Journal of Chromatography, 166 (1978) 403–410

CHROM. 11,396

APPLICATION OF A COPPER ELECTRODE AS A DETECTOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received June 5th, 1978)

SUMMARY

A novel detection system for use in high-performance liquid chromatography, based upon a post-column reaction between eluted organic copper ligands and a copper ion solution is described. The reaction is subsequently monitored in a flow-through micro cell with a copper ion-selective electrode. The detector has been investigated using the separation of five amino acids on a reversed-phase system and the effect of changing the controlling parameters has been noted. Preliminary studies have shown that the detector can also be used successfully for both ion-pair and ion-exchange based separations.

INTRODUCTION

Various detection systems have been proposed for use with high-performance liquid chromatography (HPLC). A number of these are available commercially: these include refractive index, UV absorption, fluorescence and electrochemical (polarographic) detection. Amongst other reported proposals Schultz and Mathis[1] have described the use of a liquid membrane nitrate ion selective electrode. This has been used to detect nitrate, nitrite and various phthalate ion isomers. Ions, other than nitrate, are detected by virtue of the low selectivity coefficient of the electrode.

As an alternative approach to that of measuring a property of the eluting solutes a number of workers have proposed the use of post-column reactions. In these systems the solute is involved in a reaction which is carried out after the separation and before some form of detector. Lawrence and Frei[2] have recently carried out a review of such systems.

In the detection system described in this paper a solid-state copper electrode is used in a flow-through cell to monitor the loss of free copper ion activity after the post-chromatographic reaction between an eluted ligand and an added copper ion solution. This solid state electrode has two main advantages over the membrane elec-

trode used for direct measurement by Schultz and Mathis[1]: it has a very rapid response and it can be safely used in aqueous alcohol solvents, which are often used in HPLC.

The development of the electrode system allows the detection of non-UV absorbing copper ligands such as amino acids. In addition it shows selectivity for such copper binding compounds, which in consequence leads to more ready interpretation of chromatograms. The main disadvantage of the detection systems is associated with the post-column volume introduced by the reaction coil. This can, if excessive, lead to peak broadening and subsequent loss in resolution, which is especially noticeable for rapidly eluting peaks. The effect of the reaction tube on the peak width is dependent on its length, the fourth power of its internal diameter and the velocity of the solutions moving through it [3,4]. Obviously the length and bore of the post-column tubing, which is in the form of a coil, should be kept to the minimum consistent with the rate of reaction in order to maximise the reaction and to minimise band spreading.

EXPERIMENTAL

Apparatus

The flow diagram is shown in Fig. 1. Mobile phase and copper reactant were delivered by two constant-flow syringe pumps (Varian Model LC 4100). For the major part of the work the mobile phase was delivered at 0.5 ml/min; the copper reactant flow-rate was, however, varied as described later.

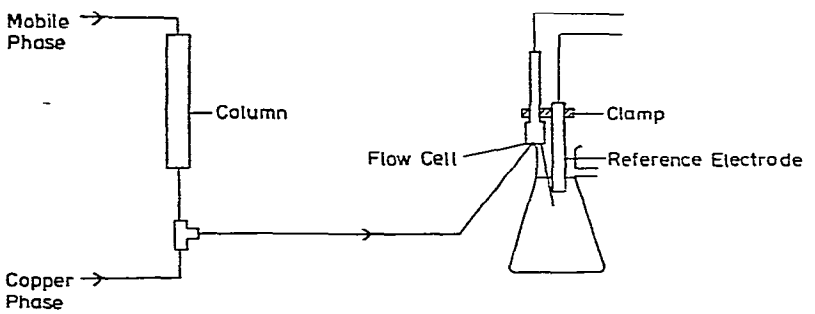

Fig. 1. Flow diagram for HPLC electrode detector.

The copper reactant and column eluent were merged in a Kel-F "T" piece (Chromatronix Cheminert fitting CJ 3031) followed by mixing in a PTFE reaction coil and passage into the electrode cell. The reaction mixture then passed to a beaker containing an Orion double junction reference electrode (Model 90-02-00) with aqueous potassium nitrate solution (100 g/l) in the outer chamber. The electrodes were connected to a Philips ion activity meter (Model PW 9414), set to record the cell e.m.f. in millivolts.

The flow cell (Fig. 2) was constructed from a Kel-F block which was threaded to allow easy changing of the electrode sensing membrane. Access to the flow chamber was provided by narrow-bore (0.3 mm I.D.) PTFE tubing. The outlet tubing contained a fine platinum wire to ensure electrical continuity between the two electrodes. The complete system was wrapped in an earthed aluminium shield in order to reduced baseline instability.

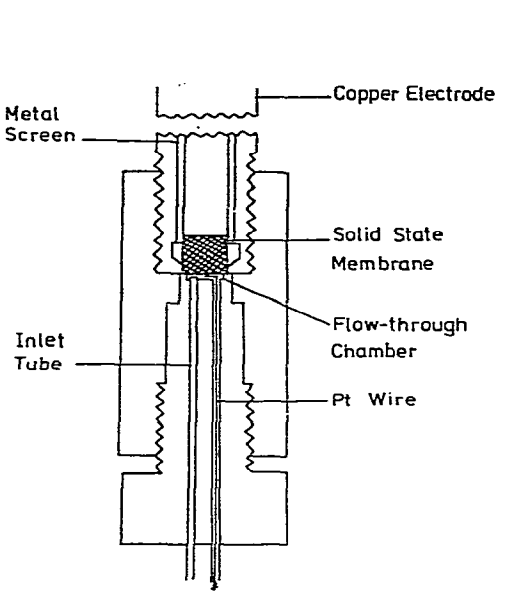

Fig. 2. HPLC electrode detector cell.

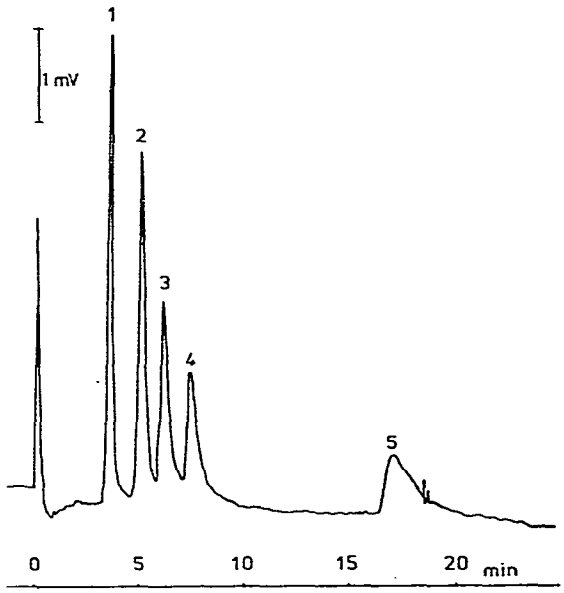

Fig. 3. Separation and detection of amino acids with reversed-phase chromatography. 1 = Glycine; 2 = valine; 3 = methionine; 4 = isoleucine; 5 = β-phenylalanine.

Column

Stainless-steel columns (150 × 5.0 mm I.D.), were packed by the procedure previously reported[5]. Spherisorb S5-ODS (Phase Separations, Queensferry, Great Britain) was used for both reversed-phase and ion-pair separations. A strong cation exchanger, prepared from LiChrosorb SI-60 (5 μm particle size, BHD, Poole, Great Britain) by the reported method[5] was also used. Samples were injected using the stop-flow technique through silanized glass wool onto a stainless-steel frit.

Copper reactant

The copper reactant was 10^{-4} *M* copper sulphate in 10^{-1} *M* ammonium acetate and 10^{-1} *M* potassium nitrate with the addition of formaldehyde (0.2 g/l).

Amino acid test mixture

An amino acid test mixture was used to investigate the parameters of the system. Composition of this mixture was glycine (0.093 g/l), DL-valine (0.175 g/l), L-methionine (0.144 g/l), DL-isoleucine (0.110 g/l) and β-phenylalanine (0.152 g/l).

RESULTS AND DISCUSSION

Reversed-phase chromatography of amino acids

In order to investigate better the parameters of this system an aqueous chromatographic separation is preferred, *i.e.* one in which the mobile phase will have no effect on the response of the electrode. For this work a column packed with Spherisorb ODS, with distilled de-ionized water as the mobile phase at a flow-rate of 0.5 ml/min,

was used. The amino acid test mixture was successfully separated on this system, eluting in the order: glycine, valine, methionine, isoleucine and β-phenylalanine, as reported in the original work[6,7] and shown in Fig. 3.

Composition of copper reactant solution

As the pH of the combined copper reactant and column eluent influences the degree of binding between the amino acid and the cupric ions, it is necessary to add a pH buffer to the copper phase. Static electrode work had shown that pH 6.7 was the optimum value for the copper–glycine reaction. This pH can be maintained by the addition of 10^{-1} *M* ammonium acetate buffer to the copper solution; use of ammonium formate, which results in a pH of 6.4, gave a reduction in detector response. In order to minimise the deterioration of the electrode sensing disc by oxidation[8], formaldehyde (0.2 g/l) was also added to the copper solution. Finally, to prevent the electrode responding to any changes in the ionic strength of the column eluent, the copper solution contained 10^{-1} *M* potassium nitrate. This addition also reduces electrode noise, presumably because of the increased conductivity of the copper solution.

It was found that the copper electrode would give a response to the amino acids even without the addition of the copper reactants, presumably by direct reaction on the disc[9]; this, however, led to a very rapid deterioration in electrode response. The electrode response was restored by cleaning the disc with fine emery paper.

Effect of copper concentration and flow-rate

Obviously the amount of copper present in the system will influence the actual detector response for the various amino acids. Investigation of this parameter showed that with a flow-rate of 0.5 ml/min the maximum response for the amino acid test mixture was obtained in the region of 10^{-4} *M* copper sulphate. Although the optimum copper concentration varies with the quantity of amino acid injected, this value was used as the standard in further investigations.

Varying the flow-rate of the copper reactant will effect both the final copper ion concentration, hence the metal to ligand ratio, and the residence time of solutions in the reaction coil and electrode cell. The response, for all the amino acids, increased as the flow-rate was decreased, reaching a maximum at approximately 0.025 ml/min, at which point it fell rapidly. This increase in response was, however, accompanied by a loss in resolution. A flow-rate of 0.5 ml/min provides a suitable compromise between these two factors.

Calibration

Detector response, measured in terms of peak height, for all the amino acids except glycine is linear up to 2.5 μg injected; for glycine the linear range extended only to 1 μg injected (Fig. 4). All the amino acids could, however, be detected substantially above this range. The theoretical relationship between concentration and detector response can be derived from the Nernst equation of electrode response provided certain assumptions are made.

The Nernst equation in this case is:

$$E = E^0 + \frac{RT}{2F} \cdot \log_e a_{Cu^{2+}} \qquad (1)$$

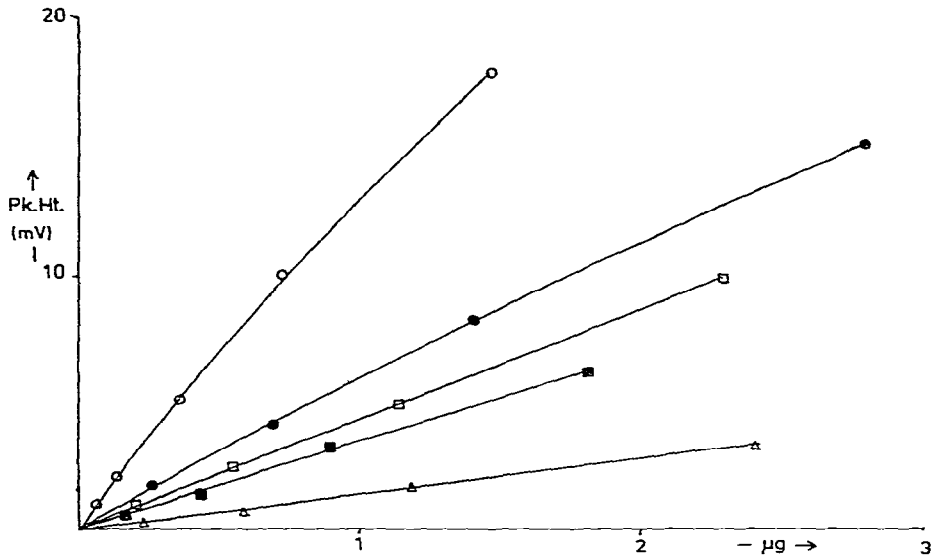

Fig. 4. Response of electrode detector to five amino acids. ○ = Glycine; ● = valine; □ = methionine; ■ = isoleucine; △ = β-phenylalanine.

where E is the electrode potential, $E°$ is the standard potential of the cell and $a_{Cu}{}^{2+}$ is the free copper ion activity. The linear portion of the calibration graph is found with low amino acid concentration (HL) and the main reaction is thus the formation of the 1:1 complex*

$$Cu + HL \rightleftharpoons CuL + H \tag{2}$$

The initial electrode potential, E_i, is given by

$$E_i = E° + k \cdot \log_e [Cu_i] \tag{3}$$

and the final electrode potential, E_f, by

$$E_f = E° + k \cdot \log_e [Cu_f] \tag{4}$$

Thus

$$\text{Peak height} = \Delta E = E_i - E_f = k \cdot \log_e [Cu_i]/[Cu_f] \tag{5}$$

From eqn. 2

$$[Cu_f] = [Cu_i] - [CuL]$$

Thus

$$\Delta E = k \cdot \log_e ([Cu_i]/[Cu_i] - [CuL]) = k \cdot \log_e \left(\frac{1}{1 - [CuL]/[Cu_i]} \right)$$

* For the sake of clarity formal charges are omitted.

or

$$\Delta E \approx k \cdot ([CuL]/[Cu_i] + [CuL]^2/2[Cu_i]^2 + [CuL]^3/3[Cu_i]^3 + \ldots)$$

For $[CuL]/[Cu_i] < 1$, then $\Delta E \approx k \cdot [CuL]/[Cu_i]$. Since $[Cu_i]$ = constant, then $\Delta E \approx k' \cdot [CuL]$.

At low amino acid concentrations and for high values of the stability constant for the copper–amino acid reaction, then

$[CuL] \approx$ total amino acid concentration

Thus

Peak height $= \Delta E \approx$ constant $\times$ total amino acid concentration

At higher amino acid concentrations both the power terms in the expansion and the formation of the 1:2 copper–amino acid complex become important considerations and lead to the deviations from linearity which were observed in the range of 3–7 μg.

Detection limits for the five amino acids tested, calculated from 3 $\times$ baseline noise, were: glycine (10 ng injected), valine (20 ng), methionine (30 ng), isoleucine (40 ng) and β-phenylalanine (100 ng). These values increase with retention time, reflecting the larger peak widths observed (Fig. 3).

Ten injections of the amino acid test mixture gave the following coefficients of variation for peak heights: glycine (1.0%) valine (1.2%), methionine (2.3%), isoleucine (3.4%) and β-phenylalanine (4.2%). These values fall within the expected range for any chromatographic procedure involving the stop-flow syringe injection (4 μl) used here. Similar values were obtained with the repeated injection of the amino acid mixture at a tenfold higher concentration.

Effect of ethanol

The inclusion of ethanol in the mobile phase is necessary for the successful operation of ion exchanges based on microparticulate silica[5]. If the detector is to be used with such packings it must function in aqueous ethanol solutions. This was examined with the test mixture using ethanol and water, in varying ratios, as the mobile phase. Increasing the amount of ethanol gave rise to progressive reductions in retention time and subsequent losses in resolution for all the acids except glycine. The height of the virtually unretained glycine peak, however, remained effectively constant (0–10% v/v ethanol) which confirms the suitability of the detector for use with aqueous ethanol solvents.

Ion-exchange chromatography of amino acids

In conventional amino acid analysers the samples are separated on cation-exchange columns and are usually detected by a post-column reaction with ninhydrin[10]. A strong cation exchanger bonded to LiChrosorb SI-60 was used to separate a mixture of nine amino acids as shown in Fig. 5. The mobile phase in this instance was water–ethanol (95:5) containing potassium nitrate at the 0.025 *M* level. adjusted to

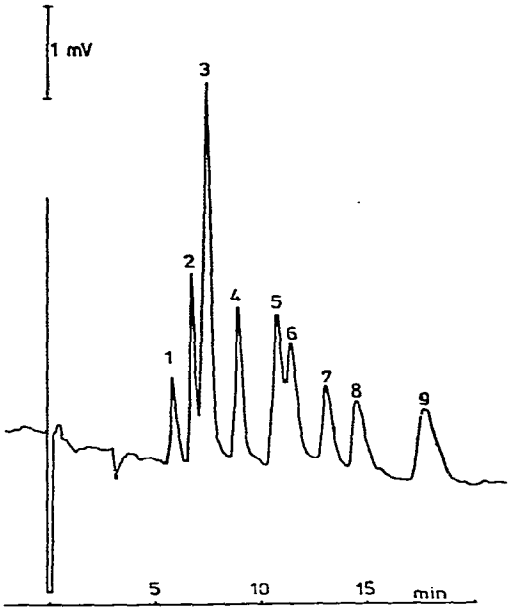

Fig. 5. Use of detector with ion-exchange chromatographic separation of amino acids. 1 = Aspartic acid; 2 = threonine; 3 = serine; 4 = glycine; 5 = valine; 6 = methionine; 7 = isoleucine; 8 = leucine; 9 = β-phenylalanine.

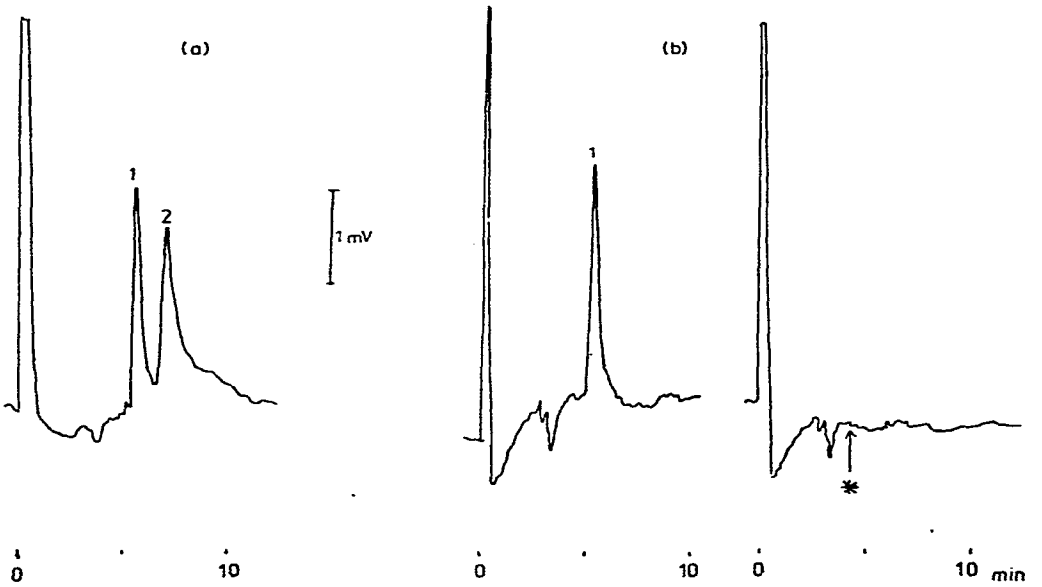

Fig. 6. Use of detector with ion-pair chromatographic separation of diamines. (a) Separation and detection of *p*-phenylenediamine (1) and toluenediamine (2). (b) Illustration of the selectivity of the detector for the *p*-isomer. *, Position of *m*-phenylenediamine peak as established using UV detection.

pH 3 with nitric acid. The copper reactant was 10^{-4} *M* copper sulphate in 10^{-1} *M* ammonium acetate with the addition of 0.25 g/l formaldehyde. (As potassium nitrate is present in the mobile phase it was not added to the copper reactant. It is necessary to make the mobile phase acid in order for an adequate separation to be achieved.)

*Ion-pair chromatography of diamines**

The separation and determination of toxic amines in hair colouring preparations by ion-pair chromatography has been demonstrated[11]. Phenylenediamine and toluenediamine were separated on a Spherisorb ODS column using water–methanol (30:70) containing 0.02 *M* ammonium formate at pH 5.6 as the mobile phase, and subsequently detected with the electrode (Fig. 6a). The composition of the copper reactant was the same as that used for reversed-phase chromatography. The selective nature of the detector is illustrated in Fig. 6b. Although it can readily detect 30 ng of *p*-phenylenediamine, the *meta*-isomer is not detected even at a 50-fold higher concentration. This is because it has the "wrong" steric configuration for binding with copper.

CONCLUSION

This work has demonstrated that a copper ion-selective electrode can be successfully used in a post-column reactor with reversed-phase, ion-exchange and ion-pair chromatography. Reproducibility was acceptable for the injection system employed and detection limits were good. As the binding reaction is rapid, only a short reaction coil is required and in consequence peak broadening was minimal. The selectivity of the detector for copper ligands was well demonstrated by the greatly differing responses to *p*- and *m*-phenylenediamine. The detector could be employed to determine other copper binding species such as the antibiotics tetracycline and penicillin. Use of other electrodes, of the rapid response type, may enable the system to be extended.

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* *Editor's note:* The referees did not think that ion-pair chromatography is the right term here. It may also be simple adsorption of the ionised diamines on the bonded phase.