Journal of Chromatography, 470 (1989) 131–137
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 267

UTILITY OF COPPER-CONTAINING ELECTROLYTES FOR ISOTACHOPHORESIS OF AMINO ACIDS

FREDERICK S. STOVER
Central Research Laboratories, Monsanto Co., 800 N. Lindbergh Blvd., St. Louis, MO 63167 (U.S.A.)

SUMMARY

Isotachophoretic separation of free amino acids using Cu^{2+}–diethylene triamine (Den) in high-pH leading electrolytes is investigated. Large changes in relative step heights of free amino acids are seen. Mobility order depends on both Cu^{2+} concentration and Cu^{2+}/Den ratios. Mobility differences seen with CuDen addition can be ascribed to both polyvalent counterion effects and specific interactions forming tertiary complexes. These electrolytes offer a new dimension for mobility variation of complexing amino acids and peptides.

INTRODUCTION

A major drawback to the isotachophoresis (ITP) of free amino acids is that the separations are essentially one dimensional. Leading electrolytes with pH values from 8.6 to 9.6 generally are employed to impart anionic mobilities to neutral amino acids sufficient for isotachophoretic migration[1]. The useable pH range is restricted by decreased ionization at lower pH values and by OH^--migration at higher pH values. Thus, the usual practice of altering effective mobilities by changing leading electrolyte pH is limited for mixtures of amino acids. Also, using pH 8.6–9.6 electrolytes, several pairs of amino acids often form mixed zones (*e.g.* Asp–Glu, Ser–Thr, Tyr–Met and Val–Trp). Hirokawa *et al.*[2] recently evaluated the separability of 26 amino acids under typical electrolyte conditions, and noted the frequent occurrence of mixed zones.

A second, widely used method of obtaining improved ionic mobility differences is addition of complexing agents to samples or leading electrolytes[3–5]. Everaerts *et al.*[1] added Cu^{2+} to amino acid samples in an effort to obtain separation of the cationic complexes. Except for histidine, copper–amino acid complexes were not sufficiently stable to be detected as cationic species in a pH 5.4 electrolyte. A more successful approach involved addition of propionaldehyde to an anionic pH 7.5 electrolyte[6]. Schiff base formation between amino acid and aldehyde resulted in lower amino group pK_a values. While some improved separations were obtained, many amino acid pairs remained unresolved.

Recently, "pre-capillary" derivatization of free amino acids with dinitrophenol[7] and citraconic anhydride[8] has been reported. Advantages of these approaches include the ability to operate at lower pH values and use of specific detectors.

0021-9673/89/$03.50

We report here investigations of anionic amino acid ITP utilizing labile equilibria between copper complexes in the leading electrolyte and free amino acids. Such systems offer totally aqueous electrolytes, no derivatization step and the possibility of tailoring separations by altering the distribution of counterions in the leading electrolyte.

EXPERIMENTAL

ITP was performed using an LKB (Bromma, Sweden) 2127 Tachophor with a 200 mm × 0.8 mm PTFE capillary and an LKB 2127-140 conductivity detector. Separation currents were 250 μA and detection currents were 100 μA. Leading and terminating reservoirs were refilled with electrolytes after every two to three runs. HCl (Ultrex® grade) was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.); $CuCl_2$ (Gold Label® grade), 95% diethylene triamine (Den), and hydroxypropyl methylcellulose (HPMC) were obtained from Aldrich (Milwaukee, WI, U.S.A.); L-histidine (His), L-alanine (Ala), glycine (Gly), L-glutamic acid (Glu), L-aspartic acid (Asp), L-phenylalanine (Phe), L-valine (Val), β-alanine (Bala) and 2-amino-2-methyl-1,3-propanediol (ammediol) were obtained from Sigma (St. Louis, MO, U.S.A.); 2.5 *M* NaOH and $Ba(OH)_2$ were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). All chemicals were reagent grade unless otherwise specified and used as received.

RESULTS AND DISCUSSION

The use of Cu^{2+} complexation in high-pH electrolytes to alter amino acid mobilities is complicated by the hydrolysis of $Cu(aquo)^{2+}$. We recently measured formation constants for tertiary complexes of Cu^{2+} with N-methyliminodiacetic acid (MIDA) and various amino acid analogues[9]. It was found that the formation constant for the reaction

$$Cu(MIDA) + AA \rightarrow Cu(MIDA)AA \quad (1)$$

generally is within an order of magnitude of the constant for complexation with the bare metal ion

$$Cu + AA \rightarrow CuAA \quad (2)$$

where AA is an amino acid or derivative and charges have been omitted for clarity. These results indicated that the use of copper complexes in leading electrolytes could maintain solubility while providing a site for amino acid complexation. This is often the case when using complexing metal counterions. For example, the $HCl–ZnCl_2$–His leading electolyte used in phosphonate analysis[5] probably contains $Zn(His)_2$ as the complexing counterion. Similar tertiary equilibria have been used successfully for the separation of amino acid enantiomers by liquid chromatography[10] and capillary zone electrophoresis[11].

There are several criteria to consider when selecting a secondary ligand, X, for use in an ITP electrolyte where the equilibrium

$$CuX + AA \rightleftarrows Cu(AA)X \quad (3)$$

TABLE I
OPERATING SYSTEMS
Additive to leading electrolytes: 0.2% HPMC.

Leader (all pH 9.0)		*Terminator*	
No.	*Composition*	*No.*	*Composition*
L1	10 m*M* HCl + ammediol	T1	10 m*M* β-alanine adjusted to pH 9.0 with $Ba(OH)_2$
L2	10 m*M* HCl + Den	T2	10 m*M* L-histidine adjusted to pH 9.0 with $Ba(OH)_2$
L3	5 m*M* $Cu(Cl)_2$ + 5 m*M* Den + NaOH		
L4	5 m*M* $Cu(Cl)_2$ + Den		
L5	3 m*M* $Cu(Cl)_2$ + 4 m*M* HCl + Den		
L6	1 m*M* $Cu(Cl)_2$ + 8 m*M* HCl + Den		

governs the effective mobility. (1) The complex CuX must have a high enough stability to prevent hydroxide formation at pH 9. (2) Since copper must act as a counterion in the anionic electrolyte, the complex CuX should have a net positive or zero charge. (3) Since the strongest coordinating sites on copper occupy square-planar positions[12], X should be no more than tridentate to allow strong amino acid interaction with CuX. (4) To maximize the amount of CuX available for AA binding, X should preferentially form 1:1 over 1:2 complexes. This also will minimize $Cu(OH)_2$ precipitation if equimolar Cu–X concentrations are employed. (5) Formation of an acidic or basic complex, CuHX or Cu(OH)X, with a pK_a of 8.5–9.5 would eliminate the need for a buffering counterion B, which may form a mixed complex Cu(B)X. A candidate ligand that fulfills these requirements is Den.

Table I lists the electrolytes employed in this study. Electrolyte system L1–T1 is commonly used in amino acid ITP. Use of polyvalent Den as a counterion could cause changes in AA mobilities due to electrophoretic and relaxation effects[13]. Electrolyte L2 was tested to determine the extent of these retardations. Electrolyte L3 was designed based on the above criteria for Cu(AA)X formation to give maximum Cu–AA interactions. Finally, electrolyte L4, using Den instead of NaOH to adjust pH, was tested as an intermediate strength system.

Separation of mixtures with electrolyte systems L1–T1, L2–T1,L3–T2 and L4–T2 are given in Figs. 1 and 2. Inspection of these results shows that use of Cu + Den in the leading electrolyte has a profound effect on AA mobilities. For electrolytes containing Cu, the decrease in His mobility is so great that it can be used as a terminating ion for the other seven AAs. A plot of AA relative step heights, on a log scale, for electrolytes L1–L4 is shown in Fig. 3. Large decreases in mobilities for His, Gly and Asp are observed with Cu-containing electrolytes. Benefits of using L3 or L4 include improved Glu and Asp separations and better resolution of Gly from Phe and His. Ala, Val and Phe remain mixed with other AAs in all four electrolytes. Disadvantages to using the Cu-containing systems include higher impurity levels which interfere with some AAs, longer analysis times and, for certain pairs, poorer resolution.

Use of Den alone (L2) shows modest changes in the separation patterns obtained

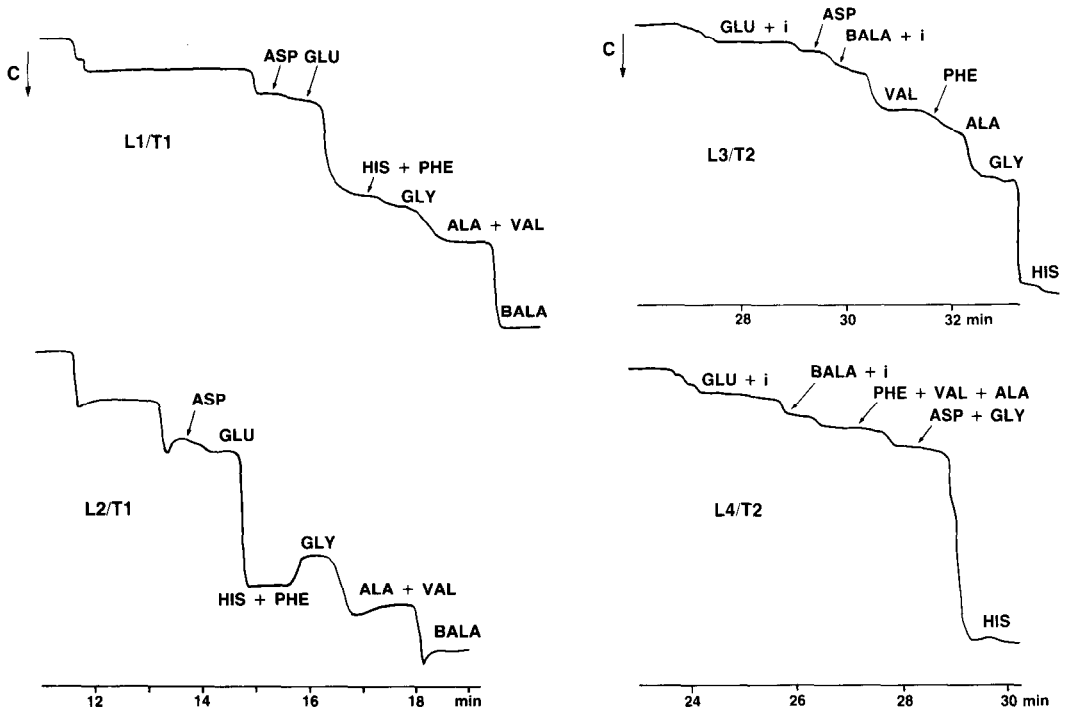

Fig. 1. Conductivity traces for injections of a mixture containing 0.14 mg/ml each of Asp, Glu, His, Phe, Gly, Ala and Val. Top: 10 μl mixture in L1–T1 electrolytes; bottom: 5 μl mixture in L2–T1 electrolytes. C axis is decreasing conductivity.

Fig. 2. Conductivity traces for injections of (top) 5 μl amino acid mixture in L3–T2 electrolytes and (bottom) 3 μl in L4–T2 electrolytes. Same mixture as in Fig. 1 but with His replaced by Bala. i = Electrolyte impurities. C axis is decreasing conductivity.

due to electrophoretic and relaxation effects of the polyvalent counterion. All amino acids are retarded with reference to Bala. The most interesting feature of the L2 electrolyte is the enforced migration of His and Phe ahead of Gly.

Mobility effects resulting from variation in Cu^{2+} content of the leading electrolyte were investigated using electrolyte systems L5–T2 and L6–T2. These electrolytes, along with L2 and L4 form a consistent set with Cu^{2+} concentrations of 0, 1, 3 and 5 m*M*. Separation of AA mixtures in 1 and 3 m*M* Cu^{2+} electrolytes with Den adjustment of pH is shown in Fig. 4. Fairly large changes in AA mobilities and migration orders are obtained with the 3 m*M* system, but electrolyte L6 with 1 m*M* Cu^{2+} gives identical separations as a Den-only electrolyte (L2).

To gain insights into the active counterion compositions of the leading electrolytes, calculations were performed to determine copper speciation in electrolytes L2–L6. Motekaitis and Martell's computer program BEST[14] and literature values for stability constants [15] were used for the calculations and the results are given in Table II. Equilibrium calculations show that leading electrolyte compositions depend on the method of pH adjustment. Use of excess Den (L4) instead of NaOH results in formation of $Cu(Den)_2$ at the expense of CuDen and Cu(OH)Den.

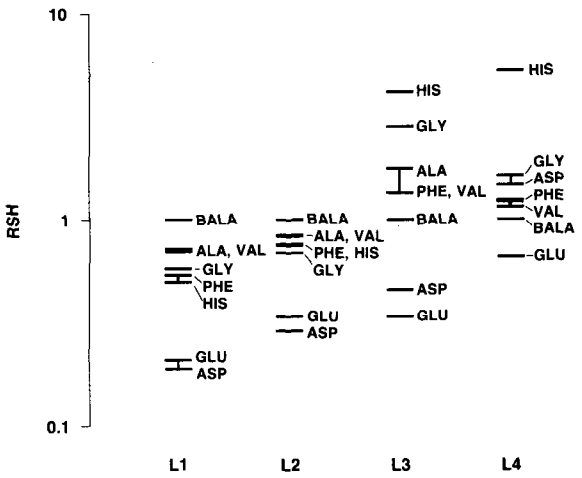

Fig. 3. Plot of relative step heights, on a log scale, referenced to Bala for individual amino acids in leading electrolytes L1–L4. Vertical bars represent mixed or non-resolved zones.

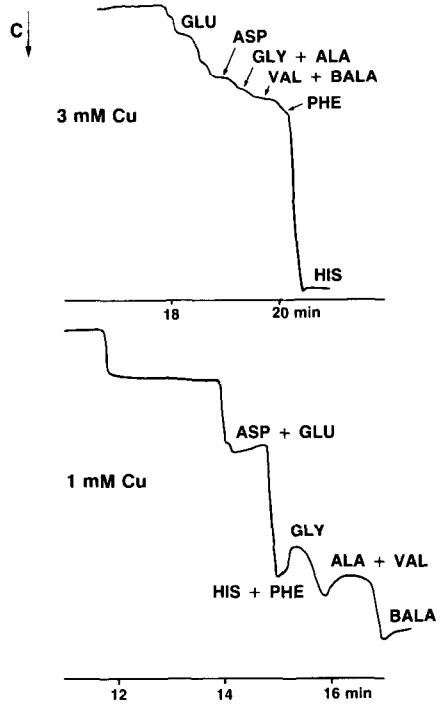

Fig. 4. Conductivity traces for injections of (top) 1 μl mixture with Bala in L5–T2 electrolytes and (bottom) 5 μl mixture with His in L6–T1 electrolytes. C axis is decreasing conductivity.

TABLE II
CALCULATED LEADING ELECTROLYTE COMPOSITIONS

Leader	*Total [Cu] (mM)*	*Total [Den] (mM)*	*[CuDen] (mM)*	*[Cu(Den)$_2$] (mM)*	*[Cu(OH)Den] (mM)*	*[HDen+H$_2$ Den] (mM)*
L3	5.0	5.0	3.0	–	2.0	–
L4	5.0	8.8	1.0	3.4	0.6	0.4
L5	3.0	8.8	0.1	2.8	0.1	2.8
L6	1.0	7.8	–	1.0	–	5.4
L2	–	7.2	–	–	–	6.6

The counterion with the strongest presumed AA binding is the binary CuDen complex. Electrolyte L3, with the highest CuDen content, gave the lowest absolute His mobility as measured from adjusted terminator resistances. However, the relative step height of His was higher in L4, due to significant Cu(Bala)Den formation in L3.

Reducing the total Cu concentration to 3 m*M* (L5) also causes considerable change in counterion distributions. Although this electrolyte contains Cu(Den)$_2$ as the primary counterion, enough complexation power is retained so that His can be used as a terminator. Apparently, 0.1 m*M* CuDen is sufficient to cause changes in AA mobilities. Further reduction in total Cu to 1 m*M* (L6) yields an electrolyte with performance similar to the non-Cu-containing L2 electrolyte. Thus, a Cu(Den)$_2$ level of 1 m*M* causes no mobility change and this species is inactive.

Unique mobility profiles are seen with all electrolytes except the L2–L6 pair. Changes in effective mobilities are a combination of electrophoretic and relaxation effects from changing $+1/+2$ counterion distributions and specific binding effects with $CuDen^{2+}$. The power of this approach to tailoring separations is demonstrated by the fact that only Ala and Val fail to separate from other amino acids using the electrolytes tested here.

Discontinuities in leader conductivity signals were observed with Cu-containing electrolytes L3–L5. Shown in Fig. 5 are anomolous conductivity changes seen at 12–16

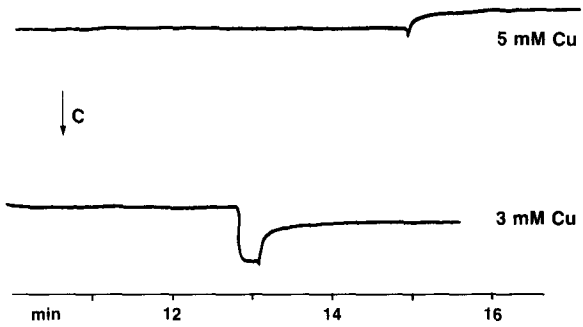

Fig. 5. Conductivity record of Cu-containing leading electrolyte signals prior to leader/terminator boundary. Top trace: L4; bottom trace: L5. Signals recorded at 100 μA and same decreasing conductivity scale (C axis) as previous figures.

min in the L4 and L5 electrolytes. Discontinuities occur near the expected time of the Cl^- leading boundary, with some shift due to bulk leader transport number changes. These discontinuities are suspected to be caused by CO_3^{2-} migrating in an enforced or zone manner. This is supported by the fact that no discrete zone or zone length increase was observed near AA zones when 5 μg CO_3^{2-} was injected in the L3–T2 electrolyte system. Thus CO_3^{2-} may act as a pseudo-leader in these systems and cause the lengthy analysis times observed at high Cu content.

Most probably all twenty common protein amino acids cannot be successfully separated by ITP using these types of electrolytes. The seven amino acids tested here are among those that bind copper most strongly. Less dramatic mobility shifts are anticipated for other amino acids. However, electrolyte systems suggested here offer a new method of altering ITP separations of free amino acids. In addition, such systems could be useful for obtaining improved separations of related compounds such as peptides and proteins.

REFERENCES

1 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis—Theory, Instrumentation and Applications*, Elsevier, Amsterdam, 1976, pp. 311–322.
2 T. Hirokawa, T. Gojo and Y. Kiso, *J. Chromatogr.*, 369 (1986) 59.
3 D. Kaniansky and F. M. Everaerts, *J. Chromatogr.*, 148 (1978) 441.
4 P. Gebauer, P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 199 (1980) 81.
5 F. S. Stover and J. H. Wagenknecht, *Anal. Chim. Acta*, 135 (1982) 347.
6 F. M. Everaerts, J. L. Beckers and Th. P. E. H. Verheggen, *Isotachophoresis—Theory, Instrumentation and Applications*, Elsevier, Amsterdam, 1976, p. 319.
7 D. Kaniansky, V. Madajová, J. Marak, E. Šimuničová, I. Zelenský and V. Zelenská, *J. Chromatogr.*, 390 (1987) 51.
8 C. J. Holloway, *J. Chromatogr.*, 390 (1987) 97.
9 F. S. Stover and B. L. Haymore, unpublished data.
10 J. M. Broge and D. L. Leussing, *Anal. Chem.*, 58 (1986) 2237.
11 P. Gozel, E. Gassmann, H. Michelsen and R. N. Zare, *Anal. Chem.*, 59 (1987) 44.
12 H. Sigel and R. B. Martin, *Chem. Rev.*, 82 (1982) 385.
13 D. Kaniansky, V. Madajová, I. Zelenský and S. Stankoviansky, *J. Chromatogr.*, 194 (1980) 11.
14 R. J. Motekaitis and A. E. Martell, *Can. J. Chem.*, 60 (1982) 2403.
15 R. M. Smith and A. E. Martell, *Critical Stability Constant*, Vol. 1, Plenum Press, New York, 1974, pp. 1–61.