The Analyst COMMUNICATION

Indirect amperometric detection of underivatized amino acids in microcolumn liquid chromatography with carbon film based ring–disk electrodes

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Received 3rd April 2000, Accepted 8th May 2000
Published on the Web 22nd May 2000

An indirect amperometric detection of underivatized amino acids has been developed using a carbon film based ring–disk electrode (CFBRDE) in microcolumn liquid chromatography (LC). Bromide present in the mobile phase could be efficiently oxidized to bromine at the upstream (disk) electrode, and was subsequently detected at the downstream (ring) electrode. Most of the underivatized amino acids that are electroinactive under conventional amperometric conditions react rapidly with the electrogenerated bromine, the concentration of amino acids can therefore be indirectly determined by continuously monitoring the reduction current of bromine. The signal monitored at the downstream electrode was largely dependent on the bromide concentration in the mobile phase. Under optimized conditions, the response linearly increased with the concentration for most of the amino acids over a concentration range of 1–100 μM, with a correlation coefficient of 0.990–0.993. The detection limits for most of the amino acids were below 1 μM (0.2 pmol). It was demonstrated that detection with a ring–disk electrode offers the advantages of achieving a much higher collection efficiency caused by a decrease in flow rate in the microcolumn LC.

Introduction

Electrochemical detection (ECD) is an attractive approach for the determination of many compounds by microcolumn LC owing to its ease of implementation, high sensitivity and relatively low cost.[1–3] In contrast to most optical detection methods, the detector is easily miniaturized without loss in sensitivity because the extremely slow flow rate in microcolumn LC leads to an improvement in the electrolysis efficiency. The major drawback of ECD is its inherent selectivity, which generally limits analysis to easily oxidized or reduced species. For the determination of amino acids, as there are only six amino acids, tyrosine, tryptophan, histidine, cysteine, cystine and methionine that are electroactive at a carbon electrode, a pre- or post-column derivatization procedure is necessary for preparing suitably electroactive derivatives for the different classes of amino acid. A commonly used derivatization reagent is *o*-phthalaldehyde (OPA), which can react with primary alkyl amines in the presence of an alkyl thiol. The OPA derivatives are electrochemically active and can be amperometrically detected.[4,5] However, the derivatizations generally introduce sources of contamination or error into the analytical procedure.

Recently, several transition metal electrodes have also been developed for direct amperometric detection in HPLC based on the electrocatalytic oxidation of the amino acids in an alkaline medium at these electrodes. Johnson *et al.*[6,7] reported on the detection of trace underivatized amino acids with gold and platinum electrodes.[6,7] Baldwin *et al.*[8] demonstrated the utility of a copper-based electrode for the constant amperometric detection of amino acids in LC. Recently, the use of pulsed electrochemical detection techniques at gold and platinum electrodes has been widely accepted as a stable and sensitive method for the determination of amino acids by HPLC.[9]

The use of electrogenerated reagents for indirect detection of some nonelectroactive species is an alternative approach that has also gained practical use. King and Kissinger[10] first reported on a post-column reaction for the detection of fatty acids with electrogenerated bromine.[10] Two separated flow cells were used in the system; the bromine was generated in an upstream cell, after a reaction with the unsaturated fatty acids the excess bromine was detected in a downstream cell. Bromine can be electrogenerated directly in the mobile phase, and reacts rapidly with a large number of compounds that are difficult to detect using conventional detectors. In this work, we report the feasibility of this approach for the detection of some underivatized amino acids in a microcolumn LC system. By using a carbon film based ring–disk electrode (CFBRDE) coupled to the microseparation system, much higher collection efficiencies are achieved, thus providing better sensitivity than with conventional HPLC methods. Some principal experimental parameters governing the performance are characterized.

Experimental

Reagent grade potassium bromide (KBr) and amino acids were purchased from Wako (Osaka, Japan) and were used without further purification. Stock solutions of the amino acids were prepared with water, which was purified by a Milli-Q system (Millipore Co., France). Prior to LC analysis, the sample solutions were made up fresh by dilution with water. A 50 mM phosphate buffer solution (pH 7.5) containing 10 mM KBr was employed as mobile phase. The pH of the buffer solution was adjusted with 0.1 M phosphoric acid and 0.1 M NaOH.

Fig. 1 shows a schematic representation of the CFBRDE. The electrodes were fabricated by photolithography and dry-etching techniques[11] from a carbon film of pyrolyzed 3,4,9,10-perylenetetracarboxylic dianhydride on the plastic films (with a thickness of about 200 μm). The disk electrode (3 mm in diameter) was separated from the ring electrode (1 mm width) by a gap of 0.5 mm. For the amperometric measurements, a CFBRDE was installed in a radial thin layer flow cell (BAS Japan, Tokyo, Japan) with a 25 μm thick Teflon gasket (the dead volume was 1.25 μl). The disk electrode was held at +1.2 V *vs.* Ag/AgCl to generate the bromine electrochemically, while the ring electrode, which serves as the collection electrode (amperometric indicator), was held at 0.2 V *vs.* Ag/AgCl. The

DOI: 10.1039/b002603o

electrode potentials were controlled by an ALS/CHI Model 802 dual potentiostat (ALS/CH Instruments, Tokyo, Japan).

The microseparation system consisted of an LC-100 micro LC pump (BAS Japan), a Rheodyne (Cotati, CA, USA) Model 7520 injector with a sample loop of 0.2 μl, and an Inertsil (GL Sciences, Tokyo, Japan) ODS-3 separation column (150 × 0.3 mm id, 3 μm). The flow rate was maintained at 4 μl min^{-1}. The experiments were carried out at room temperature.

Results and discussion

The reagent bromine is electrochemically generated at the upstream (disk) electrode with a constant current (I_g), and then is reduced at the downstream (ring) electrode to yield a collection (reduction) current (I_c). In indirect amperometric detection mode, the analyte of interest reacts with the electrogenerated bromine and the concentration of analyte is determined by monitoring the decrease in the bromine at the detection electrode. One important factor concerning the analytical performance of the detector is the collection efficiency of the CFBRDE, which is defined as the ratio of collection current (I_c) to generation current (I_g). The collection efficiency of the ring–disk electrode was dependent on the electrode configuration, the electrode potentials for generation or collection, and the flow rate. In the absence of analyte entering the reaction detector, the response of I_c is increased proportionally to I_g. Our previous work studied the reaction of amino acids with electrogenerated bromine using voltammetric and hydrodynamic voltammetric techniques.[12] It was confirmed that the maximum collection current was obtained at a generation potential (WE_1) of +1.2 V *vs.* Ag/AgCl and a collection potential (WE_2) of +0.2 V *vs.* Ag/AgCl, respectively.[12] Under fixed generation and collection potentials, the collection efficiency was highly dependent on the flow rate. As can be seen in Fig. 2(A), the collection efficiency was *ca.* 0.02 at a flow rate of 1.0 ml min^{-1}, while it increased to *ca.* 0.42 when the flow rate was reduced to 4 μl min^{-1}. The dependence of peak response (the decrease from constant collecting current) for injected analyte on the flow rate was also examined with a micro-flow injection analysis (FIA) system. The typical flow rate dependence for the analytical signal of 1 pmol (50 μM) methionine is shown in Fig. 2(B). Apparently, the slower flow rate leads to an increase in the reaction time between the amino acids and the bromine over the electrode surface, and consequently yields a very high coulometric efficiency. A sufficient time for the reaction of the amino acids with the bromine would eventually lead to improvement in the signal-to-noise ratio (S/N) in the indirect detection mode. Thus ECD with CFBRDE is particularly useful in a microcolumn LC system.

Apart from the operation conditions (such as flow rate and generation or collection potentials) the reagent concentration in the mobile phase will also greatly affect the response of amino acid. The peak responses at the CFBRDE for different amounts of methionine injected were subsequently examined in the chromatographic system by switching to different KBr concentrations. The results are summarized in Table 1. Both the dynamic linear range and the limit of detection were found to be dependent on the concentration of bromide in the postcolumn additive reagent. As seen in Table 1, the upper limit of the range may be higher when using higher concentrations of KBr, but the greatest dynamic range (two orders of magnitude) is for the 10 mM concentration. In addition, some unexpected noise, due to some impurities or the instability of the bromine generation/collection, was observed when the concentration of KBr was very high. This could lead to a decrease in the S/N ratio of the system. In consideration of the above factors, 10 mM KBr was chosen and was used throughout the experiments. This provided a dynamic range for most of the amino acids of at least two orders of magnitude.

Fig. 3 illustrates a chromatogram for a standard mixture containing 25 μM each of serine, histidine, methionine, isoleucine, leucine, tyrosine and phenylalanine. The detection was carried out at the CFBRDE by setting WE_1 and WE_2 at +1.2 and +0.2 V *vs.* Ag/AgCl, respectively. A well resolved chromatogram was obtained by a reversed phase Inertsil ODS-3 column using a mobile phase containing 50 mM phosphate buffer and 10 mM KBr.

Other amino acids, such as alanine, tryptophan, aspartic acid, lysine and valine at the CFBRDE were also detectable in the indirect amperometric mode. The analytical results in terms of the dynamic range, the detection limits and the relative standard deviations and the retention times are summarized in Table 2. Generally, the detector exhibited a linear response for most amino acids in the range from 1 to 100 μM. A calibration curve was constructed by determining the peak height for three replicated injections of each different sample. The relative standard deviations (RSDs) ranged from 2% (glycine) to 4.8% (phenylalanine) for 25 μM of each sample (n = 5). Based on a S/N ratio of 3, the absolute detection limits for most of the

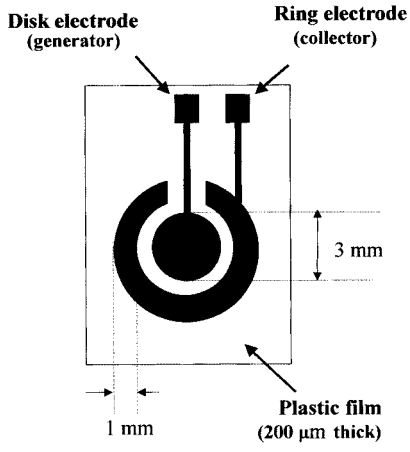

Fig. 1 Schematic diagram of a CFBRDE.

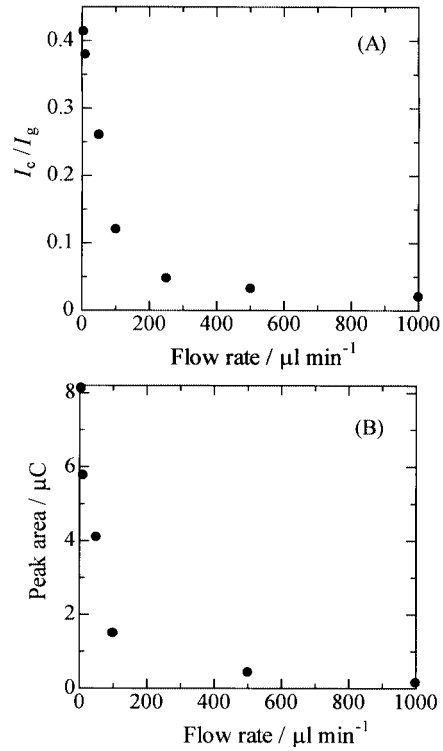

Fig. 2 (A) Dependence of flow rate on the collection efficiency of bromine at a CFBRDE. (B) Peak area for response of 50 μM of methionine as a function of flow rate. WE_1 = +1.2 V *vs.* Ag/AgCl; WE_2 = +0.2 V *vs.* Ag/AgCl; sample injection volume: 0.2 μl; mobile phase: 50 mM phosphate buffer containing 10 mM KBr.

Table 1 Effect of KBr concentration in the mobile phase on the analytical dynamic range and the detection limit for the detection of methionine at the CFBRDE in a micro-FIA system[a]

KBr/mM	Linear range/μM	Detection limit[b] μM	pg
10	1–100	0.3	9.0
50	10–250	5	149
100	25–400	15	448

[a] WE_1 = +1.2 V *vs.* Ag/AgCl; WE_2 = +0.2 V *vs.* Ag/AgCl; sample injection volume: 0.2 μl; mobile phase: 50 mM phosphate buffer containing KBr; flow rate: 4 μl min^{-1}. [b] Detection limits are estimated based on S/N ratio ⩾3.

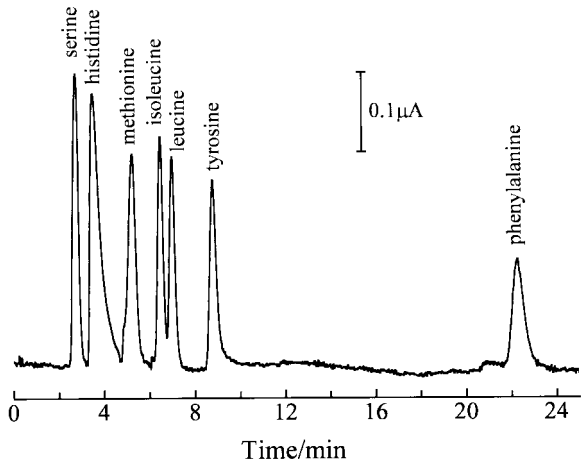

Fig. 3 Chromatogram of a standard mixture containing 25 μM each of serine, histidine, methionine, isoleucine, leucine, tyrosine and phenylalanine. Flow rate: 4 μl min^{-1}. The other conditions were the same as in Fig. 2.

amino acids were below 1 μM (0.2 pmol). The sensitivities with the indirect amperometric mode were found to be comparable to those obtained with a direct amperometric mode based on the electrocatalytic reaction at some metallic electrodes.[6–8] The equivalent values for the absolute detection limit (in pg) are also presented in Table 2. With constant or pulsed amperometric detection methods, the sensitivities were largely dependent on the structure of the amino acids. Amino acids, such as glutamic acid, proline and cysteine were very poor, probably because of a larger inhibitory effect due to their larger aliphatic side chains.[8,9] In the present approach, however, the sensitivities for these amino acids were almost the same as others. Unfortunately, not all the amino acids listed in Table 2 could be completely separated under the conditions, as shown in Fig. 3.

CFBRDEs can be used as disposable-type electrodes. All the tested electrodes exhibited a good electrode-to-electrode reproducibility, because their size and shape were precisely controlled by the lithographic technique. For a long-term determination, for example, to detect 25 μM of methionine over 24 h, the reproducibility gave an RSD below 6% (n = 100).

It has been shown that a CFBRDE can offer advantages in both versatility and sensitivity for the indirect detection of trace underivatized amino acids in microcolumn LC. The concept of

Table 2 Indirect amperometric detection of amino acids at a CFBRDE in microcolumn LC[a]

Compound	Detection limit[b] μM	pg	Linear range/μM	r^2	RSD[c] (%)
Alanine	0.3	5.3	1–100	0.993	2.3
Cysteine	0.4	9.7	1–150	0.991	4.5
Glycine	0.2	3.0	1–100	0.993	2.0
Glutamic acid	0.3	8.8	1–150	0.991	3.3
Histidine	0.8	25.1	5–150	0.990	4.2
Isoleucine	0.4	10.5	1–150	0.990	3.6
Leucine	0.4	10.5	1–150	0.990	3.8
Methionine	0.3	9.0	1–100	0.993	3.7
Phenylalanine	1	33.0	5–150	0.990	4.8
Proline	0.5	11.5	1–100	0.992	3.0
Serine	0.3	6.3	1–100	0.993	2.1
Threonine	0.3	7.1	1–100	0.992	2.5
Tryptophan	2	81.7	5–150	0.990	5.3
Tyrosine	0.3	10.9	1–150	0.992	3.1
Valine	0.4	9.4	1–150	0.991	2.9

[a] WE_1 = +1.2 V *vs.* Ag/AgCl; WE_2 = +0.2 V *vs.* Ag/AgCl; sample injection volume: 0.2 μl; mobile phase: 50 mM phosphate buffer containing 10 mM KBr; flow rate: 4 μl min^{-1}. [b] Detection limits are estimated at S/N ratio ⩾3. [c] Relative standard deviations obtained from five repeated injections (25 μM) of each samples.

direct amperometric detection is based on the electrocatalytic oxidation of compounds containing aliphatic amine and hydroxy groups at noble metal electrodes in strong alkaline media. Hence, alcohols, glycols and carbohydrates are also detectable at metal electrodes. The indirect method proposed in this study, however, does not respond to these compounds. Thus, it may be more selective for amino acids as compared with the direct detection methods. The combination of direct and indirect methods may be an interesting approach for both sensitive and selective determination of amino acids in real samples.

The authors thank Mr. M. Asano of BAS Japan for helpful discussions concerning the electrochemical detection.

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