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Journal of Chromatography B, 708 (1998) 27–38

JOURNAL OF CHROMATOGRAPHY B

Determination of glutamate and aspartate in microdialysis samples by reversed-phase column liquid chromatography with fluorescence and electrochemical detection[1]

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Received 22 September 1997; received in revised form 22 December 1997; accepted 29 December 1997

Abstract

Five different systems for fast determination of aspartate and glutamate in microdialysis samples are described: (I) a high-speed HPLC using a gradient pump with a sharp elution profile, (II) a column switching technique, (III) an isocratic pump with a low-pressure switching valve for one-step gradients, (IV) microbore chromatography using injections of acetonitrile as a wash-out step, (V) on-line connection of microdialysis and HPLC/derivatization. In all cases, automated precolumn derivatization with *o*-phthalaldehyde-2-mercaptoethanol reagent were used. Both fluorescence and electrochemical detection techniques were evaluated in terms of reproducibility, sensitivity, interference, maintenance and troubleshooting. The electrochemical detection method required a second derivatization step with 0.2 *M* iodoacetamide to remove excess of a thiol moiety and regular recalibrations after each six to ten injections. Under these conditions the correlation coefficients for electrochemical vs. fluorescence detectors were 0.918 for Asp and 0.988 for Glu for 65 microdialysis samples. Coefficients of variation for six analyses between calibrations were below 3% for both detectors. The limits of detection for both amino acids were about 0.4 pmol for electrochemical detection with a thiol scavenger step, 50 fmol for fluorescence detection using conventional columns and about 20–30 fmol for the microbore system. All systems are suitable for detecting basal levels of Asp and Glu in 5–10 μl microdialysis samples from a rat brain where typical concentrations lie around 1–10 pmol or more. It is concluded that a microbore setup with one isocratic pump and an autosampler optimized for injections of washing solvent between samples is the most practical and economical. The system allows analysis of minute sample volumes down to 1–2 μl. © 1998 Elsevier Science B.V.

Keywords: Glutamate; Aspartate

1. Introduction

Glutamate (Glu) is well recognized as a major excitatory neurotransmitter in the central nervous system [1,2]. It is hypothesized that the massive efflux of Glu and aspartate (Asp) observed in different neuropathological models of brain injury [3–5] causes an uncontrolled excitotoxic stimulation of postsynaptic (mainly NMDA) receptors, membrane depolarization and energy depletion, which in turn leads to neuronal cell death [6]. However, recent studies indicate that besides Asp and Glu, other neurotransmitters or their metabolites can also be involved in the mechanisms of ischaemic brain damage [7]. Furthermore, Asp and Glu play im-

[1] Part of the work was done at CMA/Microdialysis AB, Stockholm, Sweden.

0378-4347/98/$19.00
PII S0378-4347(97)00677-4

portant roles in cell metabolism. For instance, Glu is an intermediate in energy metabolism, a precursor of the main inhibitory neurotransmitter γ-aminobutyric acid and detoxifies ammonia via the formation of glutamine.

The technique of in vivo microdialysis [8,9] allows sampling and continuous monitoring of extracellular pools of neurotransmitters and other small molecules.

Microdialysis has been shown to be an elegant tool in studies of amino acid outflow [10], ischaemia [11], hypoglycaemia [12], epilepsy [13] and brain lesions [14].

In most cases, amino acids were separated after precolumn derivatization with an *o*-phthalaldehyde 2-mercaptoethanol (OPA-MCE) reagent using reversed-phase liquid chromatography as originally described by Lindroth and Mopper [15]. The method was recently modified for an automated derivatization procedure [16] and for microdialysis samples [17]. It was shown that, besides fluorescence (FL), electrochemical (EC) detection of OPA derivatives is possible [18,19], although with some precautions. A typical separation of 15–25 physiological amino acids found in microdialysis samples can be achieved by gradient elution in 30–40 min. In this mode, Asp and Glu elute at the beginning of the chromatogram with retention times of some 5–10 min. This means that elution can occur almost under isocratic conditions. Thus, if just these two amino acids are of interest, there is no need for additional separation. On the contrary, a fast and simple analysis would be more beneficial, especially due to the high sampling frequency during a microdialysis experiment. A simple sharpening of the gradient profile will reduce the analysis time down to approximately 10 min, including reequilibration. However, a more rapid and economical separation is possible by omitting the gradient pump and using different switching techniques instead. The use of microbore columns allows detection of Asp and Glu in volumes of microdialysates as low as 1–2 μl which corresponds to approximately 1 min sampling intervals.

In the present paper, methods for fast automated Glu and Asp determination, based on column switching (a second high-pressure valve), a one-step gradient (3-way low-pressure valve) and flushing by a second injection of an organic solvent (for microbore columns) are described. Furthermore, the possibility of an on-line connection of microdialysis to the liquid chromatograph and corresponding on-line derivatization with OPA is discussed. The sensitivities of fluorescence and electrochemical detectors are compared.

2. Experimental

2.1. Instrumentation

A CMA/200 refrigerated microsampler (CMA/Microdialysis, Stockholm, Sweden) was used, either in a standard version or equipped with extra valves as described below. Automated derivatization with one reagent (for FL detection) or two reagents (for EC detection), as well as the timing of extra valves, were controlled by standard software supplied with the autosampler. A modified version of the program was used for in-between injections of acetonitrile in order to wash the microbore column. Amino acid derivatives were detected by a Spectroflow 980 fluorescence detector (Kratos Analytical, Ramsey, NJ, USA), equipped with a 5 μl detector cell, a CMA/280 fluorescence detector (CMA/Microdialysis) equipped with 6 μl cell (System IV) or by an LC 4B/17A Electrochemical detector (Bioanalytical Systems, West Lafayette, IN, USA). For the Spectroflow detector, the excitation wavelength was set at 330 nm, the emission cut-off filter at 418 nm. The CMA/280 is a fixed wavelength detector operating at maximal excitation and emission wavelengths in the ranges 340–360 nm and 395–545 nm, respectively. The electrochemical detector included a glassy carbon electrode operating at +0.7 V vs. an Ag/AgCl reference electrode. Data were recorded using an SP-4290 integrator (Spectra-Physics, San Jose, CA, USA) or EZChrom data acquisition system (Scientific Software, San Ramon, CA, USA). All mobile phases were degassed by a CMA/260 degasser (CMA/Microdialysis).

Columns used in Systems I, II and V were 60×4 mm I.D. analytical with 5×4 mm I.D. guard cartridges packed with Nucleosil 5C18 (Knauer, Berlin, Germany). In System III, a 100×3.2 mm I.D. Biophase Phase II, C-18, 5 μm particle size (Bioanalytical Systems) was used. For microbore

separations (System IV), a 100×1 mm I.D. columns packed with C18 silica, 5 μm particle size (BAS, Tokyo, Japan), were used. Five different separation systems were studied.

2.1.1. System I

A system for fast gradient elution comprising an SP-8800 ternary pump (Spectra- Physics) mixing a binary gradient according to the following scheme: (min-%A/%B) 0-100/0; 2-100/0; 4-0/100; 6-100/0.

2.1.2. System II

The CMA/200 autosampler was fitted with a second high-pressure valve and the gradient pump was replaced by two isocratic pumps (Knauer, model 64). The instrumental setup is schematically depicted in Fig. 1a. As can be seen, the analytical column is mounted in the loop position of the second valve, which is directly connected to the detector. This will allow flushing of the column with buffer B in the opposite direction (backflush) to the flow of buffer A. The switching was programmed with standard CMA/200 software as follows: 80 s after the injection, the second valve was switched to the backflushing position for 30 s. Then the valve was switched back to reequilibrate the column with buffer A and the autosampler started derivatization of the next sample. This step was completed in 130 s, which gives a total analysis time of 4 min.

2.1.3. System III

The gradient pump used in System I was replaced by an isocratic pump (CMA/250 LC Pump) fitted with a low-pressure electronically driven three-way valve (CMA/Microdialysis) as shown in Fig. 1b. The valve switched between buffers A and B according to the standard CMA/200 software: Elution started with buffer A for 5.5 min. Then the valve was switched to B for 30 s and subsequently switched back to buffer A. The whole sequence between two injections, including derivatization and reequilibration (run-to-run analysis time), was less than 10 min for the fluorescence detector and 12 min for the electrochemical detector.

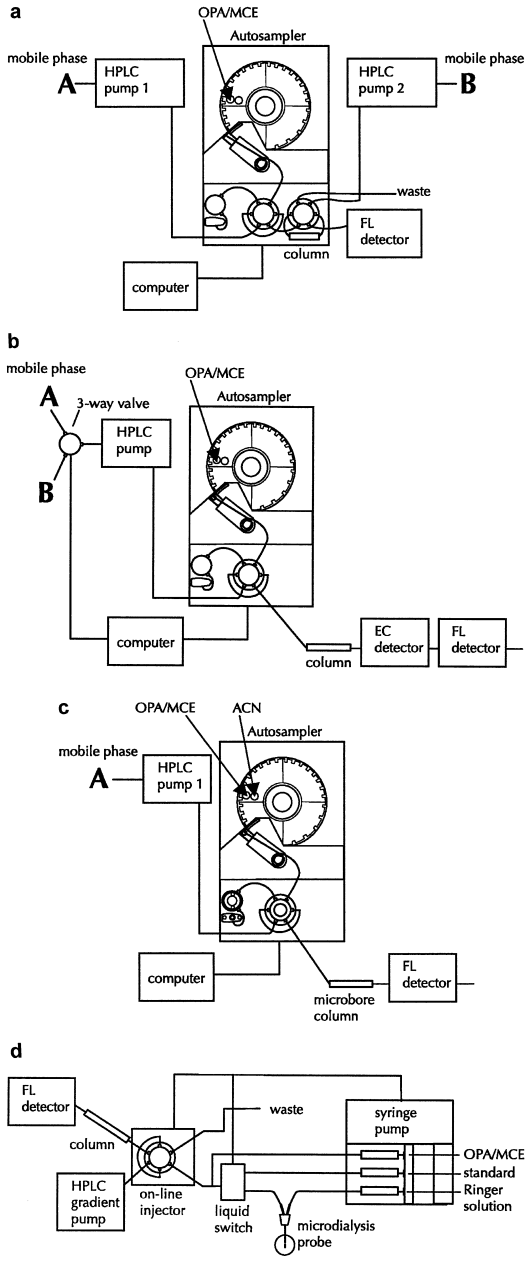

Fig. 1. Schemes of the HPLC systems used for fast Asp and Glu determinations: (a) System II: a column switching system including two pumps and two high-pressure 6-port valves; (b) System III: an isocratic pump provided with a low-pressure valve allowing mixing of one-step gradients, EC and FL detectors connected in series; (c) System IV: an isocratic setup for microbore separations, wash-out step made by injection of acetonitrile, (d) System V: on-line connection of in vivo microdialysis to HPLC.

2.1.4. System IV

The microbore version for Asp and Glu determinations (see Fig. 1c) was based on one isocratic pump (MicroLC pump 20, BAS, Tokyo, Japan), a CMA/280 fluorescence detector and a CMA/200 autosampler. The latter had modified software enabling automated injections of an organic solvent (acetonitrile, ACN) onto the column before each new derivatization. After 150 s (set as the analysis time), the autosampler flushed the sampling needle (this takes approximately 120 s) and then pipetted 10 μl ACN from a 1.5 ml vial placed in the calibrator position of the autosampler. Following a further 45 s in the 'wait in loop' position, the ACN was injected onto the column. The same analysis time (150 s) was used during the washing out step of retained amino acids and reequilibration of the column before starting the next sample derivatization.

2.1.5. System V

This system features an on-line connection of microdialysis to HPLC. The microdialysis setup consisted of a CMA/100 microinjection pump equipped with three 1 ml syringes, a CMA/111 syringe selector, a CMA/10 microdialysis probe (4 mm membrane length) implanted into the rat striatum and a CMA/160 on-line injector (all from CMA/Microdialysis) connected to HPLC System I, as shown in Fig. 1d. The CMA/200 autosampler was not used. Derivatization of the microdialysis samples was accomplished by mixing the perfusates with the OPA-MCE reagent in the capillary T-union and loading them directly into the injector loop. 10 min fractions were loaded into the sampling valve at a flow-rate of 1 μl/min, followed by 5 s injection intervals needed for the introduction of samples onto the chromatographic column.

2.2. Chemicals, reagents and mobile phases

Amino acid standards (protein, AA-S-18; acidics and neutrals, AN; acidics, neutrals and basics, ANB), *o*-phthaldialdehyde (OPA), 2-mercaptoethanol (MCE) and boric acid were obtained from Sigma (St. Louis, MO, USA). Sodium acetate, sodium phosphate, EDTA, methanol, acetonitrile and tetrahydrofuran were supplied by E. Merck (Darmstadt, Germany). Iodoacetamide (98%) was purchased from Janssen Chimica (Beerse, Belgium).

A stock solution of the OPA reagent was prepared as follows: 13.4 mg OPA was dissolved in 5 ml of 50% 0.2 *M* borate buffer (pH 9.5) and 50% methanol. Then 28 μl MCE was added. The working solution was prepared fresh daily by diluting the stock reagent 4 times with borate-methanol buffer. This gave 5 m*M* OPA and 20 m*M* MCE solutions. A scavenger reagent was prepared by dissolving 185 mg iodoacetamide in 1 ml methanol. The final solution was prepared daily by diluting 5 times with 0.1 *M* borate (pH 9.5) buffer to give 200 m*M*. This reagent should quantitatively derivatize samples with a total amino acid content of up to 1 m*M*, at about 50 pmol/μl for an amino acid in a biological sample.

Usually, 10 μl of a microdialysis sample was mixed with 4 μl OPA-MCE reagent and 4 μl iodoacetamide (needed only for electrochemical detection) and 16 μl was injected onto the column. For applications on microbore columns, 1000 μl of a commercially available reagent (OPA incomplete, Sigma) was mixed with 14 μl MCE solution diluted in methanol (1:10). Microdialysis samples from 1 to 10 μl were derivatized with 1–3 μl OPA incomplete-MCE reagent, depending on microdialysis experimental conditions (sampling area, membrane length, perfusion speed). Reaction times were 60 s for each reagent, the temperature was set to +4°C.

Mobile phase A for Systems I and II consisted of 0.1 *M* sodium acetate buffer, pH 6.95, 2.5% THF and 10% methanol. Mobile phase B was methanol. The flow-rate for both eluents was 1.2 ml/min. For System II, the maximum speed of analysis was achieved by increasing the flow-rates for buffer A to 1.5 ml/min and for buffer B to 3 ml/min. Buffer A for System III contained 0.1 *M* sodium acetate, pH 6.0, 0.5 m*M* EDTA, 7% ACN. Mobile phase B contained 90% ACN and 10% 0.1 *M* acetate buffer. Flow-rate was 0.5 ml/min. Mobile phase for System IV was 0.1 *M* acetate buffer, pH 6.0 including 10% ACN. The flow-rate was 70 μl/min. For the on-line mode, the most convenient separation system was the one with a gradient pump operating as a one-step gradient switching, as in System III.

3. Results and discussion

3.1. Speed and reliability of analysis, detection limits, impurities and interference as evaluated by fluorescence detection

The chromatogram in Fig. 2a shows the separation of 2.5 pmol Asp and Glu in a mixture of other physiological amino acids (ANB standard). The backflushing technique applied in System II allows the fastest separation cycle of only 4 min. A third broader peak in the chromatogram corresponds to the methanol flowing out from the column during the reequilibration period. This sensitivity is generally adequate for the detection of Asp and Glu in most of the microdialysis perfusates whose volumes range between 5 and 20 μl. The limit of detection of 50 fmol is achievable by operating the fluorescence detector at maximum excitation energy and photomultiplier signal. Further improvement of detection limits down to approximately 20–30 fmol Asp and Glu/10 μl sample can be achieved using the microbore columns (System IV), as illustrated in Fig. 2b. This corresponds to 15.4–23 fmol Asp and Glu injected onto the microbore column. Compared to the previous system, the micro-LC setup has a number of practical advantages: (1) Only one isocratic pump, with no extra valves, is required for Asp/Glu separations. (2) Decreasing the flow-rates from 1.5 ml/min for two pumps down to 70 μl/min for only one mobile phase causes a dramatic reduction of waste of organic modifier and other chemicals. (3) Microdialysis samples as small as 1–2 μl can be analyzed as shown in Fig. 7e. The total analysis time is about 8 min (Fig. 2c) which allows analysis of a fully loaded autosampler (60 samples) within one working day (8 h).

The calibration curves for both Asp and Glu peak heights were linear over the range 0.025–25 pmol/10 μl. The coefficients of variation for 39 standard (2.5 pmol/10 μl) samples analyzed within the course of 24 h were 8.24% for Asp and 8.13% for Glu. The between-assay variation, expressed as the average standard deviation for 2.5 pmol/10 μl standard injections and 4 independent sets of 24 h runs, was 0.236 pmol/10 μl (range from 0.206 to 0.271) for Asp and 0.207 pmol/10 μl (range from 0.165 to 0.231) for Glu. The coefficients of variation of Asp and Glu peak heights for the calibration standard (2.5 pmol/10 μl) injected at the beginning of six independent runs were 11.43% for Asp and 4.78% for Glu, respectively.

Some of the most common problems in highly sensitive analyses of amino acids are associated with contaminants and impurities which may interfere with Asp and Glu peaks. For microdialysis experiments, it is essential to keep all devices and solutions under strict control against any contamination by biological material (bacterial, blood, etc.). For example, it was observed that Asp and Glu could be detected after only 1 week in a bag of Ringer solution which had been opened, resealed and stored at laboratory temperature. A newly opened bag of sterile Ringer did not show any such contamination. Other frequent sources of impurities are water, chemicals and glassware used for the preparation of buffers and reagents. A derivatized blank sample of 5 μl Ringer solution (Fig. 3a) can be easily become contaminated when pipetted with a plastic tip which has been touched with fingers (Fig. 3b). Proteins of various origin can precipitate and permanently adsorb on the top of column. Each injection of the OPA-MCE reagent will result in the elution of a number of amino acid derivatives easily detectable at sub-pmol levels.

Several physiological interferences such as sulphur-containing amino acids and small peptides can co-elute with Asp and Glu peaks. However, the concentrations of these compounds when recovered by microdialysis from the extracellular space are on average 2–3 orders of magnitude lower than Asp and Glu concentrations. If there is still some risk for *S*-amino acids interfering with the Asp/Glu assay (microdialysates from other organs such as liver, etc.), the slower System III with a better resolution should be used. Fig. 4a shows the separation of Phospho-Ser, Asp and Glu in an amino acid standard mixture (AN, 9 pmol of each injected). The chromatogram of 12 *S*-amino acids and small peptides (glutathione, cysteic acid, glutathione sulphonic acid, serine-O-phosphate, cysteine sulphinic acid, serine-*O*-sulphate, γ-glutamyl-glutamine, homocysteic acid, γ-glutamyl-taurine, homocysteine sulphinic acid, γ-glutamyl-glutamate, γ-glutamyl-glycine), 9 pmol of

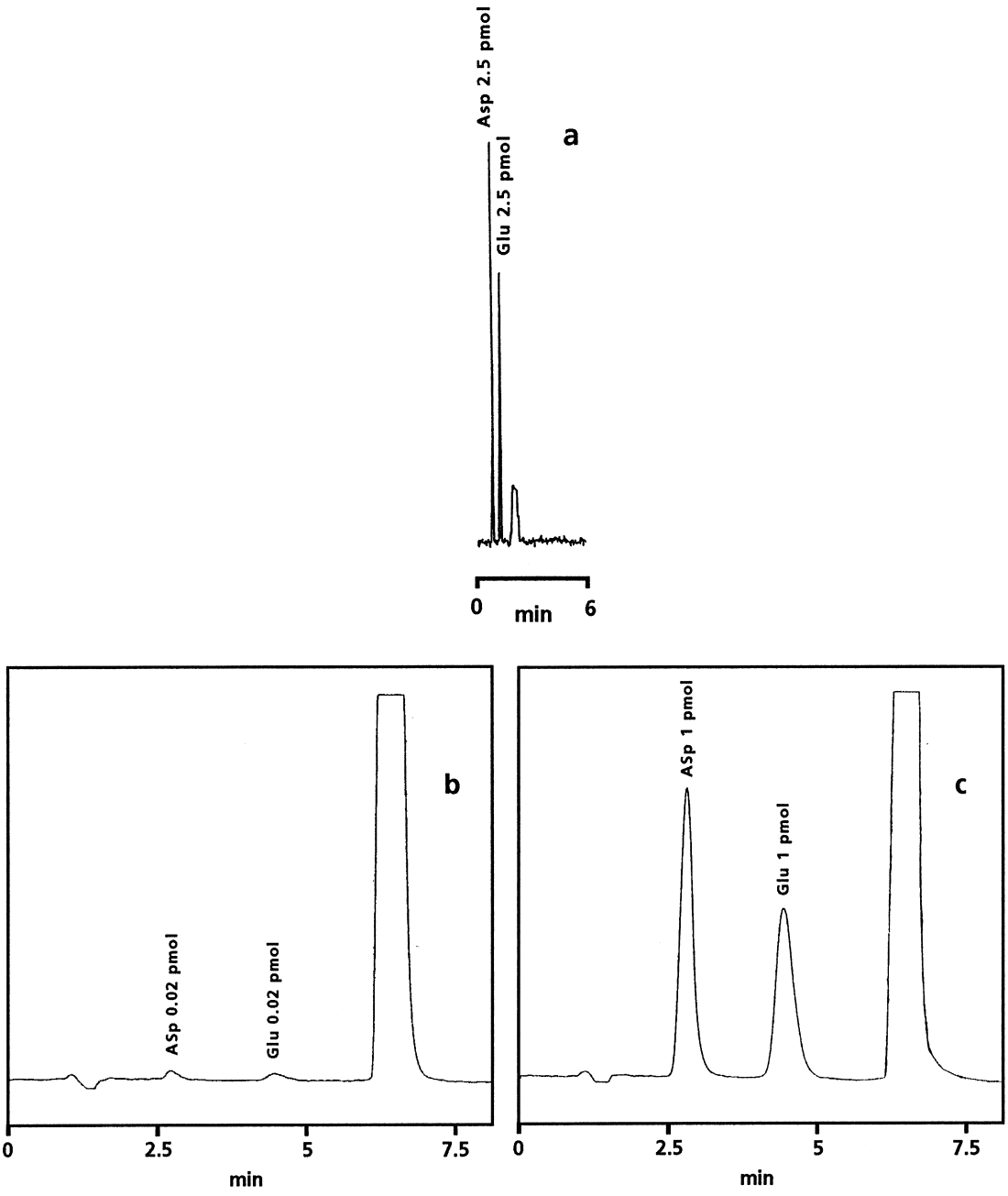

Fig. 2. (a) Chromatogram of a 10 μl standard mixture of physiological amino acids (ANB) containing 2.5 pmol each of Asp and Glu, separated on System II/Kratos detector; (b) the limit of detection of 20 fmol for Asp and Glu in a 10 μl sample volume was achieved on a microbore column (System IV/CMA/280 detector); (c) a typical chromatogram of 10 μl amino acid standard mixture (AA-S-18), containing 1 pmol each, derivatized with 3 μl OPA-MCE. A 10 μl volume was injected into a microbore System IV.

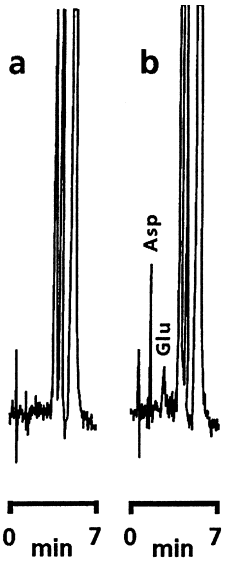

Fig. 3. (a) Chromatogram of derivatized blank (5 μl Ringer solution) sample separated on System III/CMA/280 detector; (b) same as (a) but with a blank contaminated during pipetting from a tip touched with fingers.

each injected, is shown in Fig. 4b. Finally, the chromatogram in Fig. 4c shows the separation of a mixture of two amino acid standards, 4.5 pmol injection per compound. As seen, Asp and Glu peaks are satisfactorily separated from *S*-amino acid standards. A large peak eluting at 7 min represents the rest of the amino acids and the acetonitrile (buffer B) from the flushing step.

3.2. Electrochemical detection: The importance of two reagent derivatization

Electrochemical detection of OPA amino acid derivatives by liquid chromatography (LCEC) was first reported in 1983 [18] but did not achieve wide acceptance. There are several reasons and explanations for this fact. Firstly, electrochemical cells are generally more complicated to maintain compared to the optical systems. Troubleshooting of any HPLC method based on electrochemical detection requires qualified and experienced personnel. Secondly, elution gradients based on methanol, acetonitrile or other organic solvents, which are necessary for the complete elution of amino acids, are very unfavourable for the signal from the electrochemical detector and its baseline stability, especially at higher attenuation. Finally, the OPA-thiol reagent, which is 'transparent' in fluorescence light, will give a large tailing front peak and a thiol peak eluting somewhere in the middle of the chromatogram. Organic thiols are readily oxidized at +0.7 V on a glassy carbon electrode causing rapid poisoning and deterioration. Conversely, the excess of a thiol moiety towards OPA and amino acids is needed for a quantitative reaction. One compromising solution can be inactivation of the unreacted thiol in the second derivatization step with a late eluting amine or with a thiol-scavenging compound. Fig. 5a and Fig. 5b show the separation of Asp and Glu in a standard amino acid mixture (AA-S-18, 4.5 pmol injected) as their OPA derivatives (a) without and (b) with a second derivatizaton step with 0.2 *M* iodoacetamide. As seen, a full separation of the Asp and Glu peaks without any loss in response signal was achieved with the thiol-scavenger. A dramatic suppression of the frontal peak and a better baseline are also notable. However, background noise is still relatively high and caused an increase in the limit of detection (defined as signal/noise ratio of 2) to about 0.4 pmol for both amino acids. A chromatogram of 10 μl Ringer solution derivatized by 2 reagents is also depicted (Fig. 5c). A massive peak at the end of each chromatogram represents the detector's response to the acetonitrile flush during the one-step gradient, as described for System III. As seen, after just one 30 s flush with 90% ACN, it took about 5 min for the electrochemical detector to stabilize back to the previous baseline level. This time probably could be shortened by pumping the buffer at higher flow-rates during the reequilibration period.

3.3. Fluorescence vs. electrochemical detection

From the technical point of view there is no difference between precolumn derivatization with one or with two reagents, since completely automated instrumentation was used. The second reagent step prolongs derivatization time by approximately 2 min, giving a final time of about 5 min for the sample preparation. This procedure can be performed during the reequilibration of the column and the EC detector. FL detection, in contrast, does not require a second reagent and the detector is insensitive to the

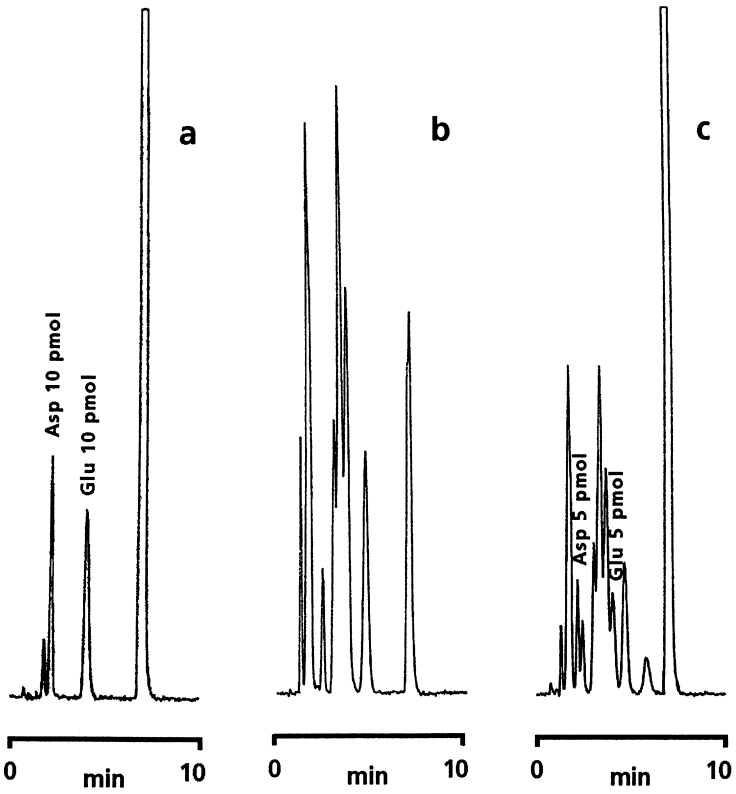

Fig. 4. Chromatograms of: (a) a standard mixture of amino acids (AN) at concentrations of 10 pmol each in 10 μl, derivatized with 4 μl OPA-MCE and 4 μl iodoacetamide reagents, 16 μl was injected into System III/Kratos detector; (b) a 10 μl sample of *S*-amino acids and small peptides (described in the text); (c) a 5 μl amino acid standard as in (a) spiked with 5 μl *S*-amino acid standard as in (b). Asp and Glu peaks are sufficiently separated.

buffer switching; thus, the derivatization can be completed within 2 min.

The backflushing technique (System II) can further reduce the run-to-run time to less than half of the time needed for EC detection. The limit of detection is at least five times lower for the FL detector than for the EC detector using two-reagent derivatization. At a given concentration of the OPA-MCE reagent, the linear calibration range was 4 orders of magnitude for both detectors. However, the EC detector give a sharper calibration curve slope: 0.95 for Asp and 0.8 for Glu compared to 0.48 and 0.45, respectively, on the FL detector. EC detection is less reliable in terms of reproducibility in a long run where calibrations are not made regularly every 2–3 h. However, this problem can be overcome if the LCEC system is recalibrated after each ten injections. In this case, the relative standard deviation, expressed as the average of four groups each consisting of six standard injections between calibrations, was nearly the same for both detectors: ±2.74% for Asp and ±2.83% for Glu detected by EC detector. For FL detection, the corresponding values were ±2.35% and ±2.71%, respectively. The correlation coefficients for EC vs. FL detectors were 0.918 (Asp) and 0.988 (Glu) for 65 microdialysis samples, as discussed later.

In conclusion, any dramatic differences between FL and EC detectors were observed particularly in applications for microdialysis samples, although the EC detector generally has a more laborious and variable operational performance. On the other hand, the isocratic LCEC systems are one of the most widespread instruments in neurobiological laboratories. They can easily be adapted for the analysis of the main neurotransmitters recovered during in vivo

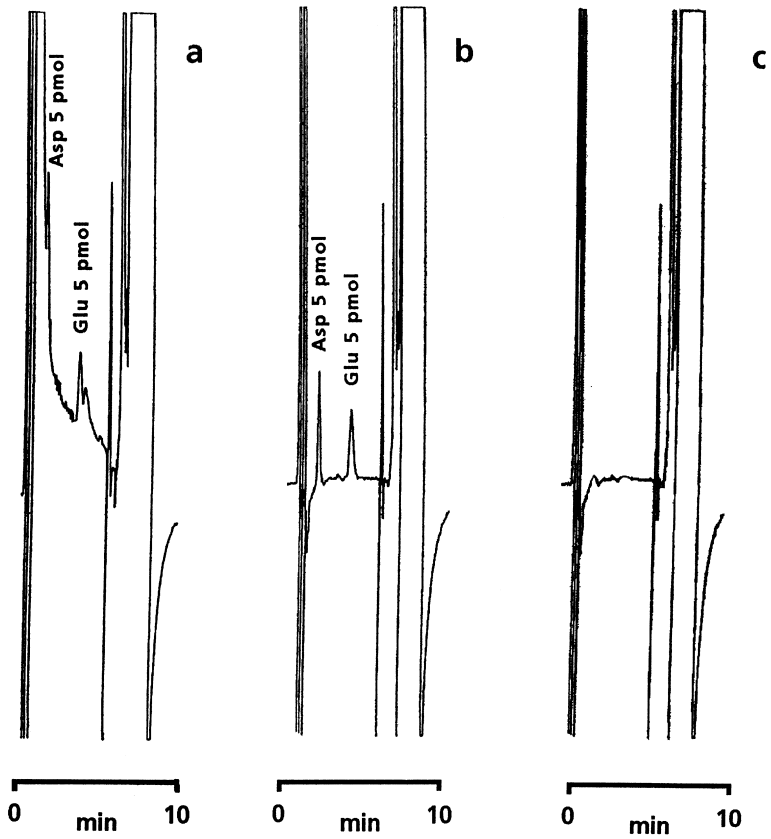

Fig. 5. Chromatograms of a standard mixture of amino acids (AA-S-18), at concentrations of 5 pmol each in 10 μl, derivatized: (a) without a thiol-scavenger by adding 4 μl OPA-MCE and 4 μl 0.1 *M* borate; or (b) with a scavenger by adding 4 μl OPA-MCE and 4 μl iodoacetamide solution; (c) a blank sample (Ringer solution). System III with an electrochemical detector was used.

microdialysis: catecholamines, serotonin, acetylcholine, GABA, Asp and Glu.

3.4. Microdialysis/HPLC on-line

An interesting alternative to 'off-line' microdialysis experimental protocols is to connect the outlet of the microdialysis probe directly to the analytical instrument (see Fig. 1d). Recently, a number of reports have been published describing microdialysis linked on-line to biosensor devices for measuring glucose [20,21], lactate [22], glutamate [23] and other metabolic molecules, indicating an increasing importance of this new technology. However, thus far, no more than one analyte can be detected by these devices. For simultaneous determinations of several compounds or for those where a biosensor approach is not feasible, there is still a need for chromatographic or other separation techniques. In practice, this means separating the perfusate flow into fractions with the result that speed and sensitivity of the analysis are the limiting factors. The fractions are loaded directly into the loop of a high-pressure valve and injected onto the chromatographic column, as originally described for on-line determinations of dopamine and metabolites [24].

For Asp and Glu analyses, the situation is further complicated by the necessity of precolumn derivatization and the instability of the reagent and derivatives at room temperature. The dependence of the fluorescence yield expressed in peak heights for 5 pmol Asp and Glu samples, derivatized either with a laboratory prepared OPA-MCE reagent or with a ready-made (Sigma complete) reagent for 6 h, is

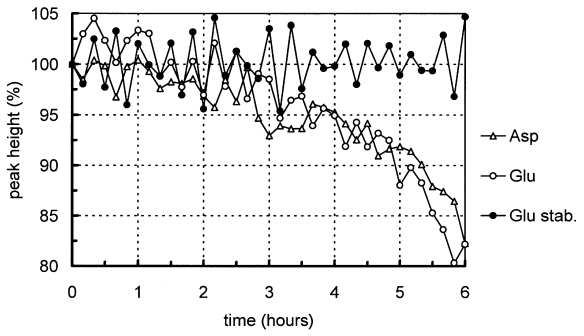

Fig. 6. Stability of Asp and Glu derivatized on-line by laboratory-prepared OPA-MCE reagent (curves for Asp and Glu) and by a reagent stabilized with Brij-35 (Glu stab.) over a period of 6 h.

depicted in Fig. 6. The commercially available reagent is stabilized with a non-ionic detergent (Brij-35) which prolongs its stability as reported earlier [25]. As can be seen, for an unstabilized reagent, the peak heights for both amino acids differed by no more than ±5% of the initial peak heights during the first 4 h. After this period the peaks continued to decrease successively, reaching about 80% of the initial levels after 6 h. On the contrary, the reagent containing Brij-35 was completely stable under these conditions as illustrated for glutamate (Glu stab.) levels. Both reagents were stored in a syringe at laboratory temperature. The on-line method is recommended as a simpler and cheaper alternative for

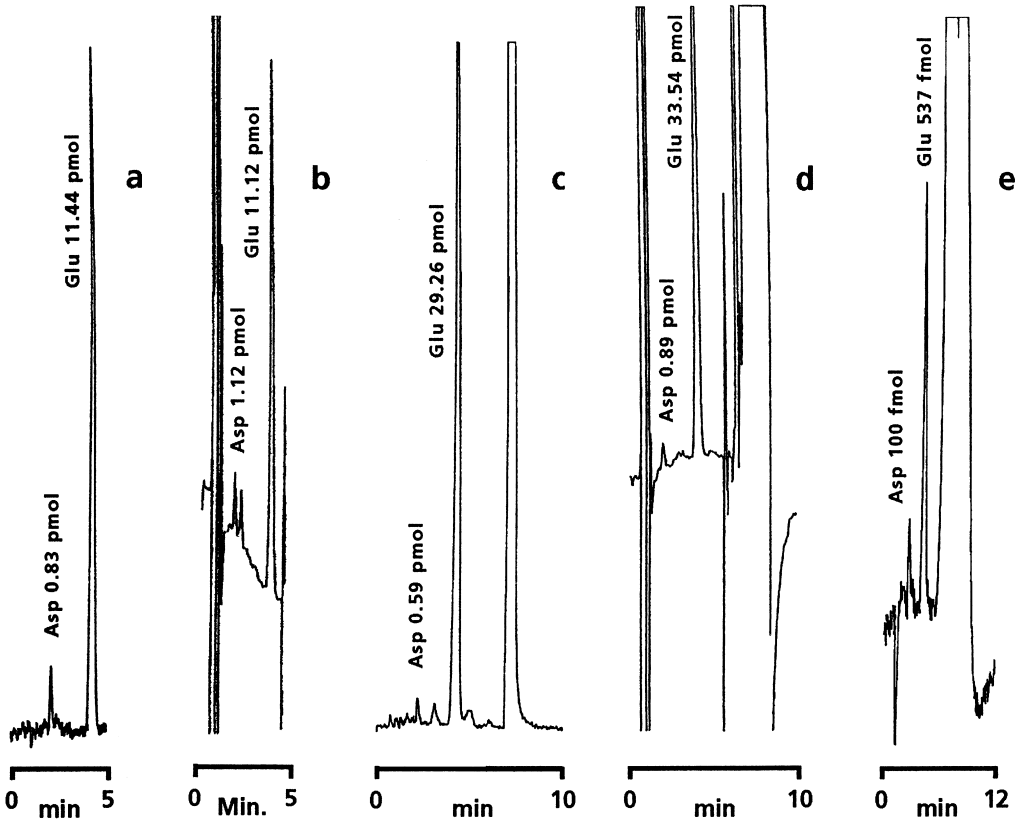

Fig. 7. Chromatograms of microdialysis samples detected simultaneously by FL and EC detectors in System III: (a) FL detector, 10 μl sample from striatum of a conscious rat, derivatization as in Fig. 4a; (b) EC detector, same sample as in (a); (c) FL detector, 10 μl sample from an acute experiment and possibly still damaged tissue as reflected by high Glu concentration; (d) EC detector, same sample as in (c); (e) a typical chromatogram of only 2 μl microdialysis sample from rat striatum determined on microbore System IV with fluorescence (CMA/280) detector.

amino acid derivatizations in microdialysis, offering still better reproducibility and easier manipulation than manual OPA derivatization.

3.5. Microdialysis samples

Chromatograms of microdialysis samples collected from rat brain are shown in the figures corresponding to the chromatographic systems III and IV. In System III, both EC and FL detectors were connected in series allowing simultaneous analysis of the same samples. For experiments on conscious animals, a guide cannula was first implanted into the rat lateral striatum under halothane anaesthesia. Following 24 h recovery, a CMA/12 probe with a 2 mm membrane length was inserted into the guide cannula. The probe was perfused with Ringer solution at 2 μl/min.

2.5 h after probe implantation, the baseline concentrations of Glu and Asp were 11.44 and 0.83 pmol/10 μl, respectively, for FL (Fig. 7a), and 11.12 and 1.12 pmol/10 μl, respectively, for EC detection (Fig. 7b). This Glu/Asp ratio is in agreement with published data of ratio ranges from 1.25 [10] to 10.5 [9]. The correlation between fluorescence and electrochemical detectors for Asp and Glu analyses was further evaluated for 65 microdialysis samples (Fig. 8a,b) at basal and stimulated conditions as described elsewhere [26]. The correlation coefficients were 0.918 for Asp and 0.988 for Glu. The EC detector overestimated Asp and Glu concentrations in some five cases which additionally indicates the greater lability of this method compared to fluorescence detection.

Under some circumstances, abnormally high Glu levels are observed, as shown in Fig. 7c,d. Glu concentration was about 50 times higher than Asp. Some additional extra peaks eluting between Asp and Glu could also be detected. One possible explanation might be severe mechanical damage to the brain tissue during probe implantation which could cause constant leakage of Glu from intracellular stores and from the blood. This phenomenon is observed particularly in acute preparations and when using probes with a high recovery. This is the case in the experiment illustrated in the figure where a CMA/12 probe with a 4 mm membrane was used.

There is always a risk of external contamination, from improper handling of probes, perfusion instruments, pipettes and media, as discussed above. The latter is even more important for microbore chromatography and derivatization of minute samples. The chromatogram in Fig. 7e shows a typical microdialysis sample collected from rat striatum during 1 min intervals. A CMA/12 microdialysis probe

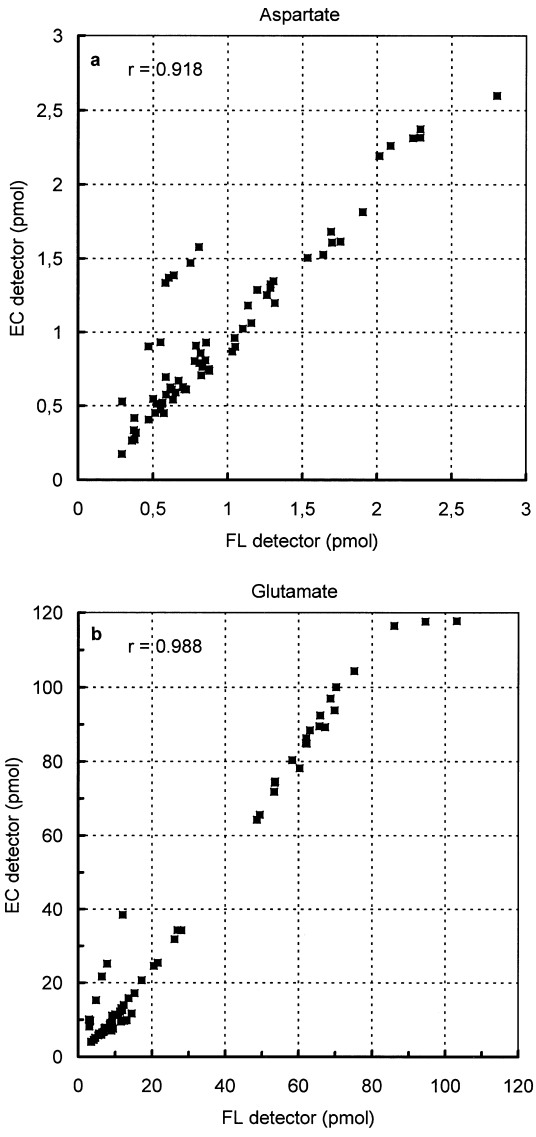

Fig. 8. Correlation between fluorescence (FL) and electrochemical (EC) detectors for: (a) Asp; and (b) Glu. Detectors were connected in series for simultaneous analysis of 65 microdialysis samples from rat brain.

with a 2 mm membrane was perfused at 2 μl/min with a Ringer solution. Here, the concentrations of both Asp and Glu are in the fmol range, i.e. at levels where most analysers would almost always detect some background contaminants interfering with the assay.

Finally, in order to investigate neuronally released Glu and Asp, it is essential to attain the highest reproducibility of both microdialysis sampling and HPLC analysis. The issues related to selective discrimination between neuronal and glial portions of Glu overflow have been studied using various pharmacological manipulations [27,28] but still remain an open question.

Acknowledgements

The author thanks Dr. M. Sandberg, University of Göteborg, Sweden, for the kind gift of a sample of *S*-amino acids and small peptides.

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