at that wavelength.

RESULTS AND DISCUSSION

Because the copper complexes of amino acids and their derivatives absorb radiation, their detection is simple, unlike the HPLC analysis of amino acids alone. In the latter case, detection must be effected at about 200 nm, which limits the range of suitable mobile phases. Alternatively, pre- or post-column derivatization is required in order to label the solutes with chromophores or fluorophores. In this study the copper ions present in the mobile phase form the chromophore essentially *in situ*. Moreover, the presence of copper ions significantly alters the partition coefficients, and hence the retention tiimes, of the amino acids and their N-methylated derivatives. The retention of these compounds is longer when copper ions are added to the mobile phase. This is an important facet of the investigation and it will be dealt with in a subsequent publication.

Table I shows the capacity factors of various N-methylamino acids and their parent compounds, with the exception of N-methyltryptophan, N-methylphenylalanine and N-methyltyrosine. The latter amino acids are fairly hydrophobic and, in order to elute them in a reasonable time, methanol was added to the mobile phase. Table I shows the selectivity, α, between the parent and substituted amino acids. As expected, the N-methylamino acids elute after the parent compounds, because of the increase in hydrophobicity. The trend of increasing α with hydrophobicity is suggested by the data in the table. It is noteworthy that the elution order of the two groups of solutes is identical.

Fig. 1 shows the separation of some N-methylamino acids with a mobile phase containing $5 \cdot 10^{-4}$ M copper(II) ions. It can be seen that the symmetry of the late peaks is not very good and the efficiency is poor. This behaviour is frequently encountered with metal additives and it may reflect the equilibrium processes that take place in the system.

Fig. 2 shows a chromatogram of a mixture of some amino acids and N-methylamino acids. As the molar absorptivities are roughly the same for both species, they can be separated and analysed in a single chromatographic run, without the need for special procedures or instrumental modifications.

The linearity of the detector response as a function of the concentration of N-methylamino acids must be established, as it is essential for quantitative analysis.

TABLE I

CAPACITY RATIOS, k', OF N-METHYLATED AMINO ACIDS AND THEIR PARENT COMPOUNDS

The mobile phase contained $5 \cdot 10^{-4}$ M Cu(II). Temperature 35°C. Detection at 235 nm.

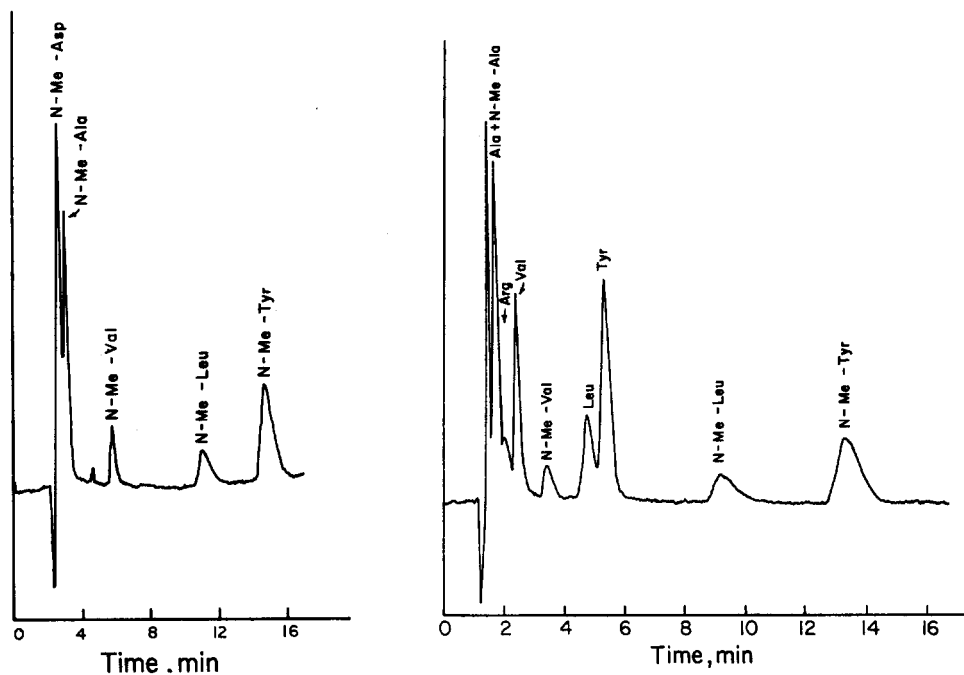| N-Methylamino acid	k'	Amino acid	k'	α
N-Me-Asp	0.21	Ser	0.10	1.4
N-Me-Ser	0.24	Asp	0.17	1.4
N-Me-Ala	0.46	Ala	0.29	1.6
N-Me-Arg	0.78	Arg	0.50	1.6
N-Me-Val	1.60	Val	1.07	1.50
N-Me-Leu	6.53	Leu	3.14	2.08
N-Me-Tyr	8.53	Tyr	3.36	2.54

Fig. 1. Separation of five N-methylamino acids. The concentration of all solutes was 10^{-3} *M*. The mobile phase contained $5 \cdot 10^{-4}$ *M* Cu(II). Detection at 235 nm. Temperature 35°C.

Fig. 2. Separation of some amino acids and N-methylamino acids. Conditions as in Fig. 1.

TABLE II

LINEARITY OF DETECTOR RESPONSE AS A FUNCTION OF N-METHYLAMINO ACID CONCENTRATION

The mobile phase contained $5 \cdot 10^{-4}$ *M* Cu(II).

Solute	Slope	Correlation coefficient	Response index
N-Me-Asp	100.5	0.9999	1.01
N-Me-Ser	11.3	0.7966	0.77
N-Me-Ala	59.7	0.9081	1.27
N-Me-Arg	14.9	0.9928	1.02
N-Me-Val	10.2	0.9990	1.00
N-Me-Leu	4.3	0.9939	1.05
N-Me-Tyr	36.83	0.9994	1.01
N-Me-Phe	33.41	0.9992	1.01
N-Me-Trp	81.68	0.9999	1.00

Complete data are given in Table II where, in addition to the correlation coefficients of the linear regression of absorbance on concentration, the slopes of the lines and the response index[10] are indicated. With the exception of the serine and alanine derivatives, the dependence of the response on the concentration is linear. The Scott response index[10] is another measure of detector signal linearity:

$$A = KC^r \tag{1}$$

where A is the detector signal (absorbance units in the present case), K is a constant, C is the solute concentration and r is the response index. Again, with the exception of the above two solutes the system behaves in a linear fashion. The reasons for the non-linearity in the response of these two solutes are not clear to us. Any dependence on the capacity ratio, however, can be ruled out. N-Methylaspartic acid elutes before N-methylserine and N-methylalanine, yet its response is linear and, as will be shown shortly, its limit of detection is much lower.

Further inspection of Table II shows that the slopes of the regression lines are indepedent of the capacity ratio, and in general they are roughly of the same order of magnitude. From the slopes one can estimate the molar absorptivities, ε, of the N-methylamino acid–copper complexes using the following relationship:

$$\varepsilon = \frac{SWF}{VC_s B} \tag{2}$$

where S is the slope absorbance/molarity, W is the peak width (cm), F is the flow-rate (ml/min), V is the volume injected (ml), C_s is the recorder chart speed (cm/min) and B is the optical path length (cm). Table III shows the calculated molar absorptivities of all the solutes studied here. The high ε value of the tryptophan derivative is due to the inherently high absorbance of the indole moiety. In general, however, the ε values are similar to those of the parent compounds[8]. As expected no relationship is found between the absorptivities and the capacity ratios. The various amino acid side-chains affect the electronic structure and the hydrophobicity of the complex differently.

Since our previous contribution[8] we have become aware of another earlier publication discussing ε values of amino acid–copper complexes. Wilson et al.[11] measured the molar absorptivities when the amino acids were in excess of the copper ions. Assuming a 2:1 complex (CuL$_2$) they found ε values of about $6.1 \cdot 10^3$–$6.7 \cdot 10^3$ for some amino acids and $3.8 \cdot 10^3$ for histidine. In our work, most of the data used

TABLE III

MOLAR ABSORPTIVITIES, ε, CALCULATED FROM EQN. 2, OF N-METHYLAMINO ACIDS

Solute	ε	Solute	ε
N-Me-Asp	$6.4 \cdot 10^3$	N-Me-Leu	$2.1 \cdot 10^3$
N-Me-Ser	$1.1 \cdot 10^3$	N-Me-Tyr	$6.0 \cdot 10^3$
N-Me-Ala	$3.1 \cdot 10^3$	N-Me-Phe	$3.7 \cdot 10^3$
N-Me-Arg	$2.0 \cdot 10^3$	N-Me-Trp	$12.4 \cdot 10^3$
N-Me-Val	$1.5 \cdot 10^3$		

to calculate ε consisted of cases where the concentrations of the amino acids were lower than or equal to that of the copper. Even when relatively high concentrations of amino acids were injected, the sample is diluted on entering the column. Thus, the complex formed in the column is probably 1:1. The ε values in Table III seem to substantiate this point.

Wilson *et al.*[11] indicated that the molar absorptivities are a function of the amino acid concentration for a given copper concentration. The non-linear behaviour of the calibration graphs for N-methylalanine and N-methylserine might be due to this concentration effect.

The variance in the ε values in Table III calls, perhaps, for some explanations. Undoubtedly some of the difference is due to the nature of the side-chain, *e.g.*, aromatic *versus* aliphatic. In addition, errors in the measurements and/or approximations made in developing eqn. 2 might also explain the difference in the ε values of similar complexes such as alanine, leucine and valine. However, the possibility of specific chromatographic processes that may compete differently for the free solute. and the complexed one cannot be discounted.

Table IV shows the detection limits, defined as a signal-to-noise ratio of 2:1, of the present system. No attempt was made to optimize the chromatographic procedure and the values given are intended to demonstrate the feasibility of the method. Comparison with our previous work[8] shows that the detection limits of the methylamino acids are about the same as those of the present compounds. The behaviour of N-methylserine and N-methylalanine is again different and their detection limits are significantly higher than those of the other solutes. We do not know, at present, whether this is an artifact of our system or an intrinsic characteristic of these two amino acids. While not as sensitive as the fluorescamine detection, the present approach is attractive in many applications such as peptide synthesis. It should be pointed out that no relationship between the detection limit and the capacity ratio seems to exist. This is of practical importance as the peaks of strongly retained solutes are relatively wide and small.

TABLE IV

DETECTION LIMITS OF SOME N-METHYLAMINO ACIDS

The mobile phase contained $5 \cdot 10^{-4}$ M Cu(II).

Solute	Detection limit	
	mg per 10 ml	*Molarity*
N-Me-Asp	3.61	$2.46 \cdot 10^{-6}$
N-Me-Ser	3090	$2.60 \cdot 10^{-3}$
N-Me-Ala	155	$1.50 \cdot 10^{-4}$
N-Me-Arg	77.6	$4.13 \cdot 10^{-5}$
N-Me-Val	37.9	$2.90 \cdot 10^{-5}$
N-Me-Leu	137.0	$9.50 \cdot 10^{-5}$
N-Me-Tyr	42.1	$2.16 \cdot 10^{-5}$
N-Me-Phe	37.2	$2.08 \cdot 10^{-5}$
N-Me-Trp	2.79	$1.28 \cdot 10^{-6}$

Applications of the method

    The synthesis of N-methylalanine acids can be monitored using liquid chromatography with mobile phases containing copper ions. Fig. 3 shows the result of the synthesis of N-methylleucine using the procedure of Benoiton and co-workers[3,4]. The synthesis was done by an inexperienced graduate student. It can be seen that the reaction was not carried out to completion and about 50% of the parent leucine is left in the reaction mixture. When an experienced researcher prepares the N-methyl derivative, the final product is pure, at least within the limits of detection of the chromatographic method.

    The integrity of the N-methylamino acid during the synthesis of a peptide is of obvious importance. In the course of our research in a different area, the peptide Boc–Phe–Gly–N-MeLeu–Met–NH$_2$ (Boc = butyloxycarbonyl) was prepared. It was of interest to examine the completeness of the coupling steps.

    Fig. 4 shows a chromatogram of the peptide hydrolysate. Comparison with a synthetic mixture shows that the N-methylleucine was incorporated in the right proportion to the other amino acids. The peak at about 4 min is of unknown nature or origin.

    A different peptide synthesized by us is Boc–Phe–N-MePhe–Sar–Leu–Met–NH$_2$, which is a partial sequence of an analogue of a peptide known as Substance
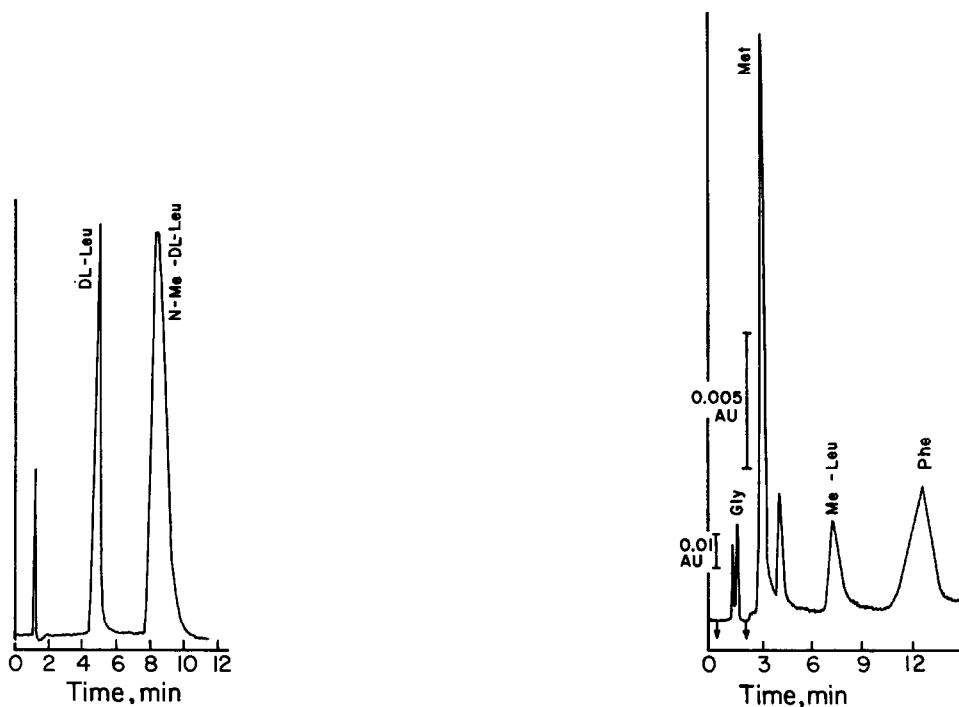
Fig. 3. Chromatogram showing the products of the reaction for the synthesis of N-methylleucine. The aqueous mobile phase contained $5 \cdot 10^{-4}$ M Cu(II). Detection at 235 nm. Temperature, 35°C.

Fig. 4. Chromatogram of hydrolysis products of the peptide Boc–Phe–Gly–N-MeLeu–Met–NH$_2$. Conditions as in Fig. 1 except temperature, 60°C. The arrows indicate a change in detection.
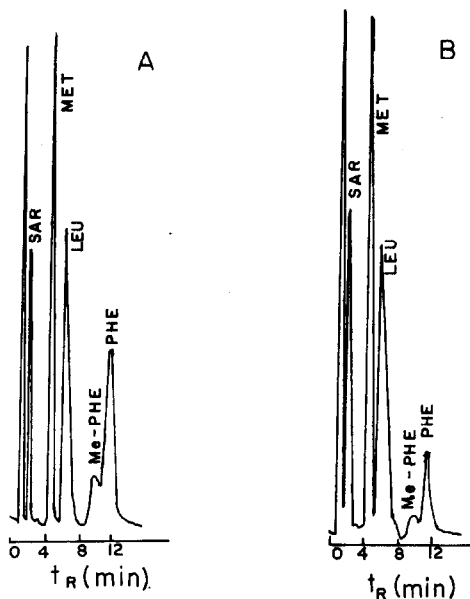
Fig. 5 (a) Chromatogram of a synthetic mixture of Sar, Met Leu, N-MePhe and Boc–Phe. Mobile phase: methanol–water (1:4) containing $5 \cdot 10^{-4}$ M Cu(II). Concentration of all amino acids: $1 \cdot 10^{-3}$ M. (b) Chromatograms of hydrolysis products of Boc–Phe–N-MePhe–Sar–Leu–Met–NH$_2$. Conditions as in (a).

P. Fig. 5a shows a chromatogram of a synthetic mixture of the five amino acids. The concentration of all solutes was identical. The peptide was hydrolysed in hydrochloric acid and the hydrolysate was chromatographed. The result is shown in Fig. 5b. The chromatogram shows that the ratio of Sar, Met and Leu is about 1:1:1. The size of the N-MePhe and Phe peaks shows incomplete hydrolysis of the Boc–Phe–N-MePhe part of the peptide. The incomplete hydrolysis of the above hydrophobic pair of amino acids is well known. Chromatography can be used to monitor the kinetics of such a hydrolysis reaction.

In conclusion, it can be seen that copper-aided chromatography can be extended to the analysis of N-methylamino acids. The copper ions in the mobile phase form a complex with the solutes that can be monitored at 235 nm. The detector signal is linear and sufficiently sensitive to make the method applicable in a number of disciplines. The attraction of the method is in its simplicity, as any conventional liquid chromatographic system can be used when copper ions are added to the mobile phase. In addition, the substituted amino acids can be analysed together with their parent compounds. At the end of the analysis the chromatograph can be washed free from copper ions and used for other purposes.

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