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APPLICATION OF A COPPER TUBULAR ELECTRODE AS A POTENTIOMETRIC DETECTOR IN THE DETERMINATION OF AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A copper tubular electrode (CTE) has been used as a potentiometric detector in the determination of amino acids by reversed-phase high-performance liquid chromatography. The reference electrode was an electrically grounded tubular platinum electrode inserted in the flow stream. Comparison with a variable-wavelength UV absorbance detector showed that the CTE gave comparable response time and sensitivity, but was much more selective, showing response only to copper-binding molecules. The CTE has been applied to the analysis of urine and an intravenous amino acid preparation; no sample pre-treatment was required, other than filtration. Microgram quantities of amino acids were readily detected by using the CTE.

INTRODUCTION

Various detection systems have been used for the determination of amino acids by high-performance liquid chromatography (HPLC). Detection by UV absorbance at the usual wavelength of 254 nm is not optimal for amino acids (due to their low absorptivity), and it is necessary to employ a wavelength of 200 nm for satisfactory results[1]. Use of such a short wavelength introduces problems of solvent purity. More sensitive detection may be achieved by using the fluorescence or chemiluminescence properties of amino acid derivatives, particularly 5-dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives[2,3] formed by use of pre- or post-column reactions.

The capacity of amino acids to form complexes with metal ions is well recognised[4] and has been applied to HPLC through the use of a copper-selective membrane electrode for detection in the analysis of amino acids[5]. Amino acids were indirectly detected by monitoring the reduction in free copper ion activity resulting from post-column reaction between the eluted amino acids and an added copper ion solution. This system, although sensitive, has a number of inherent disadvantages, including peak broadening resulting from the excessive post-column volume introduced by the reaction coil, slow electrode response and a high susceptibility of the electrode to poisoning by complexing agents[6,7].

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In this paper, we describe the use of a copper tubular electrode (CTE) as a sensitive and selective detector in the analysis of amino acids by HPLC. Alexander and Maitra[8] have recently reported the use of copper wire and CTE electrodes as sensitive universal detectors in continuous-flow systems, in which they were found to be superior to the copper-selective membrane electrode for direct quantitation of amino acids. This system is shown to be useful as a detector for amino acids without post-column reaction of the eluted amino acids with copper ions.

EXPERIMENTAL

Instrumentation

A schematic diagram of the flow system used is shown in Fig. 1. In this system, a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 solvent pump, a Waters Model U6K injector, a Waters Model 450 variable-wavelength detector and the electrode detector were linked serially in flow to each other. The electrode detector (see Fig. 2) consisted of two tubular electrodes, one of copper and one of platinum, both of which were connected to an Activon voltage offset controller (± 1.5 v), and the platinum electrode was grounded. The outputs from the UV detector and the voltage controller were linked to a two-pen Omniscribe Model B5271 recorder, which had a 10-mV input.

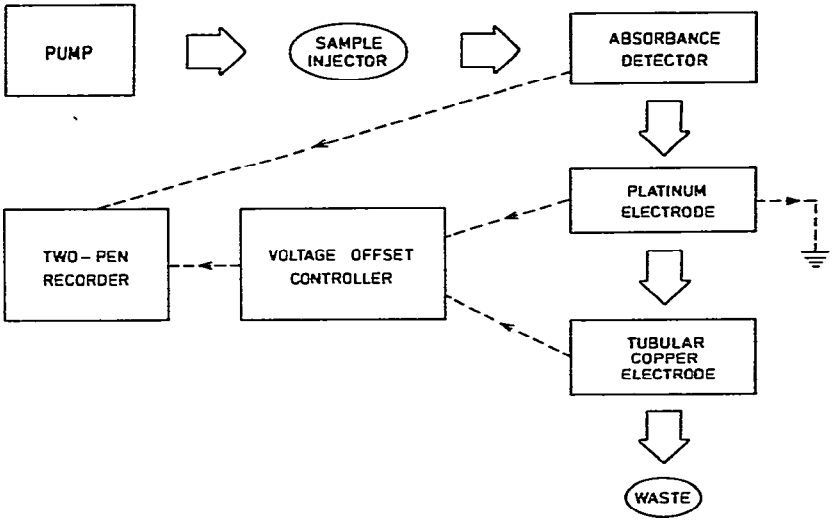

Fig. 1. Schematic diagram of flow system for HPLC analysis with a UV absorbance detector in series with the platinum reference electrode and the CTE: The arrows represent the flow-path; – – – represent electrical connections.

The components of the electrode detector system were as follows. The internal-flow CTE was constructed from copper rod 0.5 cm in diameter and 2 cm in length. This rod was precisely drilled to give an I.D. of 0.75 mm for the flow-path, the length of which was varied over the range 0.5–5.0 mm (1 mm was the most frequently used). The platinum electrode was a 1-cm length of platinum tubing (0.75 mm I.D.) sand-

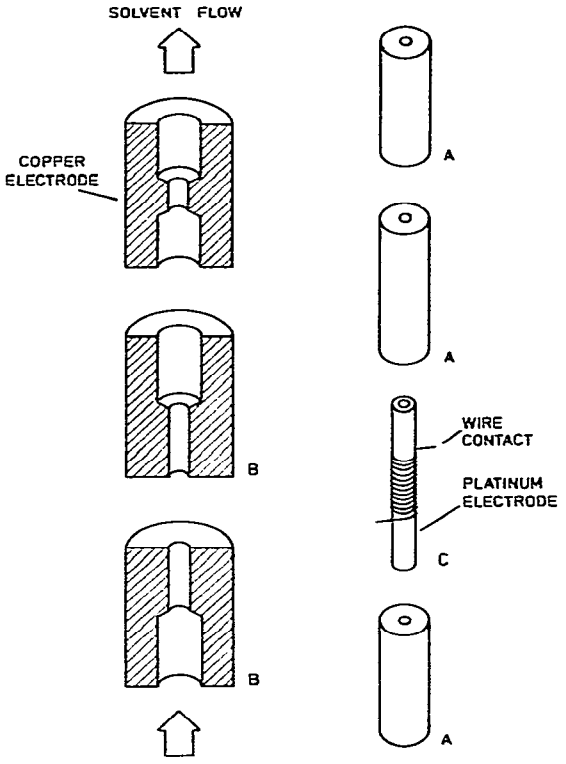

Fig. 2. Diagram showing construction of the CTE and platinum reference electrode. A = PTFE Flow tubing; B = PTFE moulding; C = platinum electrode.

wiched between two pieces of PTFE tubing (see Fig. 3), which served both mechanically to support the platinum electrode and to provide a means of attaching the flow tubing used to connect the various components of the system.

Reagents and stock solutions

Amino acids were obtained from various sources: glycine from BDH (Poole, Great Britain); valine from Koch-Light (Colnbrook, Great Britain); *l*-isoleucine from

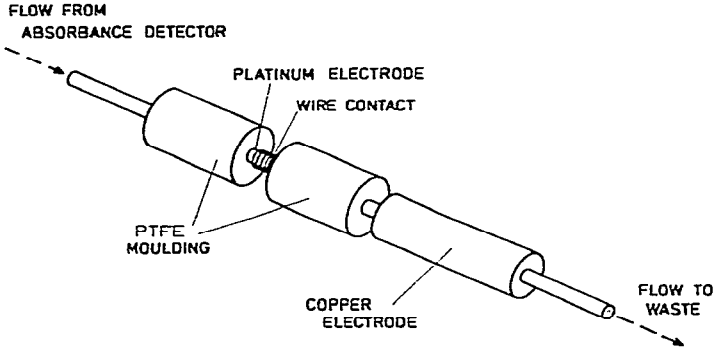

Fig. 3. System used for connecting the platinum reference electrode and the CTE.

BDH; methionine from NBC (Cleveland, OH, U.S.A.) and phenylalanine from Merck (Darmstadt, G.F.R.). The amino acids were used without further purification, and standard solutions were prepared immediately before use by dissolving weighed amounts in a buffer solution (pH 6.7) prepared from AR-grade sodium hydroxide and potassium dihydrogen phosphate (BDH).

Volucon standard buffers were used to calibrate the pH meter.

The mobile phase used for the HPLC separation of amino acids was prepared by mixing 50 ml of 1 *M* NaH_2PO_4, 19.2 ml of 1 *M* NaOH and 10.0 ml of 40% (w/v) formaldehyde in a 1-l volumetric flask; the solution was then diluted to the mark to give a final pH of 6.7 ± 0.1. All water used for the chromatographic procedure was distilled and passed through a Millipore Q water-purification system before use, and methanol was triply distilled in all-glass apparatus.

The urine control sample was obtained from Travenol Labs. (Costa Mesa, CA, U.S.A.) and was freshly reconstituted before use. The pharmaceutical intravenous amino acid solution was also obtained from Travenol, under the trade name "Synthamin 17", and contained nine essential and six non-essential amino acids in concentrations ranging from 400 mg to 21 g per litre; this solution was diluted by a factor of 25 for chromatographic runs. Both urine and the intravenous solution were filtered through a 2.5-μm Millipore filter before injection into the chromatograph.

Chromatographic procedure

Separation by HPLC was accomplished at 25°C on a μBondapak C_{18} column (30 cm × 3.9 mm I.D.; Waters Assoc.). The column was standardised by using the manufacturer's recommended procedure[9] and gave counts in the region of 4000 theoretical plates. The mobile-phase flow-rate was set at 1 ml/min (unless otherwise stated), producing a back pressure of 1000 p.s.i. The absorbance detector was operated at 200 nm with a sensitivity setting of 0.1 a.u.f.s.

In this study, all eluted compounds were detected both by the UV detector and the CTE, and the peaks were displayed on the two-pen recorder (chart speed of 1 cm/min). Each data point shown represents the mean of triplicate injections.

To prevent a decrease in sensitivity of the CTE due to poisoning, the electrode surface was periodically regenerated by rapid flushing with 5 ml of 8 *M* HNO_3 followed by 20 ml of distilled water and 20 ml of methanol. When the electrode was stored in the flow system, a solvent consisting of methanol–water (50:50, v/v) adjusted to pH ≈5 was used to prevent deterioration of the electrode.

RESULTS AND DISCUSSION

Mobile phase

The effect of pH on the response of the CTE was discussed in a previous paper[8], wherein it was shown that sensitivity for amino acid detection was greatest at high pH values. In this work, the optimum pH for the mobile phase was 6.7, which represented a compromise between maximum sensitivity and the prevention of electrode poisoning.

Fig. 4 shows a typical chromatogram of a separation of five amino acids with the CTE as detector.

A slightly acidic mobile phase was necessary to discourage the formation of

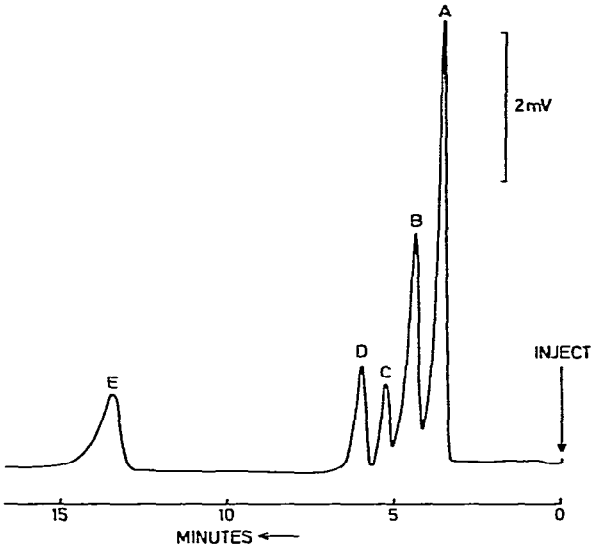

Fig. 4. Chromatogram of an amino acid mixture (1 μl) injected into a reversed-phase column coupled to the electrode detector system. Peaks: A = glycine (6.6 μg); B = valine (5.2 μg); C = methionine (2.8 μg); D = isoleucine (3.9 μg); E = phenylalanine (1.9 μg).

insoluble cupric hydroxides, carbonates and phosphates on the inner surface of the CTE. The ready formation of such compounds in alkaline media would result in potential drift, leading to error in the measured concentration[10].

The purpose of formaldehyde in the mobile phase was to repress negative deviation of the baseline, which occurred most prominently before the peaks due to such sulphur-containing amino acids as methionine and cysteine. The exact mechanism whereby formaldehyde can eliminate such negative baseline deviations is not clear, but its reducing properties may assist in improving the rate of exchange of the amino acids on the electrode surface. It is known[8,10] that the mild reducing properties of formaldehyde prevent oxide film formation at electrode surfaces, and it was added to the mobile phase for the same reasons as previously reported[8,10] for use with copper membrane electrodes, that is, to give baseline stability. Formaldehyde did not affect the amino acid peak heights as shown in Fig. 4.

Reference electrode

The platinum tubular electrode shown in Figs. 1, 2 and 3 was used for two purposes. First, it acted as an auxiliary electrode to reduce electrical noise; secondly, it served as a reference electrode to the CTE. Since it was electrically grounded, the reference used was the earth potential. This configuration was adopted only after considerable experimentation with conventional reference electrodes; however, we found that the electrically grounded platinum electrode gave greatest sensitivity and least baseline noise.

Electrode flow-path

With glycine as representative amino acid, the electrode response was studied

as a function of the length of the electrode flow-path for solutions of three different concentrations of glycine (5.8, 11.5 and 17.4 $\mu g/\mu l$). In all instances, the electrode signal reached a maximum for a flow-path of 1.0 mm.

Poor response for very short flow-paths is considered to be due to the relatively short residence times of the amino acid ligands in the electrode, whereas reduced response in the longer-path-length electrodes is due to sample-dispersion effects. The optimum flow-path length of 1 mm was adopted in all further work.

Flow-rate

The effect of flow-rate on response time and sensitivity was studied by removing the HPLC column, replacing it with a suitable connector and then injecting glycine solution (3.4 $\mu g/\mu l$) at various flow-rates. Values of $t_{max.}$, the time required to reach the maximum of the chromatographic peak, were measured for each flow-rate; the results are shown in Table I.

TABLE I
RESPONSE CHARACTERISTICS OF THE CTE AND ABSORBANCE DETECTORS AT VARIOUS FLOW-RATES

Flow-rate (ml/min)	*Peak height (mm)*		$t_{max.}$ *(sec)*	
	UV detector	*CTE*	*UV detector*	*CTE*
0.5	122	98	24.2	27.0
1.0	90	88	13.3	15.8
2.0	57	74	6.9	8.5
3.0	42	63	4.6	5.5
4.0	37	58	3.5	4.0

In agreement with our earlier findings[8], the CTE response improved with increasing flow-rate; however, this was accompanied by a decrease in sensitivity due to the decreased residence time of the amino acid in the electrode. The response time of the CTE compared favourably with that of the absorbance detector, whereas the sensitivity of the CTE (as measured by peak height) was less dependent on flow-rate than was that of the absorbance detector. It is clear that the optimum flow-rate would represent a compromise between response and sensitivity; in this work we used a flow-rate of 1.0 ml/min.

The relationship between flow-rate and resolution was also examined to provide additional information on the response of the CTE. Injections of 1 μl of a solution containing 11.5 $\mu g/\mu l$ of glycine and 8.8 $\mu g/\mu l$ of isoleucine were made at various values of solvent flow-rate and the resolution (R_s) of the two resulting peaks was calculated by using the conventional formula[11]. For values of $R_s < 0.8$, peaks are considered to be only partially resolved, whereas $R_s > 2$ indicates resolution with at least two peak-base widths between peak maxima. The results are shown in Fig. 5.

A decrease in resolution with increasing flow-rate was observed for both the absorbance detector and the CTE, but the resolution achieved with the absorbance detector declined much more rapidly than for the CTE. It is noteworthy, however, that, at all flow-rates tested, the UV detector gave slightly superior resolution.

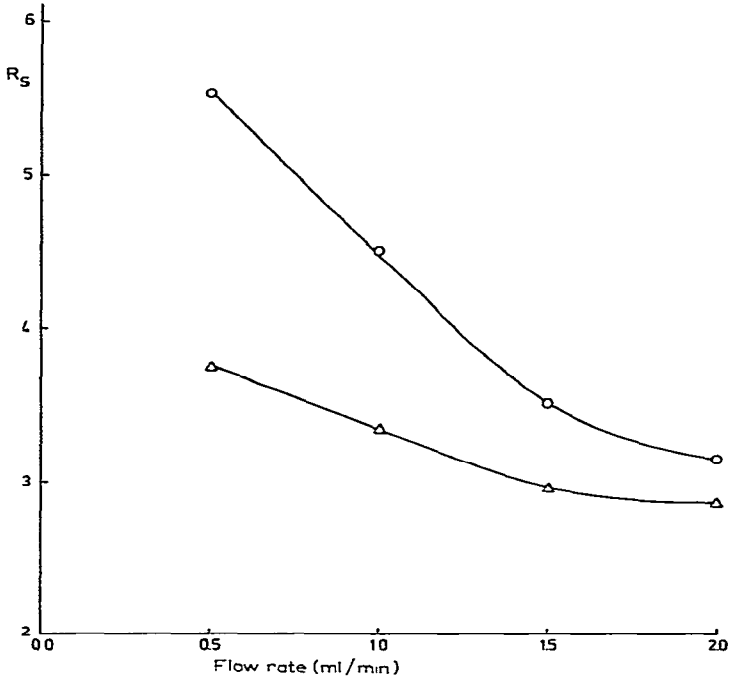

Fig. 5. Resolution (R_s) of glycine and isoleucine at different flow-rates obtained using the electrode detector (△) and the UV absorbance detector (○).

Calibration

Calibration curves for glycine, valine and isoleucine were prepared by using both the absorbance detector and the CTE. Linear plots were obtained with the UV detector for the three amino acids in the concentration range 0–25 μg/μl. The calibration plots obtained for the CTE were non-linear and are shown in Fig. 6; the electrode response follows the elution order glycine, valine, isoleucine. The shapes of the CTE calibration plots are similar to those obtained by Loscombe *et al.*[5] in their work with a copper-selective membrane electrode.

The precision of the electrode response was estimated by 10 replicate injections of a solution containing glycine and isoleucine at concentrations of 1.74 and 1.30 μg/μl, respectively. Coefficients of variation of 1.4% for glycine and 2.5% for isoleucine were obtained; these results compared favourably with those obtained by using the absorbance detector, which gave coefficients of variation of 4.0 and 3.6%, respectively, for the same 10 injections.

By using the definition of detection limit as three times the baseline noise, the calculated detection limits for glycine, valine and isoleucine were 75, 200 and 300 ng, respectively, in a 1-μl injection.

We are currently developing a combined recorder offset and signal-amplification system that will permit detection of smaller amounts of amino acids than stated above. Initial studies have shown that a ten-fold increase in sensitivity over the above values can be easily attained.

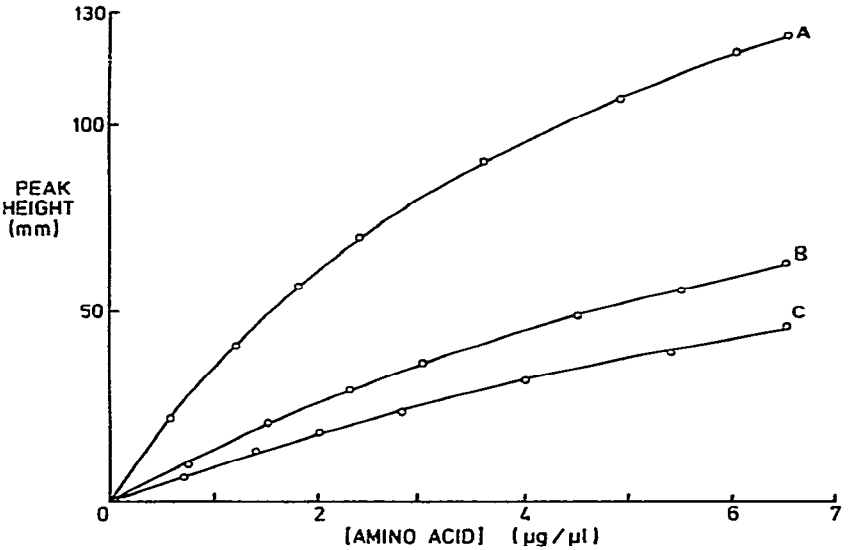

Fig. 6. Response of the electrode detector to different amino acids. Curves: A = glycine; B = valine; C = isoleucine.

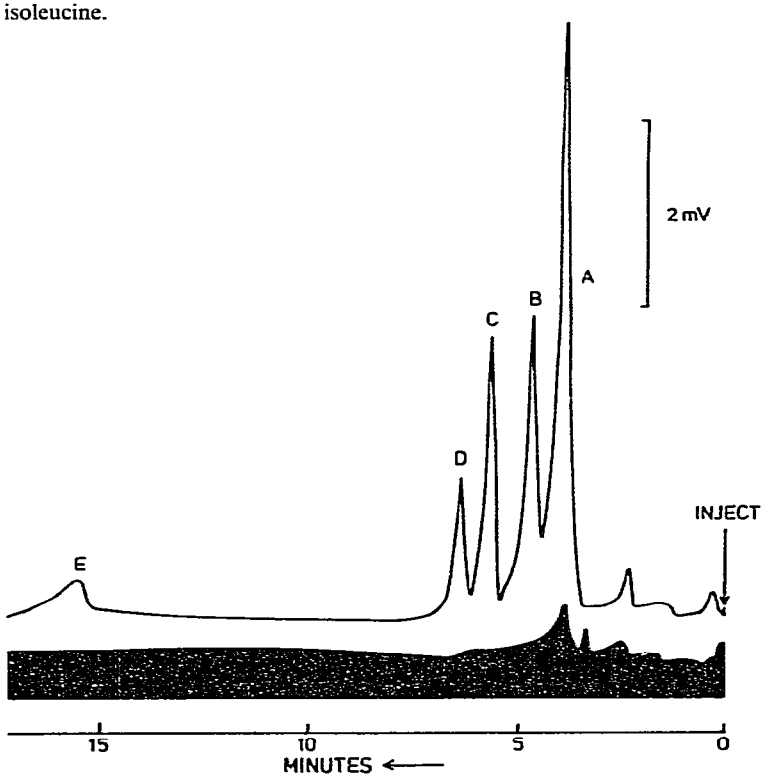

Fig. 7. Use of the electrode detector for chromatographic analysis of an undiluted urine sample (dark trace) and a "spiked" urine sample (upper trace) containing the following amounts of amino acids added per μl of urine: A = glycine (7.5 μg); B = valine (5.3 μg); C = methionine (7.6 μg); D = isoleucine (4.1 μg); E = phenylalanine (0.7 μg). Conditions: 1-μl injection; flow-rate 1 ml/min.

APPLICATIONS

The suitability of the CTE for analysis of urine and a pharmaceutical preparation was investigated. The freshly reconstituted urine sample was not pre-treated in any way except for filtration (Millipore filter) before injection. The chromatograms obtained for the urine sample using both detectors are shown in Figs. 7 and 8; these figures also show the chromatograms obtained from urine with five amino acids added.

The chromatograms obtained by using the UV detector are characterised by a profusion of unidentified peaks, and this background renders recovery calculations of

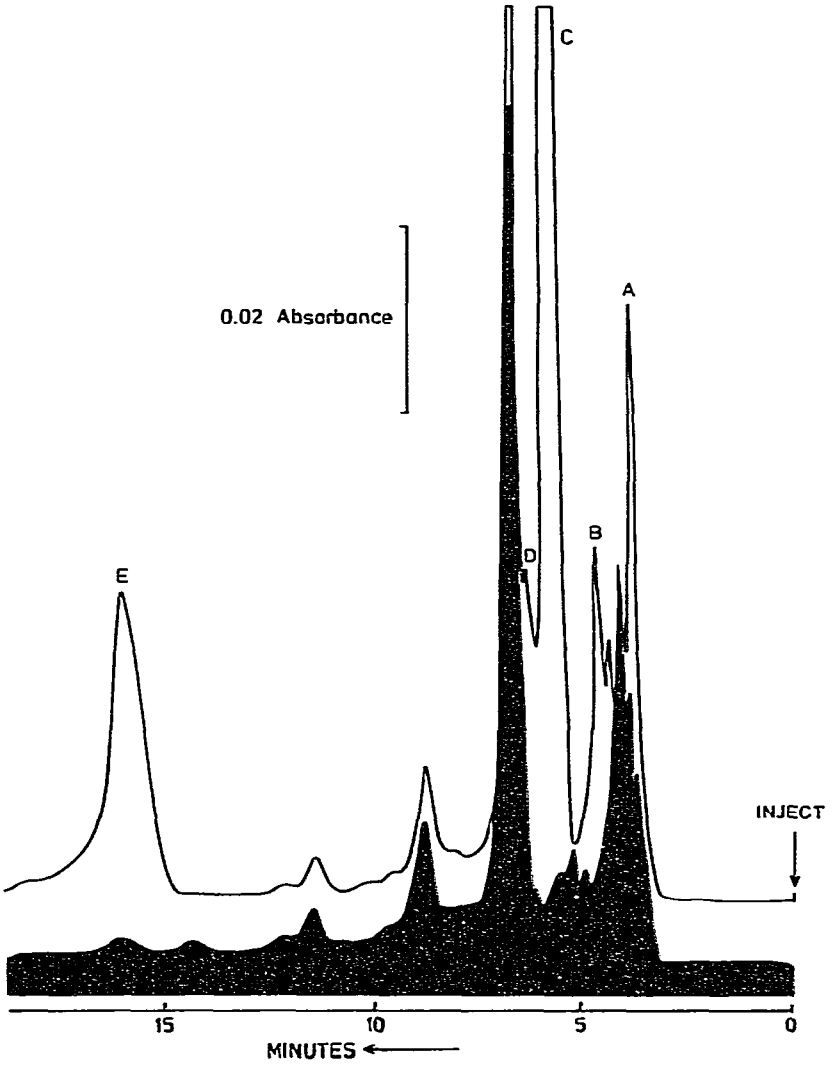

Fig. 8. Chromatographic analysis of urine and "spiked" urine samples with a UV absorbance detector (at 200 nm) under conditions shown in Fig. 7.

the added amino acids difficult. In contrast, the CTE chromatogram of the blank urine sample is very "clean", and the peaks produced by the added amino acids are easily identified. From these chromatograms, the recoveries for glycine, valine, isoleucine, methionine and phenylalanine were 96, 106, 107, 105 and 100%, respectively. The amounts of amino acids added to the sample are shown in the figures. Fig. 7 illustrates the main advantage of the CTE, that is, its selectivity, which eliminates the need for major pre-treatment of the sample.

An intravenous solution containing 15 amino acids and excipients (such as sodium metabisulphite, sodium acetate and sodium chloride) was analysed using the CTE detector; no pre-treatment of the sample was involved, except dilution and filtration. The chromatogram obtained is shown in Fig. 9. No attempt was made to identify or quantify each amino acid, and the separation conditions were not optimised, as the purpose was merely to demonstrate the utility of the CTE for pharmaceutical analysis. However, peaks due to 11 of the 15 components can be discerned, and it is likely that the remaining amino acids are eluted together with the resolved components.

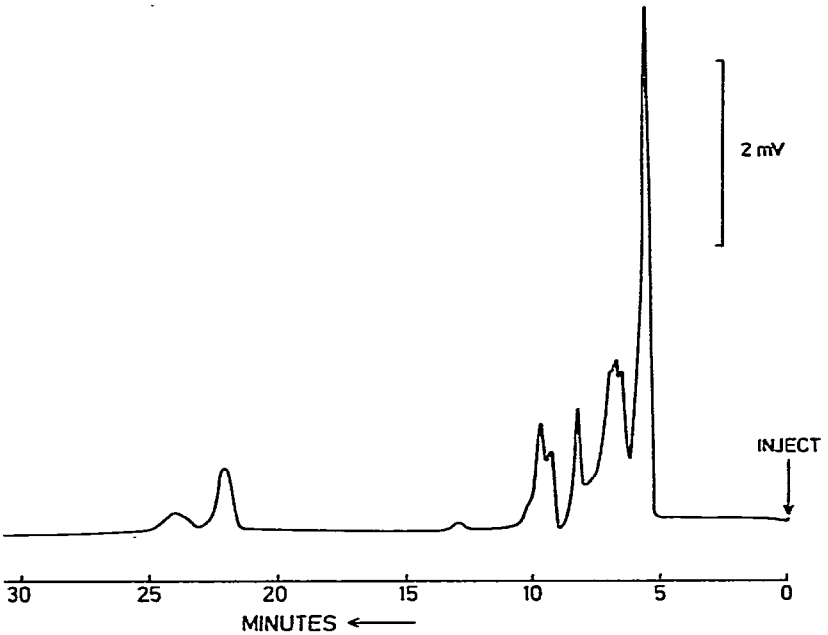

Fig. 9. Chromatographic analysis of an amino acid intravenous solution with the electrode detector. Conditions: 1-μl injection; flow-rate 1 ml/min.

CONCLUSIONS

This study has demonstrated that the CTE can be used as a selective detector in HPLC. In using such an electrode, problems associated with post-column derivatisation procedures or absorbance detection of solutes having poor UV absorption are minimised. The performance of the CTE, as indicated by its response time and sensitivity, is comparable with that of the UV absorbance detector. The major advantages

of the CTE are: first, the selectivity of the CTE is greatly superior to UV detection, and secondly the CTE can be manufactured at very low cost.

In this paper, amino acids have been used as solutes to demonstrate the application of the CTE to HPLC. Other copper-binding compounds may also be detectable. Narang and Gupta[12] have shown that sulpha drugs (sulphanilamide, sulphaguanidine, sulphathiazole, sulphamerazine, sulphadiazine and sulphapyridine) readily form complexes with copper, and Evans *et al.*[13], in their discussion of the analysis of porphyrins in biological materials by HPLC, have reported that copro- and meso-porphyrin esters readily complexed copper. In this laboratory, it has been found that the CTE responds to a variety of solutes. Further, this study opens up interesting possibilities of the use of tubular electrodes constructed from other metals as selective potentiometric detectors for HPLC systems. Further work on this aspect is in progress.

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