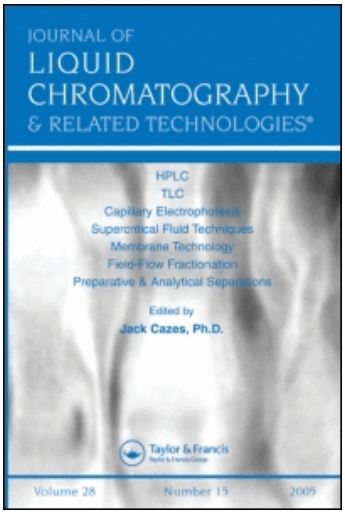

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DETERMINATION OF GLUTAMATE IN RAT BRAIN MICRODIALYSATES BY MICROBORE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A highly sensitive microbore liquid-chromatographic method with electrochemical detection has been developed for the determination of glutamate in rat brain dialysates. Isocratic separation of glutamate was achieved using a microbore reversed phase C_{18} column and a 100 mM Na_2HPO_4-methanol (60:40, v/v, adjusted to pH 5.0 with orthophosphoric acid) mobile phase. A hydrodynamic voltamogram was generated (300-900 mV) from which an optimal working potential of +600 mV was established. The absolute detection limit of glutamate was 17 fmol (signal-to-noise ratio of three). This system offers the significant

advantages of small sample consumption and improved detection limits compared with conventional liquid chromatographic applications. These factors permit greater sampling frequency and better temporal resolution between *ex-vivo* brain neurochemistry and measures of behavioural performance.

INTRODUCTION

Glutamate is a fast-acting excitatory neurotransmitter in the central nervous system[1,2] with defined actions in modulating neuronal excitability, synaptogenesis[3,4] and cerebral ischemic injury.[5,6]

The *in-vivo* cerebral microdialysis technique is currently restricted by the relatively large sample volume required for analyte detection by conventional chromatographic methods. In order to correlate changes in *ex-vivo* brain neurochemistry with behavioural output over time, it is necessary to develop highly sensitive analytical techniques. Currently, microbore column high performance liquid chromatography (HPLC) has the significant advantages of very high sensitivity and relatively small sample volume consumption compared to that of conventional analytical column applications. Microbore column technology has been exploited in the analysis of both platelet[7] and plasma[8] serotonin and in the determination of acetylcholine and biogenic amines by on-line brain microdialysis.[9]

Glutamate is neither fluorescent nor electroactive but can be made so by pre-[10] and post-column[11] o-phthalaldehyde (OPA) derivatization. In the present study, we have developed a highly sensitive pre-column OPA derivatization assay employing microbore column liquid chromatography and electrochemical detection (ED) to determine glutamate content in rat brain microdialysates with a high sampling frequency.

MATERIALS AND METHODS

Chemicals and Reagents

Glutamate was purchased from Sigma (St. Louis, MO, USA). The glutamate stock solution (0.1 mg/mL) was prepared in double deionised water and kept at 4°C. o-phthalaldehyde (OPA) and ß-mercaptoethanol (ß-ME) were supplied by Fisher Scientific (Loughborough, UK).

Liquid Chromatography

The HPLC-ED (BAS, LC-4C, Bioanalytical System, West Lafayette, IN, USA) system consisted of a Rheodyne 9125 injector (5 μL loop), a solvent delivery system (BAS, PM-80) and a reversed phase C_{18} microbore column (BAS UniJet, 150 x 1 mm I.D., particle size 5 μm). A high-efficiency pulse damper was incorporated into the system to reduce background noise. The derivatized glutamate product was oxidised using a glassy carbon working electrode (+600 mV vs. Ag/AgCl) with a special thin-layer gasket for microbore LC-ED. In order to minimize dead volume, the microbore column was connected directly between the injector and the working electrode. The detector output current was monitored using an integrator (Chromatocorder Sic, Tokyo, Japan). Isocratic separation was achieved at room temperature (23°C) with a flow rate of 40 μL/min.

The mobile phase consisted of 100 mM Na_2HPO_4 and methanol (60:40 v/v) adjusted to an apparent pH of 5.0 with orthophosphoric acid. Water and methanol were HPLC grade (BDH, Poole, UK). Before use, the mobile phase was filtered through a 0.22 μm filter (Millipore) using vacuum assistance.

Derivatization

The derivatization reaction was carried out at room temperature. OPA (27 mg) was dissolved in 1 mL methanol, 9 mL 0.4 M potassium tetraborate (adjusted to pH 10.4 with 5 M NaOH) and 5 μL ß-ME. The OPA stock reagent was covered in foil and stored at 4°C for 1 week. The OPA working solution was prepared each day by diluting 1 mL of the OPA stock reagent with 3 mL of potassium tetraborate (0.4 M). The derivatising agent (6 μL) was reacted with an equal volume of dialysate or glutamate standard for 2 min before injection onto the analytical column.

Microdialysis Procedures

The experiments were carried out in adult, Lister hooded male rats (OLAC, Bicester, UK), weighing between 280 and 350 g. The rats were anaesthetized with urethane (1.2 g/kg, i.p.) and placed in a Kopf stereotaxic frame. A microdialysis probe (BAS, dialysing length 2 mm, diameter, 0.32 mm) was placed into the right nucleus accumbens with its tip located at the following coordinates, taken from the atlas of Paxinos and Watson:[12]: AP +1.7 mm from the bregma, ML -1.5 mm, DV -7.5 from dura. Body temperature was

maintained at 37 °C with a heating pad. Implanted probes were perfused at 1.0 μL/min with an artificial CSF composed of (mM): NaCl 147; KCl 3; $MgCl_2$ 1; $CaCl_2$ 1.3; NaH_2PO_4 0.20; Na_2HPO_4 1.30; pH 7.4. Brain dialysates were collected every 5 min into 2 μL perchloric acid (0.2 M) and immediately analysed for their glutamate content. The position of the probe was verified by standard histological procedures at the end of the experiment.

Recovery and Precision

The relative recovery of glutamate (Ri) in-vitro, defines the ratio of the dialysate concentration of a substance (C_{out}) to its concentration in the medium surrounding the probe (C_{in}).

$$Ri = C_{out} / C_{in}$$

To determine intra-assay variance, quadruplicate assays were carried out on the same sample concentration at different times during the day. Inter-assay variance was determined by the same sample concentration on days one, two, four, and six. Coefficients of variation (C.V.s) were calculated from these values.

RESULTS

Figure 1 shows representative chromatograms of the lowest concentration of glutamate detectable (17 fmol), a higher concentration (850 fmol) of a standard sample (Figure 1B), and a baseline dialysate sample collected from the nucleus accumbens 60 mins after probe implantation, which contains 587 fmol glutamate (Figure 1C). The retention time for glutamate was approximately 8.0 min. Samples could be injected every 15 min without significant interference from late eluting substances. The relative recovery of glutamate (flow rate of 1.0 μL/min) was 39.1 ± 1.4% (n=5). The hydrodynamic voltammogram (Figure 2) shows the detector response of glutamate as a function of the applied voltage. The current-voltage relationship was non-linear with a clearly defined plateau region between 500 and 700 mV.

The reproducibility of the method was defined by examining both intra- and inter-assay variabilities. The intra- and inter-assay variation for the determination of glutamate at concentrations of 85, 170, 850, and 1710 fmol were acceptable with C.V.s of less than 10% (Table 1).

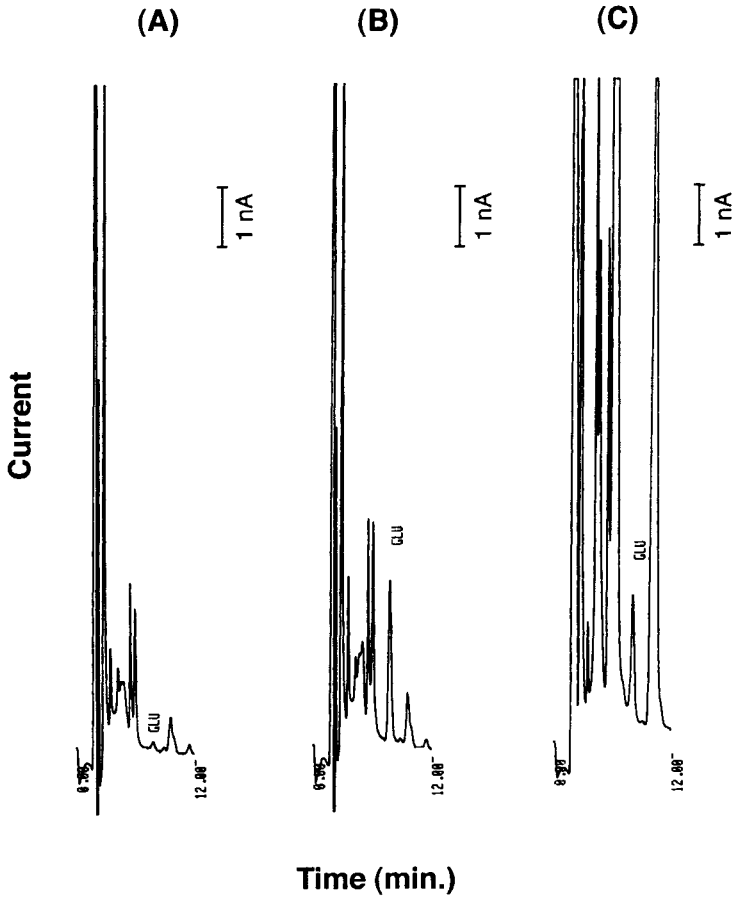

Figure 1. Chromatograms of (A) spiked glutamate (17 fmol) in artificial CSF, (B) spiked glutamate (850 fmol) in artificial CSF, and (C) a baseline dialysate sample collected from the nucleus accumbens 60 mins after probe implantation which contains 587 fmol glutamate.

To determine assay linearity, four different concentrations of glutamate (85-1710 fmol) were analysed. The peak-areas were linearly related to the concentration of glutamate and the equation for the regression line for glutamate was found to be $y = 0.57 x + 1.31$ ($r=0.999$). The slopes of the calibration graphs ($n=4$) were reproducible throughout the study: 0.55 ± 0.011 (mean ● S.D.) with a C.V. of 4.01 %.

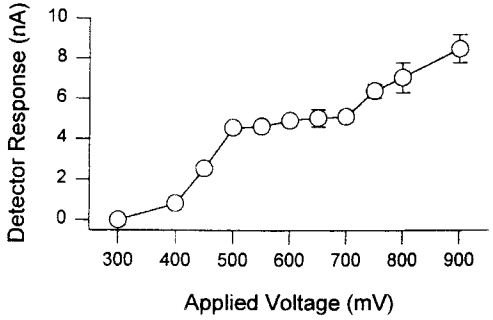

Figure 2. Hydrodynamic voltammogram of an OPA-derivatised glutamate standard (100 ng/mL). The detector response at each potential was recorded on 4 successive trials (± SEM).

Table 1

Intra- and Inter-Assay Precision and Relative Error in Glutamate Determination for Spiked Concentrations of 85, 170, 850 and 1710 fmol (n=4)

Spiked Concentrations (fmol)	Measured Concentrations (mean ± SEM)	C.V. (%)	Relative Error (%)
Intra-assay			
85	80 ± 4	4.9	-6.2
170	166 ± 14	8.2	-2.6
850	849 ± 71	8.4	-0.12
1710	1689 ± 59	3.5	-1.25
Inter-assay			
85	82 ± 4	4.8	-.32
170	172 ± 9	5.4	1.4
850	876 ± 40	4.6	3.1
1710	1708 ± 81	4.7	0.1

Precision [C.V. %] = [standard deviation (S.D.)/mean] × 100.
Relative Error (%) = [(spiked conc. - measured conc.)/spiked conc.] x 100.

The limit of detection, as defined by the lowest concentration of a standard that can be measured with acceptable precision (C.V. less than 20%), was 17 fmol (signal-to-noise ratio of three). The lowest practical limit of quantification was 51 fmol.

DISCUSSION

Microbore column liquid chromatography systems invariably exhibit lower detection limits compared with conventional HPLC-ED systems. This advantageous feature arises from (1) reduced peak broadening, (2) a smoother baseline due to reduced mobile phase consumption, and (3) higher coulometric yields due to more prolonged contact with the working electrode.

Additionally, microbore HPLC-ED systems require smaller sample volumes, making them ideal for high frequency sampling and improved temporal resolution in in-vivo brain dialysis.

In order to reduce baseline interference from the OPA-alkylthiol derivative during the electrochemical detection of excitatory, inhibitory, and other amino acids in rat brain microdialysates, the OPA-sulphite derivative has been used. In this setting, a significant improvement in interference was observed, however, the applied potential remained high at +700 mV[11] and +850 mV.[13]

In the present study, we have shown that, provided the applied potential is reduced to +600 mV, the OPA-alkylthiol derivative can be oxidised without significant baseline interference. Since no loss in detector response was encountered at this potential (Figure 2) the detection limit was lowered to just 17 fmol on column. This is a considerable improvement over a recent microbore fluorescent-based detection system which reported a detection limit for glutamate of only 67 fmol.[11]

In summary, a highly sensitive isocratic microbore HPLC-ED method has been developed for the determination of glutamate in rat brain microdialysates. This methodology offers improved detection limits and greater stability compared with conventional gradient elution LC-ED systems, and consequently will be of considerable utility for behavioural studies with well-defined events in time, such as learning.

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