Journal of Chromatography, 235 (1982) 401–409
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,311

SEPARATION OF AMINO ACIDS ON REVERSED-PHASE COLUMNS AS THEIR COPPER(II) COMPLEXES*

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(First received May 15th, 1981; revised manuscript received September 25th, 1981)

SUMMARY

We report the separation of amino acids on reversed-phase columns using aqueous mobile phases containing copper ions. Cu(II) forms complexes with the amino acids and therefore influences their retention times. More importantly the Cu–amino acid complex absorbs UV radiation with λ_{max} around 230 nm. Thus the solutes can be detected at relatively long wavelengths. The linearity of the detector signal and the detection limits were studied. Ssamples as small as 10 ng per 10 μl can be detected and the useful range of detection is over four orders of magnitude. The effects of the pH of the mobile phase were also studied: retention increases with pH, at least over the range investigated.

INTRODUCTION

The separation of amino acids is of great importance in many research areas. The number of publications dealing with this topic is voluminous. Chromatographic methods of separation have been used extensively for amino acid analysis. Perhaps foremost of all chromatographic techniques is that of ion exchange, coupled with post-column derivatization for detection purposes. In recent years other liquid chromatographic methods, in conjunction with either post- or pre-column derivatization steps with chromophores or fluorophores, have been utilized. Very little work has been reported in which amino acids derivatives have not been formed. For example, Molnár and Horváth[1] have used a reversed-phase system with extremely acidic mobile phases. Hancock *et al.*[2] have also used a reversed-phase system for the separation of amino acids. Schuster[3] has described the separation of these compounds using an amine column in the reversed-phase mode. The purpose of the present work is to describe a method which allows the separation of amino acids, as their Cu(II) complexes, on conventional reversed-phase columns, and a UV detector at *ca.* 230 nm. The amino acids are injected into the mobile phase which contains copper ions. The

* Presented at the *5th International Symposium on Column Liquid Chromatography, Avignon, May 11–15, 1981*. The majority of the papers presented at this symposium has been published in *J. Chromatogr.*, Vol. 218 (1981).

0021-9673/82/0000–0000/$02.75

presence of Cu(II) in the mobile phase affects the retention and facilitates the detection of the solutes.

Spies[4] has discussed the complex formed between an amino acid and Cu(II) and, most significantly, has studied the UV absorbance of such a complex. He found that the maximum of the absorbance is between 230 and 240 nm, depending on the amino acids, with molar absorptivities above 6000 l mol^{-1} cm^{-1}. Thus with a 1-cm absorption cell, micromolar concentrations of amino acids could be detected with conventional high-performance liquic chromatography (HPLC) UV detectors. While not as sensitive as fluoresence detectors, Cu(II)-aided detection can find use in many applications, such as in the synthesis of peptides. It is surprising, therefore, that the use of Cu(II) in amino acid analysis did not draw much attention, either in chromatography, or otherwise. Walton[5] has mentioned in his review on ligand-exchange chromatography that Cu(II), in addition to being the binding site, can be used for the detection of amino acids. Masters and Leyden[6] have described the use of copper-loaded silylated controlled-pore glass for the ligand exchange of amino sugars and amino acids. They have indicated that the presence of Cu(II) in the mobile phase can be utilized for the detection of the solutes analysed. More recently our group has been involved in the separation of amino acid enantiomers using chiral mobile phases in conjunction with reversed-phase columns[7]. The chiral reagent is a Cu(II) complex of L-aspartylalkylamide. We have noted that the presence of Cu(II) is essential not only for the enantiomeric resolution but also for the detection. A similar approach was described by Caude and Foucault[8]. None of the above-mentioned studies has investigated in detail the potential use of Cu(II) for the separation, detection and quantitative determination of amino acids.

It is of interest to mention here that the formation of amino-acid complexes with Cu(II) has been utilized to determine the amounts of amino acids indirectly. Kahn and Van Loon[9], as well as Slavin and Schmidt[10], have used atomic absorption spectrophotometers as LC detector in order to quantitate Cu(II) and hence the amino acids. A different approach was advanced by Loscombe *et al.*[11] who used a copper-selective electrode to detect the presence of amino acid–Cu(II) complex.

We describe here some initial experiments aimed at characterizing the chromatographic behaviour of amino acids in the presence of Cu(II) ions.

EXPERIMENTAL

Apparatus

All chromatographic runs were made with a Spectra-Physics SP8000 unit equipped with a variable-wavelength UV detector. The columns were 250 × 4.1 mm O.D. reversed-phase (ODS Partisil 10). The mobile phase consisted of triply distilled water containing $3 \cdot 10^{-4}$ *M* copper (II) chloride. In the pH study, the pH was controlled with an acetate buffer.

Reagents

All amino acids studied were obtained from Sigma (St. Louis, MO, U.S.A.). The water for the mobile phase and sample preparation was distilled in our laboratory.

Procedure

Chromatographic conditions: the column temperature was maintained at

34°C, except for the pH study (40°C). The flow-rate of the mobile phase was 1 ml/min. The calibration and detection study and the pH study were performed on different reversed-phase columns. Stock solutions of the amino acids were prepared and diluted as needed. Volumes of 10 μl of each sample were introduced onto the column with an injection valve.

Synthesis of a peptide

To study the utility of the method, the peptide TRF was synthesized as follows. pGlu-His-$NHNH_2$ was coupled by the "azide method" to proline amide. The peptide obtained (TRF) was purified on Sephadex G-25 using *n*-butanol–pyridine and 0.1% acetic acid as eluent. The resulting peptide was desalted by ion-exchange chromatography. Elemental analysis of the peptide (general formula $C_{16}H_{22}O_4N_5$) showed the following composition: C 51.54, H 6.18, N 22.81%. The calculated values are C 51.74, H 6.24 and N 22.63%. The specific rotation was $[\alpha]_0^{25} = 63.2$ (C-1, water). The purity of the peptide was checked using a reversed-phase HPLC system[12].

Peptide hydrolysis

TRF (2 mg) was hydrolysed in boiling 6 *N* hydrochloric acid solution (1 ml) in an evacuated sealed tube for 24 h. The sample was desiccated to dryness. Amino acid analysis: Glu:His:Pro:NH_3 = 1:1:1:1.

RESULTS AND DISCUSSION

Cu(II) ions absorb strongly in the UV region below 240 nm. Thus when Cu(II) is added to the mobile phase there is an initial time period when the detector baseline is not stable. Once the system is equilibrated (after about 1 h) the baseline remains steady and the retention times are reproducible. At this point the solutes can be introduced to the column.

The first aim of the study was to establish the retention orders of the amino acids. Table I shows the capacity ratios of 19 amino acids with different functional groups. Of the 21 so-called "common amino acids" only cystine, isoleucine and glutamine are missing from Table I. Their absence is due to the lack of immediate

TABLE I

CAPACITY RATIOS OF SOME AMINO ACIDS

Mobile phase: $3 \cdot 10^{-4}$ *M* Cu(II) in water. Detection: UV at 230 nm.

Amino acids	*k'*	*Amino acids*	*k'*
Asp	0.24	nVl	3.5
Glu	0.29	lys	3.9
Gly	0.88	Met	4.2
Ser	0.97	Dopa	4.7
Asn	1.09	Arg	7.0
Ala	1.12	Leu	7.5
Thr	1.24	Tyr	9.6
His	1.86	Phe	28.0
Val	2.47	Trp	67.0
Pro	2.95		

availability in our laboratory. Table I shows that the acidic amino acids elute first while the basic ones elute with the hydrophobic amino acids.

The amino acids, it should be noted, were injected as received without pre- or post-column derivative formation. The solutes injected formed a complex with the Cu(II) in the mobile phase. The complex when eluted was detected at 230 nm.

The linearity of the detection system as well as the detection limit are, of course, of major importance. Fig. 1 shows typical calibration graphs for some amino acids. Table II gives the slopes of the lines and the correlation coefficients for the least-squares fit. The high values of the correlation coefficients should be noted. The data in Table II are given in the order of elution. The slopes, it is seen, are not only a function of the retention time.

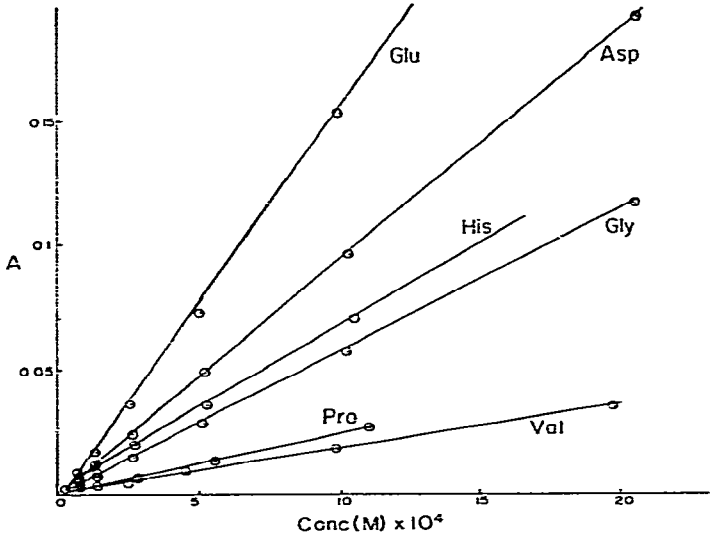

Fig. 1. Typical calibration graphs for amino acids using Cu(II)-aided detection.

TABLE II

SLOPES OF THE CALIBRATION GRAPHS FOR SEVERAL AMINO ACIDS

Amino acid	*Slope*	*Correlation coefficient*	*Response index*
Asp	93.25	0.9999	1.02
Glu	158.85	0.9994	1.10
Gly	56.51	0.9999	1.03
His	61.2	0.998	0.92
Val	17.28	0.9998	0.94
Pro	23.87	0.9999	1.10

The slopes should be related to the molar absorptivities (ε) of the amino acids. These can be obtained using Beer's law and the estimate of the solute concentration at peak maximum as described by Scott[13]:

$$\varepsilon = \frac{SWF}{VC_s b} \tag{1}$$

where S is the slope, W is the peak width (cm), F is the flow-rate (l/min), V is the volume injected (litres) C_s is the chart speed (cm/min) and b is the path length (cm). Table III shows the molar absorptivities of some solutes as calculated from eqn. 1. The variation in ε should be noted. Apparently the retention order does not seem to affect the ε values. Perhaps the different ε are indicative of the nature of the amino acid–Cu(II) complexes; *e.g.* the polar amino acids show higher molar absorptivities. Spies[4] has also found that the absorbance of the complex varies from one amino acid to another. Additional work is now being carried out in our laboratory to explain the differences in ε values. It must be stressed here the the ε values obtained from eqn. 1 are only approximations due to the assumptions made in developing that equation.

TABLE III

ESTIMATES OF MOLAR ABSORPTIVITIES (ε) OF SOME AMINO ACID–Cu COMPLEXES

Amino acid	ε ($l\,mol^{-1}\,cm^{-1}$)
Asp	3172
Glu	4449
Gly	1921
His	3550
Val	1106
Pro	2390

The values of ε reported here are, to the best of our knowledge, the only ones available in the literature for the amino acids concerned. Phan *et al.*[14] have reported molar absorptivities for Cu–arginine complexes. They found that $Cu(Arg)_2$ has a maximum absorbence at 237 nm with $\varepsilon = 7000$ l mol^{-1} cm^{-1}. They calculated that Cu(Arg) should have a maximum absorbence at $\lambda = 232$ nm with $\varepsilon = 3500$ l mol^{-1} cm^{-1}. In the present study the predominating complex is most probably. Cu–amino acid, and indeed we see from Table III that the molar absorptivities are, in general, close to the calculated value mentioned above. At a higher concentration of the amino acids studied here, the possibility of having the complex $Cu(amino\ acid)_2$ exists. The possible effect of such a species will be discussed shortly.

The utility of a method can be checked by its limit of detections and the linearity of the signal. Fig. 1 suggests that the method is linear over three orders of magnitude in the concentration of the amino acids.

To examine more closely the linearity, we employed the following equation, suggested by Scott[13]:

$$y = AC^r \tag{2}$$

where y is the detector signal, A is a constant, C is the concentration and r is the response index of the system. For truly linear detection r should be unity. Table II shows the response indices found in the present study. They vary from 0.92 for His to 1.10 for Glu and Pro. No correlation between the slope or the retention order and r was found. Note that although the response index exceeds the recommended limits of ± 0.02[13] the calibration graph is still valid as long as r is known.

A possible explanation for the deviation of r from unity might lie in the fact that as the concentration of the amino acids increases more of the tris-complex Cu(amino acid)$_2$ is formed. The concentration of Cu(II) in the mobile phase was $3 \cdot 10^{-4}$ M, while that of the amino acids varied from 10^{-6} to 10^{-3} M. At such low concentrations the predominating species is, very likely, Cu(amino acid). However, at amino acid concentrations of 10^{-3} M the possibility of forming some Cu(amino acid)$_2$ exists. If ε of Cu(amino acid) is different from that of Cu(amino acid)$_2$, then the calibration graph will show a curvature.

The detection limits were found from extrapolating the calibration graphs to signals twice the magnitude of the noise. Table IV gives these limits in units of ng per 10 μl (10 μl being the volume injected) as well as molar concentration. These limits are sufficiently low to make the method attractive in peptide synthesis. An example of how the method can be utilized will now be given.

TABLE IV

DETECTION LIMITS OF SOME AMINO ACIDS

Values in parentheses are molar concentrations.

Amino acid	*Detection limit (ng per 10 µl)*
Asp	6.65 ($5 \cdot 10^{-6}$ M)
Glu	11.8 ($8 \cdot 10^{-6}$ M)
Gly	0.413 ($5.5 \cdot 10^{-6}$ M)
His	3.1 ($2 \cdot 10^{-6}$ M)
Val	9.36 ($8 \cdot 10^{-6}$ M)
Pro	19.6 ($1.7 \cdot 10^{-5}$ M)

The tripeptide TRF was synthesized in our laboratory. Upon hydrolysis TRF yields Pro, His, Glu and NH_3 in a 1:1:1:1 ratio. Fig. 2a shows a chromatogram of a 1:1:1 synthetic mixture of the three amino acids. Fig. 2b shows the chromatogram of the above amino acids plus ammonium chloride. Fig. 2c shows the chromatogram of the hydrolysis products. Quantitation, using peak heights, showed that the ratio of the amino acids in the hydrolysate is indeed 1:1:1. The absolute purity of TRF was difficult to ascertain since the exact weight of the peptide in the sample hydrolysate was not known. It should be pointed out, perhaps, that the analysis was performed using *ca.* 2 mg of crude material.

NH_4^+ in the sample causes a negative deflection of the detector baseline. The complex $Cu(NH_3)_4^{2+}$ does absorb at 230 nm. However, the chromatographic run was carried out with an acidic mobile phase, and the equilibrium concentration of NH_3 is very small. Hence the elution of the NH_4^+ ion is characterized by a negative peak due to the dilution of the Cu(II) in the mobile phase.

Effect of the pH of the mobile phase

Amino acids can be in the form of AH_2^+, AH and A^- depending on the pH of the solution. There is evidence that the nature of the amino acid–Cu(II) complex is also pH dependent[15]. Moreover, the various species AH_2^+, AH and A^- have different retention times[16]. Consequently, it is expected that the pH of the mobile phase, in the present system, will have a large effect on the elution behaviour of the amino acids.

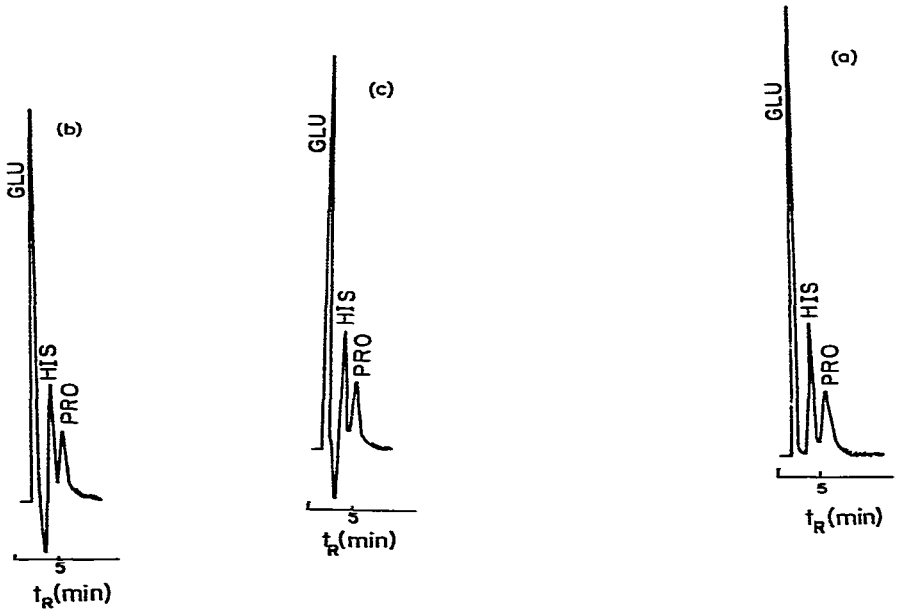

Fig. 2. (a) Chromatogram of synthetic mixtures of Glu, His and Pro. Mobile phase: $3 \cdot 10^{-4}$ M Cu(II) in water. Flow-rate: 1 ml/min. Detection: 230 nm. (b) Chromatogram of synthetic mixture of Glu, NH_4^+, His and Pro. Same conditions as in (a). (c) Chromatogram of the hydrolysis products of the tripeptide TRF. Same conditions as in (a). t_R = Retention time.

Table V shows capacity factors (k') of some amino acids, using mobile phases at different pH values. The trend is clear: lower pH values mean shorter retention times. The trend is more apparent from a plot of k' values *versus* pH. Fig. 3 shows such a plot for several amino acids. In general the change in k' values is higher at the high pH

TABLE V

EFFECT OF pH OF MOBILE PHASE ON THE k' VALUES

Amino acid	*pH*					
	6.7	*6*	*5.6*	*4.6*	*3.7*	*3.3*
Asp	0.46	0.03	0	0	0	0
Glu	0.5	0.054	0.027	0.014	0	0
Gly	0.18	0.07	0	0	0	0
Ala	0.22	0.095	0	0	0	0
His	—	—	—	0.18	0.17	0.15
Val	1.45	1.0	0.77	0.43	0.26	0.15
Pro	1.07	0.67	0.49	0.20	0.12	0.11
Lys	0.91	—	—	0.096	0.12	0.096
Nva	1.6	1	0.76	0.40	0.28	0.14
Met	2.01	1.44	1.14	0.75	0.54	0.48
Leu	4.08	3.06	2.5	1.39	1.07	1.1
DOPA	—	—	1.02	0.88	0.59	0.65
Tyr	—	—	2.17	1.47	1.03	1.18
Arg	1.82	0.95	0.38	0.20	0.12	0.14

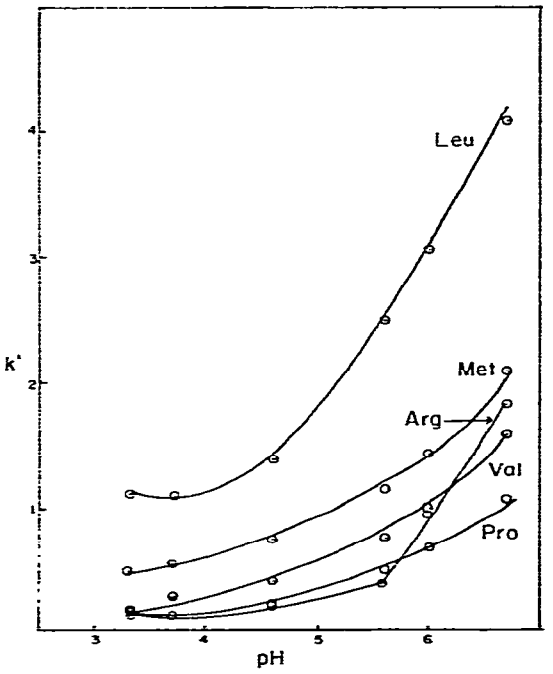

Fig. 3. Dependence of k' of some amino acids on the pH of the mobile phase.

values. This is understood in terms of the higher stability of the amino acid–Cu(II) complex in more basic solutions. The change in k' values is particularly noticeable with the basic amino acids, *e.g.* Arg. For some of the amino acids the capacity ratios are higher at pH 3.3 than at 3.7. We do not know yet whether the increase in k' is real or an artifact of measuring very low numbers. We are currently studying this behaviour.

Mobile phases of relatively high pH are advantageous not only because they retard the amino acids to a greater extent, but also because they allow better detection sensitivities. Initial work in our laboratory shows that the molar absorptivities of the amino acid–Cu(II) complexes are much higher at basic pH values. Thus better detection limits than reported here are feasible.

In summary, it is shown that separation of amino acids on reversed-phase columns is feasible with the aid of Cu ions in the mobile phase. The sensitivities achieved indicate that the approach reported here can find use in many cases such as peptide synthesis. Presently we are studying the effect of the Cu(II) concentration on the separation. In addition we are attempting to obtain the formation constant of the complex, much in the same way as we have done with nucleotides–Mg[17]. The use of metal cations to affect the resolution, to improve detection limits, and to obtain physico-chemical information further demonstrates the great versatility of chromatographic separations. Metal-aided chromatography allows the scientist to employ the full range of chemical reactions to achieve the separation.

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