Journal of Chromatography, 598 (1992) 195–201
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 027

Reductive electrochemical detection in liquid chromatography with a zinc amalgam scrubber column

Arne Bergens*,☆

Department of Analytical Chemistry, Uppsala University, P.O. Box 531, S-751 21 Uppsala (Sweden)

(First received January 23rd, 1991; revised manuscript received January 15th, 1992)

ABSTRACT

The dissolved oxygen in an acidic mobile phase can be reduced and eliminated by a scrubber column packed with zinc amalgam particles. The zinc amalgam scrubber column is mounted between the pump and the injection device and enables sensitive amperometric detection at potentials down to −0.65 V *vs.* Ag/AgCl. The high overvoltage of hydrogen on mercury allows the use of acidic mobile phases without problems with evolution of hydrogen gas. The accessible potential range is limited by the reduction of Zn^{2+} dissolved from the scrubber column. The possibilities for trace amount determinations of nitrocompounds are demonstrated in the detection of trinitrophenyl derivatives of amino acids from reaction with trinitrobenzenesulphonic acid. The detection was focused on γ-aminobutyric acid, and a detection limit of 0.1 pmol at −0.6 V *vs.* Ag/AgCl was obtained.

INTRODUCTION

The method commonly applied for the derivatization of amino acids, for example γ-aminobutyric acid (GABA), involves reaction with *o*-phthaladehyde (OPA) [1–9]. The derivatives formed from OPA are different alkylisoindoles that can be detected either by fluorescence [1–5] or by amperometric detection [6–9]. The reaction is fast, but the stability of the derivatives formed is in some cases limited, and a half-life as short as 4.1 min has been reported for the OPA-GABA derivative [7]. In the case of amperometric detection, the amine functionality in the alkylisoindole is oxidized at +0.75 V *vs.* Ag/AgCl [7–9].

Another derivatizing agent that can be used for amino acids is trinitrobenzenesulphonic acid (TNBS) [10–13]. The reaction with TNBS is quantitative in alkaline solutions and produces stable trinitrophenyl (TNP) derivatives as exemplified for GABA in Fig. 1. TNBS was introduced as a reagent for spectrophotometric determinations because the derivatives have absorbance maxima at 335–350 nm, where TNBS and most other organic substances are transparent. The TNP derivatives also possess advantageous electrochemical reduction properties [12,13]. The TNP derivatives are reduced at moderate cathodic potentials, and the reduction of an aromatic nitro group is a four-electron process. Hence, the total process of reduction of a TNP derivative yields twelve electrons and a high current response can therefore be expected.

However, reductive amperometric detection suffers from various interferences. These are dominated by dissolved oxygen in the mobile phase, which results in large baseline offset and increased faradaic noise. The common strategies used to decrease this effect include saturation of the mobile phase with an inert gas [14,15], continuous refluxing in an

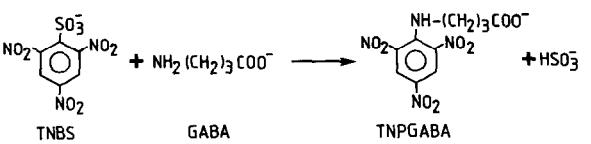

Fig. 1. Derivatization of GABA with TNBS in basic medium.

☆ Address for correspondence: Kabi Pharmacia AB, S-112 87 Stockholm, Sweden.

0021-9673/92/$05.00

inert atmosphere [16] and electrochemical reduction of the oxygen in either a scrubber cell [17] or a solvent reservoir [18]. The electrochemical reduction of oxygen seems to be a convenient and efficient technique. However, it is seldom used in practice because of the requirement for a stable high-current potentiostat. The application of an electrochemical scrubber cell seems from the literature not to have earned general use in connection with liquid chromatography (LC).

An interesting alternative to an electrochemical scrubber cell for oxygen reduction is to employ a scrubber column packed with zinc particles [19]. However, such a column would be expected to cause severe problems as a result of the evolution of microbubbles of hydrogen gas in applications that require acidic mobile phases. By treating the zinc particles with a solution of a mercuric salt, the particles become covered by a thin layer of mercury amalgam. Since hydrogen has a high overvoltage at a mercury surface, the competing reduction of H^+ is supressed. The zinc amalgam scrubber column therefore permits analysis at lower pH compared with a scrubber column packed with untreated zinc particles. The purpose of the present study was to evaluate the possibility of using a zinc amalgam scrubber column for reductive amperometric detection in LC. The application was focused on the detection and determination of trace amounts of GABA in biological samples.

EXPERIMENTAL

Apparatus

Chromatography. The high-performance LC (HPLC) system consisted of a Model 2150 (Pharmacia-LKB Biotechnology, Bromma, Sweden) double-piston pump modified with 316-grade stainless-steel connections and tubing on the inlet connectors. All tubing in the system was of 316-grade stainless steel except for a small 5 cm × 0.1 mm I.D. PTFE tube between the column and the detector cell. A three-necked round-bottomed flask was used as the mobile phase reservoir. The flask was equipped with a condenser, gas-tight connectors for the argon line and pump inlet tubing. The argon was precleaned from oxygen by passing it through a bed of copper(I) oxide catalyst (BASF, Ludwigshafen/Rhein, Germany) packed in a 42 cm × 1.2 cm glass tube. The argon was further saturated with mobile phase in a gas washing tower before entering the mobile phase reservoir. The amalgamated zinc scrubber was constructed from an empty 100 mm × 4 mm glass-lined HPLC column (SGE, Ringwood, Australia; No. 100GLE4) and mounted between the pump and the injection valve. The latter was a Valco six-port injector (Vici, Schenkon, Switzerland) with a 20-μl sample loop. All separations were performed on an RP-8 3-μm, 100 mm × 3.2 mm column obtained from Brownlee Labs. (Santa Clara, CA, USA). The column was thermostated to 35 ± 0.1°C by means of a column oven, constructed in the laboratory. The electrochemical detector was a commercially available thin-layer cell with a 50-μm gasket and a Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, USA). The potential was controlled by a Bioanalytical Systems LC-4B potentiostat. Derivatization experiments with different reaction media were performed using a Shimadzu SPD-6A (Shimadzu, Tokyo, Japan) UV detector and a Model CMA240 (Carnegie Medicin, Stockholm, Sweden) autoinjector as the injection device. All chromatograms were collected and processed with a Model SP4290 integrator (Spectra-Physics, San Jose, CA, USA).

Voltammetry. Voltammetric experiments were performed using a Model 174A polarographic analyser [EG&G Princeton Applied Research (PARC), Princeton, NJ, USA]. The electrochemical cell consisted of a 20-ml water-jacketed glass vessel with a Ag/AgCl reference electrode and a platinum-wire auxiliary electrode, all purchased from Metrohm (Herisau, Switzerland). The working electrode was a glassy carbon electrode (GCE; Metrohm, Model 6.1204.040), which could be mounted on a rotating electrode assembly (Metrohm, Model 2.628.0020), used for obtaining voltammograms under hydrodynamic conditions. The electrochemical cell was kept at 30°C by means of a conventional thermostat. The nitrogen used for deoxygenation of the supporting electrolyte was cleaned from oxygen by passing it through a washing tower containing vanadous chloride solution. Voltammograms were recorded on a Hewlett-Packard 7044B X–Y recorder.

Reagents

Zinc metal was purchased from Carl Roth (Karlsruhe, Germany) as 300-μm particles with a

specified purity of 99.99%. The mercury (II) nitrate used for the preparation of zinc amalgam was purchased from Merck (Darmstadt, Germany) and was of p.a. grade.

GABA, glycine, δ-aminovaleric acid (DAVA) and TNBS were obtained from Aldrich (Steinheim, Germany) and used as received. The amino acid standard mixture was obtained from Pierce (No. 20077; Tecator, Sollentuna, Sweden).

All organic solvents used for mobile phases were purchased from Merck and were of LiChrosolv quality. The *m*-chloroacetic acid was obtained from BDH (Poole, UK). The distilled water used in this work was further purified with a Millipore Milli-Q filtration system.

TNP derivatives of GABA, glycine and DAVA were prepared in crystalline form for use as standard substances according to Okuyama and Satake [10] and the crystals of TNP-GABA obtained showed a sharp melting point at 149–151°C, which is consistent with reported data [13].

Procedures

Preparation of the oxygen scrubber column. An 8-g portion of pure zinc particles was treated with 50 ml of 0.5 *M* nitric acid containing 2 g of mercury (II) nitrate. The mixture was stirred vigorously for 5–10 min. The solution was then removed and the zinc amalgam was washed with at least two 50-ml portions of water. The zinc amalgam was covered with water and kept in a bottle with a ground joint glass stopper to minimize exposure to oxygen. Zinc amalgam that was stored for 24 h or longer had to be treated with 2 *M* nitric acid for 2 min before use to regain maximum efficiency of the scrubber column. This precaution was taken since oxide formation on the surface of the amalgam diminished its function. The amalgam was poured in small portions into the column tube, which was filled with water and mounted in an upright position. The bottom end of the column tube was connected through its end fitting to water suction equipment, and the tube was kept filled with water throughout the packing procedure. The amalgam was sedimented on the bottom of the tube by a continuous stream of water and by vigorous knocking on the column with a spoon. With the column filled, the upper end fitting was assembled and the scrubber column was immediately mounted into the LC system.

Chromatography. The mobile phase used in the separations was a mixture of 10% (v/v) acetonitrile, 12% (v/v) 2-propanol and 78% (v/v) 0.05 *M* *m*-chloroacetic acid buffer, pH 2.7. The *m*-chloroacetic acid buffer contained 0.2 m*M* EDTA as a metal-complexing agent. Before start-up, the mobile phase was saturated with argon by vigorous bubbling for 15–30 min. The LC system was primed and running before the zinc amalgam scrubber column was prepared and mounted. Thereafter, the electrochemical detector cell was assembled and turned on. It was found necessary to leave the system running at least overnight with the mobile phase recycling at the normal flow-rate (0.70 ml/min) in order to allow the trapped oxygen in the separation column to diffuse out.

The sample solutions were deoxygenated in a 5-ml disposable syringe with a small hole made at the 5-ml mark as described previously [20]. With the injection valve in the load position and the syringe plunger above the 5-ml mark, deoxygenation of sample volumes in the range 0.5–4 ml could be made by connecting the argon to the waste tubing of the injection valve.

Voltammetry. Before any voltammetric experiments were performed, the GCE was polished with an aqueous slurry of 0.3 μm alumina powder on a damp silk cloth. The GCE was thoroughly rinsed with water in an ultrasonic bath before it was mounted on the electrode holder and inserted in the measuring cell. The supporting electrolyte was deoxygenated by saturation with nitrogen which was passed through two gas washing towers, the first containing vanadous chloride solution and the second supporting electrolyte. The electrode surface was conditioned in the deoxygenated supporting electrolyte by cycling the applied potential between −1.0 V and + 1.0 V *vs.* Ag/AgCl. The scan rate in the conditioning step was 50 mV/s, and the sweep was applied until no appreciable change in background current could be observed. After the conditioning step, the GCE was left in the supporting electrolyte and appropriate volumes of stock solutions of TNP-GABA were added. During the voltammetric experiments a 10-ml portion of supporting electrolyte was used. With the rotating electrode, the angular velocity was 157 rad/s (1500 rpm).

Derivatization. The derivatizations of GABA

standard solutions and Pierce amino acid standard solutions were performed using 1-ml Eppendorf vessels and mainly according to Caudill and Wightman [12]. The standards were prepared daily and the TNBS reagent solutions weekly with 0.05 *M* borate buffer pH 9 as solvent. All solutions were kept refrigerated when not in use. Separate experiments showed that the derivatization of a Pierce amino acid standard mixture required TNBS at an excess of 1600-fold with respect to the amount of GABA present in order to obtain the maximum yield of TNP-GABA. Therefore, the amount of TNBS used in the derivatizations was routinely 2000 times higher than the amount of GABA present in the sample. Portions of 20 μl of GABA or Pierce standard solutions were allowed to react with 200 μl of reagent solution at 40°C for 30 min. The reaction was quenched by addition of 200 μl of 4 *M* perchloric acid. The TNP derivatives were then extracted twice with 500 μl of toluene. The collected toluene phase was further re-extracted with 500 μl of borate buffer (pH 9). The 500 μl of borate buffer were finally acidified with 20 μl of 0.6 *M* perchloric acid.

RESULTS AND DISCUSSION

Introductory experiments using a similar column packed with pure zinc particles according to MacCrehan and May [19] failed because of the evolution of hydrogen gas. The microbubbels formed rendered detection at any sensitivity impossible. Attempts with back-pressure applied at the detector cell outlet also failed. Not only acidic mobile phases were tried in these experiments. Water, 0.05 *M* phosphate buffer pH 7 and the 0.025 *M* ammonium acetate buffer pH 5.4 used by MacCrehan and May were also tried with the same negative results. However, the problem of hydrogen gas evolution can be avoided if the zinc particles are treated with a mercuric salt. Amalgamated zinc is a classical reductive agent in the Jones reductor [21] for reducing, for example, Fe^{3+} to Fe^{2+} in acidic media.

The effect of the amalgamated zinc scrubber column on the background characteristics was compared with other deoxygenation techniques. The background current characteristics were studied in four different experiments. The use of an oxygen trap in the argon line was thought useful since the argon of the quality used should contain at most 5 ppm oxygen. However, the effectiveness of this oxygen trap, constructed in the laboratory, was questionable, since the background characteristics became worse when the oxygen trap was used together with argon (Fig. 2). However, a drastic improvement in the background characteristics was obtained when the zinc amalgam scrubber column was employed. The best result was obtained when the oxygen trap was used together with the zinc amalgam scrubber column. At −0.6 V, the background current decreased from 200 nA down to 30–60 nA. The background characteristics presented in Fig. 2 are typical single measurements obtained after four different preparations of the GCE. The background characteristics of a freshly polished GCE are largely dependent upon the condition of the electrode surface. A reproducible electrode treatment is therefore of great importance but difficult to obtain. The standard deviation for the data in Fig. 2 is estimated from experience to be 5–10% at a background current of 30 nA and 30% at currents higher than 100 nA. The electrode treatment also affects the faradaic noise level. In the present investigation, the levels were in the range 10–40 pA at −0.6 V. When the zinc amalgam scrubber was used the background increased sharply at potentials more negative than −0.75 V as a result of the reduction of Zn^{2+} dissolving from the scrubber column.

The lifespan of a zinc amalgam scrubber was nor-

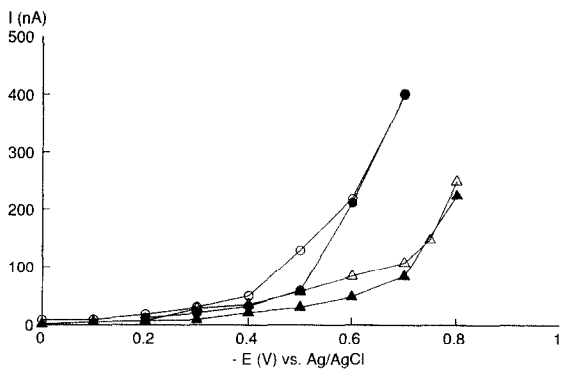

Fig. 2. Comparison of background currents for reductive detection with different methods of deoxygenation of the mobile phase: ● = saturation with argon; ○ = saturation with argon passed through an oxygen trap; △ = saturation with argon and zinc amalgam scrubber mounted; ▲ = saturation with argon passed through an oxygen trap and zinc amalgam scrubber mounted. Flow-rate 0.70 ml/min and temperature 35°C.

mally 1–3 weeks of continuous operation. It was found that the most reliable operation of the zinc amalgam scrubber was obtained if the LC system was left running at 0.70 ml/min continuously. The background current slowly increased during operation from typically 30–60 nA to 100–150 nA after approximately 1 week. Consequently, the faradaic noise became gradually worse and did not allow any measurements when the background reached 100–150 nA. The cure for these problems was to prepare a new zinc amalgam scrubber and prime the LC system with fresh mobile phase. The lifespan was prolonged if fresh mobile phase was refilled into the reservoir flask at the end of a working day. The increasing background was expected since the function of the zinc amalgam scrubber involves dissolution of Zn^{2+}. Metal impurities present in the zinc material used must also be expected to dissolve, and recycling the mobile phase will therefore lead to enrichment of metal ions into the mobile phase.

Polarographic investigation of the zinc material used showed that the major impurities were copper ($3.8 \cdot 10^{-4}$%) and lead ($2.5 \cdot 10^{-3}$%). A polarogram of a mobile phase portion that had been used and recycled over 4 days did not show any detectable amounts of copper or lead. However, the cathodic background in the polarograms increased sharply at -0.7 V, indicating a high concentration of Zn^{2+}.

The zinc amalgam scrubber column imparts two further limitations to the LC system that should be considered. The first limitation is that the zinc amalgam scrubber column liberates Zn^{2+} ions and possibly hydrogen peroxide into the mobile phase. The presence of metal ions might catalyse reactions which consume analytes of interest, and the hydrogen peroxide, which is formed as an intermediate in oxygen reduction, might interfere with sample constituents. The second limitation is the dissolved oxygen in the sample solution. The injected solutions will acquire a higher oxygen concentration than the mobile phase since it is impossible to use a similar zinc amalgam column for the deoxygenation of sample solutions. The zinc would reduce any component of interest in the sample, and the only possible method of deoxygenating samples is therefore saturation with an inert gas in a closed compartment. As a consequence of the higher oxygen concentration, an oxygen peak at the beginning of the chromatogram cannot be avoided (Fig. 3). After 20 min of purging with argon the oxygen peak is diminished to a height of about 0.25 nA with a severe tailing during the first 4 min. Further saturation with argon results in only minor improvements.

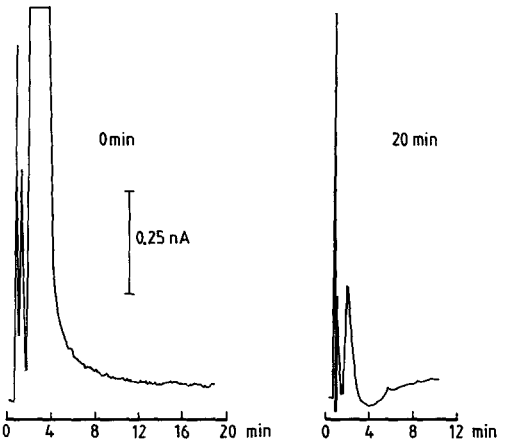

Fig. 3. Deoxygenation efficiency of the sample. A 20-μl aliquot of blank borate buffer was injected. Applied potential -0.6 V.

The electrochemical reduction of TNP-GABA has been characterized previously by Caudill and Wightman [12] as three four-electron steps with three clearly separated voltammetric waves corresponding to the reduction of each nitro group. These results could be verified with voltammetric experiments and are consistent with the commonly accepted mechanism for reduction of aromatic nitro compounds [22]. In order to find the most suitable potential setting for the amperometric detector, experiments using hydrodynamic voltammetry with a rotating GCE were performed. With the electrode rotating at 157 rad/s and the mobile phase as medium, the hydrodynamic voltammogram of TNP-GABA exhibited a maximum which was not affected by alterations in sweep rate, concentration or rotation frequency. Experiments with TNP-glycine and TNP-DAVA also showed maxima under the same hydrodynamic conditions as with TNP-GABA (Fig. 4). These maxima were most pronounced when the electrode surface was freshly polished and pretreated, which indicates that this effect is related to the condition of the electrode surface. By adding a surfactant, Triton X-100, the maxi-

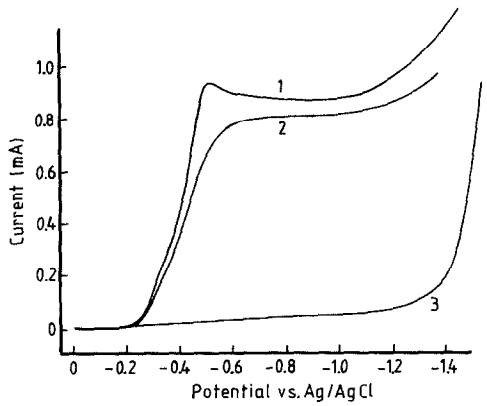

Fig. 4. Hydrodynamic voltammograms of 0.2 m*M* TNP-GABA in the chromatographic mobile phase with the electrode rotating at 1500 rpm. Curve identities are: 1 = 0.2 m*M* TNP-GABA; 2 = after the addition of one drop of 0.1% Triton X-100 solution; 3 = background current.

mum could be suppressed, indicating that the maximum in Fig. 4 originates from differences in current density at the electrode surface. The final but most crucial conclusion that can be drawn from Fig. 4 is that a potential of at least −0.6 V *vs.* Ag/AgCl is necessary to realize a mass transport controlled current under hydrodynamic conditions. However, the potential setting needs to be verified with the amperometric detector since a different GCE was used for voltammetric experiments.

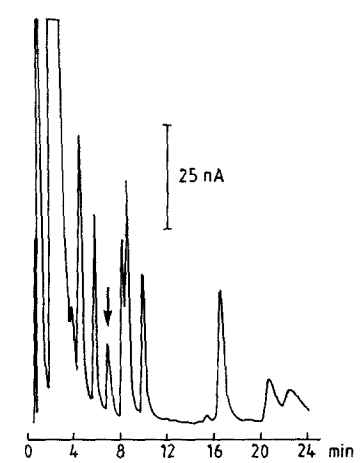

Fig. 5. Chromatogram of a Pierce amino acid standard mixture containing 10 pmol of each amino acid. Arrow indicates the TNP-GABA peak after 7.1 min. Flow-rate 0.70 ml/min and temperature 35°C.

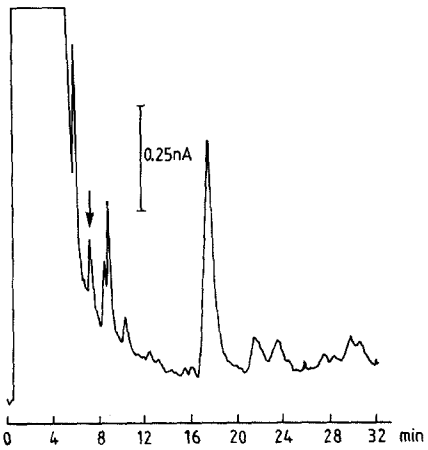

Fig. 6. Chromatogram of a Pierce amino acid standard mixture containing 0.1 pmol of each amino acid. The TNP-GABA peak is indicated by the arrow as in Fig. 7. After correction for dilution and assuming a 100% extraction efficiency, the peak corresponds to 2.6 pmol in a 10-μl sample.

As a first test of the deoxygenation technique developed, a Pierce amino acid standard mixture was investigated. A 20-μl aliquot of diluted standard mixture was treated with TNBS reagent solution as described in the Experimental section. The chromatograms resulting from injections of 10 and 0.1 pmol are shown in Figs. 5 and 6, respectively. The dominating peaks at the beginning of the chromatograms may be caused by picric acid, which is formed as a by-product in the derivatization procedure[11,12]. Similar peaks in the time interval 0–4 min were also found when a blank borate buffer was treated in the same procedure. The results in Fig. 6 indicate the possibility of achieving a detection limit as low as 0.1 pmol. However, the deoxygenation technique used here for sample solutions cannot be applied on sample volumes less than 500 μl. Furthermore, deoxygenation of small volumes causes severe technical problems. Experiments with deoxygenation of 10-μl samples with argon in closed 300-μl sample vials with sealing PTFE-lined silicone septa failed. The reason is probably the transfer time between the sample vial and the injection valve when the small sample volume resides in the syringe. Consequently, the limiting sample volume for a 500 μl minimum sample volume imparts a dilution factor as large as 50 on a sample of 10 μl volume.

In conclusion, it has definitely been proved that the zinc amalgam scrubber column is an efficient tool for reducing the oxygen concentration in the mobile phase to an acceptable level for analysis. It has also been shown that 0.1 pmol of GABA can be detected with TNBS derivatization and reductive mode amperometric detection. So far, the method seems to be useful in situations where the sample volume is not a limiting factor. However, for small sample volumes, as for example from microdialysis (typically 10 μl), problems with the work-up procedure occur. It is necessary to employ some procedure for the removal of the excess TNBS reagent. Extraction with toluene used in these first experiments may not be the ideal method for small sample volumes. The work-up procedure requires an internal standard since the many manipulations will influence the reproducibility. As an internal standard, DAVA has been used [12], and separate experiments have confirmed its advantages, with a retention time of about 12 min. Finally, the deoxygenation procedure needs to be modified for purging of small sample volumes.

ACKNOWLEDGEMENTS

I wish to thank Professor Bengt Nygård, University of Uppsala, and Dr. Anders Carlsson at Carnegie Medicin for many valuable discussions and reviewing the manuscript. I also acknowledge the assistance of Dr. Leif Nyholm with the polarographic experiments. Finally, I wish to thank Barbro Nelson for her assistance in preparation of the manuscript.

REFERENCES

1 B.N. Jones and J. P. Gilligan, *J. Chromatogr.*, 266 (1983) 471.
2 C. Sunol, F. Artigas, J. Ma. Tusell and E. Gelpi, *Anal. Chem.*, 60 (1988) 649.
3 B. N. Jones, S. N. Pääbo and S. Stein, *J. Liq. Chromatogr.*, 4(4) (1981) 565.
4 P. Böhlen and R. Schroeder, *Anal. Biochem.*, 126 (1982) 144.
5 D. L. Hogan, K. L. Kraemer and J. I. Isenberg, *Anal. Biochem.*, 127 (1982) 17.
6 M. H. Joseph and P. Davies, *J. Chromatogr.*, 277 (1983) 125.
7 S. M. Lasley, R. D. Greenland and I. A. Michaelson, *Life Sci.*, 35 (1984) 1921.
8 L. A. Allison, G. S. Mayer and R. E. Shoup, *Anal. Chem.*, 56 (1984) 1089.
9 J. Kehr and U. Ungerstedt, *J. Neurochem.*, 51 (1988) 1308.
10 T. Okuyama and K. Satake, *J. Biochem.*, 47 (4) (1960) 454.
11 G. E. Means, W. I. Cangdon and M. L. Bender, *Biochemistry*, 11(19) (1972) 3564.
12 W. L. Caudill and R. M. Wightman, *Anal. Chim. Acta*, 141 (1982) 269.
13 W. L. Caudill, G. P. Houck and R. M. Wightman, *J. Chromatogr.*, 227 (1982) 331.
14 L. Michel and A. Zatka, *Anal. Chim. Acta*, 105 (1979) 109.
15 M. O. Funk, M. B. Keller and B. Levison, *Anal. Chem.*, 52 (1980) 771.
16 K. Bratin and P. T. Kissinger, *Talanta*, 29 (1982) 365.
17 H. B. Hanekamp, W. H. Voogt, P. Bos and R. W. Frei, *Anal. Chim. Acta*, 118 (1980) 81.
18 W. A. MacCrehan and R. A. Durst, *Anal. Chem.*, 50 (1978) 2108.
19 W. A. MacCrehan and W. E. May, *Anal. Chem.*, 56 (1984) 625.
20 A. Bergens, *J. Chromatogr.*, 410 (1987) 437.
21 I. M. Kolthoff, E. B. Sandell, E. J. Meehan and S. Bruckenstein, *Quantitative Chemical Analysis*, Collier-Macmillan, London, 4th ed., 1969, pp. 829–832.
22 C. K. Mann and K. K. Barnes, *Electrochemical Reactions in Nonaqueous Systems*, Marcel Dekker, New York, 1970, pp. 348–352.