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Enhanced temporal resolution for the microdialysis monitoring of catecholamines and excitatory amino acids using capillary electrophoresis with laser-induced fluorescence detection Analytical developments and in vitro validations

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Abstract

This paper reports the development of a method based on capillary electrophoresis with laser-induced fluorescence detection for the simultaneous determination of catecholamines and excitatory amino acids on submicroliter microdialysis samples, with short analysis times (3 min or less), high sensitivity (nanomolar range, i.e., attomoles detected) and high separation efficiency (up to $1 \cdot 10^6$ theoretical plates). A continuous flow derivatization of small volumes of microdialysate (500 nl) using naphthalene-2,3-dicarboxaldehyde as derivatizing reagent is described. Thereafter, two subsequent off-line analyses are performed on each of the 30-s dialysates to determine catecholamines and amino acids. The performances of the present method are demonstrated in vitro by monitoring rapid fluctuations in the concentration of catecholamines and amino acids in the external microdialysis medium.

Keywords: Microdialysis; Derivatization, electrophoresis; Laser-induced fluoresence detection; Catecholamines; Amino acids; Glutamate; Aspartate; Naphthalene-2,3-dicarboxaldehyde; Dopamine; Noradrenaline

1. Introduction

Microdialysis has been shown to be a powerful tool to investigate many neurochemical changes occurring in vivo [1]. Because a given brain area does not contain only one, but several neurotransmitters, many of them are likely to trigger the neurobiological functions elicited by this given brain area. In order to determine the respective role of each neurotransmitter in the phenomena observed, it is important to perform a simultaneous monitoring of these compounds. Although microdialysis collects a representative sample of the extracellular fluid, the traditional approaches to microdialysis sample analysis often point out the monitoring of one (or one class) of neurotransmitter. Methods such as HPLC with electrochemical [2,3] or fluorometric [3,4] detection or such as radio–enzymatic [5] or radio–immuno assay [6] need a rather large volume of sample while being not universal enough to allow a sensitive and specific quantification of an important number of interesting compounds within a single

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analysis. As a consequence, the simultaneous determination of several compounds like catecholamines and amino acids in a single microdialysis fraction needs classically the combination of collection times of 20–30 min with perfusate flow-rates of 2–3 μl/min [7–10]. Because of the relatively long sample intervals required with these traditional analytical approaches, partial loss in temporal pattern may occur when rapid fluctuations are taking place.

To minimize the sample volume required and to increase the temporal resolution of microdialysis, capillary electrophoresis (CE) as been explored as an alternative to HPLC for analysis of dialysates [11–23]. Coupled with laser-induced fluorescence detection (LIFD), this technique requires nanoliter volumes (or less) of sample for injection while offering high sensitivity, high separation efficiency and rapid separations. Furthermore, since a derivatization reaction with a fluorescent tag is needed to detect the molecules of interest and since most of the fluorescent reagents used react with primary amines, a large number of the neurochemical constituents of brain microdialysis samples could be potentially detected. Moreover, as microdialysis samples are protein-free, it is possible to couple microdialysis with on-line derivatization systems in order to perform a continuous derivatization of very small volume of microdialysate without loss af sample [22]. As compared with other analytical methods, CE–LIFD with an on-line derivatization system allows an enhancement in mass detection limits as well as the simultaneous determination of many neurotransmitters in a single microdialysate. Furthermore, the need of very small volume of dialysate makes possible short collection times at low flow-rate, thereby leading to an increase in microdialysis temporal resolution and to a better respect of the neuronal environment [24].

This paper reports the simultaneous determination of dopamine (DA), noradrenaline (NA), glutamate (Glu) and aspartate (Asp) in 30-s microdialysis samples. By continuously derivatizating the microdialysates with naphthalene-2,3-dicarboxaldehyde (NDA), it is possible to monitor the levels of these neurotransmitters in the same 500-nl microdialysate using two successive off-line analyses. Because of the increase in the sampling rate and of the very low sample volume collected, we will thus describe: (1) rapid, very sensitive and specific determinations of catecholamines and excitatory amino acids, (2) the development of a continuous flow derivatization (on-line derivatization) for a routine derivatization of small volumes of microdialysate, (3) the evaluation of the linearity and reproducibility of the entire system and (4) the ability of the system to follow rapid changes in the sampling medium.

2. Experimental

2.1. Reagents

NDA and sodium cyanide were purchased from Fluka (Buchs, Switzerland). NA, DA, dihydroxybenzylamine (DHBA), Glu, Asp, α-aminoadipic acid (AAd), boric acid, sodium tetraborate were obtained from Sigma (St. Louis, MO, USA), monobasic and dibasic sodium phosphate from Merck (Darmstadt, Germany). The water used throughout the experiment was of HPLC-grade and obtained with a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were of the highest purity available.

2.2. Solutions

2.2.1. Buffers

Sodium borate buffer (500 m*M*, pH 8.7 used as derivatization buffer) was obtained by mixing separate solutions of boric acid (500 m*M*) and sodium tetraborate (125 m*M*). Sodium borate buffer (100 m*M*, pH 9.2 used as amino acid separation buffer) was prepared by mixing boric acid (100 m*M*) with sodium hydroxyde (10 *M*). Sodium phosphate buffers (110 and 200 m*M*, pH 7.05±0.02 used as catecholamine separation buffers) were obtained by preparing separate monobasic and dibasic sodium phosphate and mixing them to obtain the appropriate pH. The buffer solutions were filtered through a 0.45-μm pore size membrane filter (cellulose nitrate, Sartorius, Goettingen, Germany) and sonicated 10 min before use.

The 2.5 m*M* and 5 m*M* NDA solutions were prepared in methanol weekly and stored at 4°C.

For the on-line derivatization, the sodium cyanide solution was prepared by mixing (40:100, v/v) sodium cyanide solution in water (43 m*M*) with

sodium borate buffer (500 m*M*, pH 8.7). For the manual derivatization, the sodium cyanide solution was prepared by mixing (50:100, v/v) sodium cyanide solution in water (22.5 m*M*) with sodium borate buffer (500 m*M*, pH 8.7). The mixtures were prepared daily and stored at 4°C.

2.2.2. Amines and amino acids

The 1 m*M* stock solutions of NA, DA, DHBA, Glu, Asp and AAd were prepared in 0.1 *M* perchloric acid and stored at −20°C as aliquots of 1 ml. The required working solutions were obtained by further dilution with artificial cerebrospinal fluid (aCSF) (endogenous amines) or 0.1 *M* perchloric acid (internal standards).

2.3. Capillary electrophoresis system

The CE system with LIFD (IRIS 2000, Europhor Instruments S.A., now Zeta Technology S.A., Toulouse, France) is directly derived from the instrument described by Hernandez and co-workers [25]. In this respect, a collinear scheme was used for fluorescence detector. The excitation was performed by a Liconix (Santa Clara, CA, USA) helium–cadmium laser (10 mW) at a wavelength of 442 nm. The emission intensity was measured at a wavelength of 490 nm filtered by a band pass filter and a notch filter was used to attenuate background radiations. Fluorescence was detected by a photomultiplier tube (PMT), and transformed in electrical current. The relative fluorescence was then expressed in micro- or nanoamperes.

Separations were carried out with fused-silica capillaries (Polymicro Technology, Phoenix, AZ, USA) of 50 or 25 μm inner diameter (I.D.) and 375 μm outer diameter (O.D.) having a total length of 43 cm and an effective length of 23 cm. On-column LIFD was carried out through a 5-mm wide window opened by removing the polyimide cover of the capillary. Capillaries were sequentially flushed (3× 10 min) with 1 *M*, 0.1 *M* sodium hydroxyde and water prior to use. Before each electrophoretic run, the capillary was flushed with the appropriate separation buffer (1–2 min). Hydrodynamic injections were made by applying vacuum [175 and 300 mmHg (i.e., $23.3 \cdot 10^6$ and $39.9 \cdot 10^6$ Pa, respectively) for the 50 and 25 μm I.D. capillaries, respectively] at the detection end of the capillary for a fixed period of time (see separation procedures): the injection volumes were calculated according to the Hagen–Poiseulle formula: $6.86 \cdot 10^{-7} \cdot P \times (R^4/L) \cdot t$, where P (mmHg) is the applied vacuum, R (μm) the inner radius, L (cm) the total length of the capillary and t (s) the fixed period of time [26]. The capillary was maintained at 25.5°C through a Peltier effect. The instrument was controlled and data were acquired (200 points/s) with original MacIntosh software (Inforep S.A.R.L., Fontenay-le-Fleury, France) developed from LabVIEW 2 software (National Instruments Corp., Austin, TX, USA). Each electropherogram was filtered with a 10-Hz low-pass Bessel filter.

2.4. Separation procedures

Four different separation procedures were used, two for catecholamines and two for amino acids analysis. In all cases, samples were first analysed with catecholamines separation procedure and then amino acids analysis was performed. Unless otherwise specified, sample stacking procedures were used for sample injection.

2.4.1. Catecholamines

When the 25 μm I.D. capillary was used, a 200 m*M* phosphate run buffer with an applied voltage of 29 kV was employed. The sample stacking procedure consisted in a 2.5-nl (i.e., 20 s) injection of sample followed by a 60-pl (i.e., 0.5 s) injection of orthophosphoric acid (200 m*M*). With the 50 μm I.D. capillary, a 110 m*M* phosphate run buffer with an applied voltage of 12 kV was used. The sample stacking procedure consisted in a 1.1-nl (i.e., 1 s) sample injection followed by a 1.1-nl (i.e., 1 s) injection of orthophosphoric acid (10 m*M*).

2.4.2. Amino acids

The CE run buffer was 100 m*M* borate at pH 9.2 with an applied voltage of 25 kV. With the 25 μm I.D. capillary, the sample stacking procedure consisted in a 100-pl (i.e., 0.8 s) injection of sample followed by a 100-pl (i.e., 0.8 s) of orthophosphoric acid (10 m*M*) injection. With the 50 μm I.D. capillary, the sample stacking procedure consisted in a 550-pl (i.e., 0.5 s) sample injection followed by a

550-pl (i.e., 0.5 s) injection of orthophosphoric acid (20 m*M*).

2.5. Derivatization procedures

2.5.1. Manual derivatization

The derivatization conditions used were adapted from those previously described [21]. In this case, 80 μl of 0.1 *M* perchloric acid solution, 240 μl of sodium cyanide solution in borate buffer and 40 μl of 5 m*M* NDA solution were added to 720 μl of sample in aCSF. This protocole of derivatization was only used for the optimization of the catecholamine and amino acid separations on the 25 μm I.D. capillary.

2.5.2. Continuous flow derivatization

A diagram of the entire microdialysis system with on-line derivatization is shown on Fig. 1. The probe was perfused with a 500-μl Hamilton syringe mounted on a Harvard Model 22 syringe pump (syringe pump 1) containing aCSF. The connections between this syringe and the inlet of the microdialysis probe and from the outlet of the probe to the on-line derivatization system were performed by using fused-silica capillaries (75 μm I.D.×375 μm O.D., Polymicro Technology, Phoenix, AZ, USA). Using another Harvard Model 22 syringe pump (syringe pump 2), the dialysates were first mixed with 0.1 *M* perchloric acid containing internal standards (400 n*M* DHBA and 8 μ*M* AAd), then with the sodium cyanide solution in borate buffer and finally with the NDA solution. The mixing chambers (reactors) consisted of polyethylene tubing (1 cm× 300 μm I.D.×700 μm O.D.) (PE 10) and the connections between the reactors were made from fused-silica capillaries (1 cm×75 μm I.D.×375 μm O.D., Polymicro Technology). The tranfer lines from the three syringes on pump 2 to the corresponding mixing chambers consisted of 30 cm of 75 μm I.D.×145 μm O.D. fused-silica capillaries. The use of such a small O.D. allowed the introduction of this capillary in the PE 10 reactor (by making a small hole in one wall of the PE 10 tubing and by passing the capillary tubing through the hole) and then, the sealing of this connection with epoxy glue. No glue was employed for the 375 μm O.D. capillary-PE 10 connections. The calculation of the concentrations and perfusion rates of the derivatization reagents were based on the derivatization procedure previously described [21]. The internal standard solution in 0.1 *M* perchloric acid and the 2.5 m*M* NDA solution were perfused with 50-μl Hamilton syringes whereas the cyanide/borate solution was perfused with a 100-μl Hamilton syringe. Based on the 100-μl syringe, the perfusion rate of the derivatization reagents (syringe pump 2) was determined to be 4-fold slower (i.e., 8-fold slower for the 50-μl syringes) than the perfusion rate of the syringe on pump 1. Thus, the respective perfusion rates for syringe on pump 1, 50-μl syringes on pump 2 and 100-μl syringe on pump 2 were 1 μl/min, 15 μl/h and 7.5 μl/h. The outlet of the continuous flow derivatization system was inserted in a 50-μl glass microvial (Alltech, Templeuve, France), and 30-s fractions were collected and after 30 s reaction time, stored at −20°C in hermetically closed Eppendorf tubes. Prior to collection, equilibration of the derivatization system was attempted for 30 min. Derivatized samples were analysed on the same day for catecholamines. They were then stored at −20°C until the analysis of the amino acids which was performed one or two days later. The determination of amino acids (using a 100 m*M* borate separation

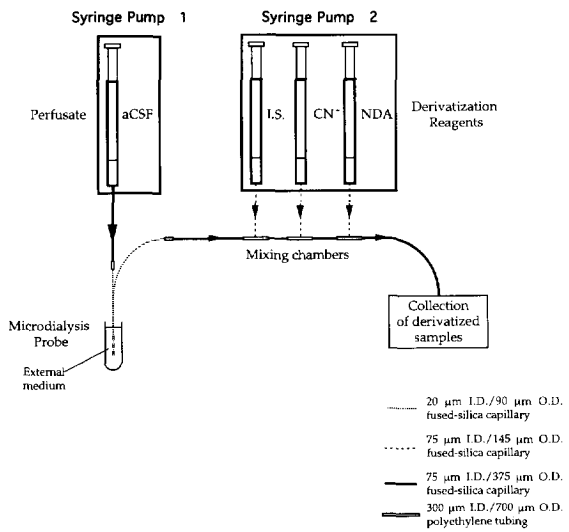

Fig. 1. Schematic representation of the on-line microdialysis derivatization system. Syringes on pump 2 contain internal standards (called I.S.), sodium cyanide solution in borate buffer (called CN^-) and NDA solution (called NDA).

buffer) was performed after the determination of catecholamines in order to avoid any contamination since the borate concentrations in the derivatization and separation media have been shown to be critical for the separation of catecholamines [21].

2.5.3. Reproducibility

The stability and reliability of the system was evaluated by collecting successive samples at different flow-rates and different collection times. The catecholamines and amino acids were derivatized at 10 n*M* and 1 μ*M* concentrations, respectively. With a perfusate flow-rate of 1 μl/min, the reproducibility was tested for 0.5-, 1- and 2-min collection times. With perfusate flow-rates of 0.25 and 0.5 μl/min, the reproducibility was tested for 2- and 1-min collection times, respectively.

2.6. Microdialysis system

2.6.1. Preparation of the dialysis probes

Concentric microdialysis probes with an active dialysis length of 4 mm were constructed from regenerated cellulose dialysis tubing (Spectra/Por hollow fiber; molecular mass cutoff 6000, 225 μm O.D.; Spectrum Medical Industries, Los Angeles, CA, USA) according to the model described by Abercrombie and Finlay [2] with modifications: fused-silica capillary tubing (20 μm I.D.×90 μm O.D., Polymicro Technology) was used as inlet and outlet of the probes instead of polyethylene tubing (PE 10) and fused-silica capillary tubing (75 μm I.D.×145 μm O.D.), respectively. This modification allows to reduce the dead volume, by the use of a smaller I.D. outlet capillary.

2.6.2. Probe and system calibration

In order to be able to estimate the derivatizated concentrations of neurotransmitters, it was necessary to determine the recovery of the microdialysis probes. This was accomplished by placing and perfusing the probe until steady state with an aCSF solution containing catecholamines (50 n*M*) and amino acids (5 μ*M*). Then the probe was placed in a free aCSF solution. This procedure allows the determination of the dead volume and of the in vitro recovery of each probe. The travel time required for the sample to move from the dialysis cannula to the outlet of the on-line derivatization system was estimated to be 1.9±0.1 min (n=4, i.e., a volume of 1.9 μl). At room temperature and using 1 μl/min as perfusate flow-rate, the in vitro recoveries of a 4-mm dialysis probe were 45±9.5, 34±7, 33±7 and 40±7% for DA, NA, Glu and Asp, respectively (n=4).

The linearity of the system was tested by perfusing the probe at 1 μl/min with aCSF while placing the probe in different catecholamines and amino acids standard solutions (concentrations ranging from 1 n*M* to 400 n*M* for NA, from 4 n*M* to 400 n*M* for DA and from 0.4 μ*M* to 40 μ*M* for amino acids).

2.6.3. In vitro response

The ability of the system to follow discrete changes in the sampling medium was examined. Step changes in external concentration were created by removing the probe from a solution containing 10 n*M* catecholamine and 1 μ*M* amino acid and placing it for 1- or 5-s into a solution containing 1 μ*M* catecholamines, 100 μ*M* Glu and 1 μ*M* Asp (i.e., no change in Asp concentration). The probe was then replaced in the solution of lower concentrations.

2.7. Expression of results

The separation efficiency was estimated by calculating the theoretical plate using the equation $N = 5.54(t/W_h)^2$, where t is the migration time and W_h the width at half maximum of the peak. Resolution between two sample components can be defined by the equation $R = 2(t_2 - t_1)/(w_1 + w_2)$ [27], where t_1 and t_2 are the migration times of the two components and w_1 and w_2 their respective baseline peak widths.

3. Results and discussion

CE–LIFD has already been used to determine neurotransmitters Glu, Asp or NA in microdialysis samples [15–18,21,22]. If the advantages of this technique were well described, they were not fully applied. The purpose of this study was to demonstrate that CE–LIFD allows (1) an improvement in microdialysis temporal resolution, and (2) the simultaneous monitoring of various neurotransmitters. In this respect, NA, DA, Glu and Asp con-

centrations were simultaneously determined in microdialysate samples with high sampling rate (30-s fractions).

3.1. CE–LIFD with manual derivatization

3.1.1. Optimization of the conditions of catecholamines separation

In a previous work, we reported a 15-min analysis time for the determination of catecholamines using a 50 μm I.D. capillary with a total length of 72 cm (effective length of 52.5 cm) and an applied voltage of 15 kV [21]. In order to decrease this time while using similar phosphate separation buffer, we reduced the I.D. of the capillary to 25 μm, the total and effective lengths of the capillary to 43 and 23 cm respectively and we increased the applied voltage to 29 kV. Fig. 2A shows that, using these new conditions, the migration times of catecholamines (DA, NA and DHBA) were 10-fold lower than those reported in the previous study, being down to ~80 s.

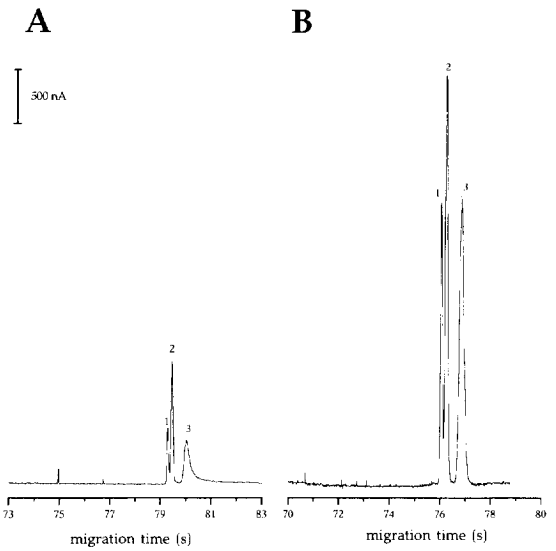

Fig. 2. Separation of 100 n*M* DA–CBI (1), NA–CBI (2) and DHBA–CBI (3) using (A) a simple hydrodynamic injection of sample (0.75 nl injected) and (B) a sample stacking procedure: the injection of 2.5 nl of sample followed by 60 pl of H_3PO_4 (200 m*M*). Separation conditions: 200 m*M* phosphate buffer, pH 7.02, as separation buffer; separation current of ~90 μA; capillary, 43 cm (effective length, 23 cm)×25 μm I.D.; applied voltage, 29kV; PMT applied voltage, 570 V. Note that the sample stacking system allows to increase the sample load and thus mass sensitivity.

Furthermore, the high separation efficiency previously reported [21] [$1.6 \cdot 10^6$ of theoretical plates for DA–cyano[*f*]benzoisoindol (CBI) derivative] was kept in the present study to reach $1.6 \cdot 10^6$ and $0.9 \cdot 10^6$ for DA– and NA–CBI, respectively (corresponding to an average baseline peak width of 0.1–0.2 s for an average migration time of 78–80 s).

3.1.2. Detection limits for catecholamines analysis

Sensitivity in CE is limited by the injection volume in the capillary which must not exceed 1% of the effective column volume [27]. For this reason, the lower size of the capillary used in the present conditions made necessary a reduction of the maximal volume to be injected and thus lead to a decrease in the sensitivity. Indeed, when compared to our previous study [21], the fluorescence signal was decreased, the maximal injection volume being only 0.75 nl (i.e., 0.6% of the effective column volume) instead of 2.5 nl in the previous conditions. With a larger volume of injection, catecholamines–CBI were no longer separated (data not shown). In order to keep a good sensitivity required for the detection of catecholamines in microdialysis samples, a sample concentration strategy was considered: we used a sample stacking with discontinuous buffer system in order to increase the sample load [28]. In our case, a zone of low conductivity was formed by injecting ortho-phosphoric acid (H_3PO_4 200 m*M*, 60 pl) after the sample. This sample stacking system improved the efficiency of the separation to $4.3 \cdot 10^6$ and $3.0 \cdot 10^6$ theoretical plates for DA– and NA–CBI, respectively and allowed to increase the sample load in the capillary above the 1% limit, since 2.5 nl of sample (i.e., 2.2% of the effective column volume) can be injected in these conditions (Fig. 2B).

Under these conditions, 720 μl of DA and NA solution derivatized at a concentration of 0.5 n*M* (final concentration after derivatization: 0.33 n*M*) produced peaks with signal-to-noise ratios of 6:1 and 11:1, respectively, with no interference from the blank (Fig. 3). The estimated injected volume being 2.5 nl, the corresponding detected amount was 0.836 amol (i.e., 836 zmol) for both catecholamines–CBI. Extrapolations based on the fluorescent signal from the electropherogram at the 0.5 n*M* level showed that the detection limits ($S/N=3$) for DA and NA were 0.25 n*M* (i.e., 413 zmol detected) and 0.14 n*M* (i.e.,

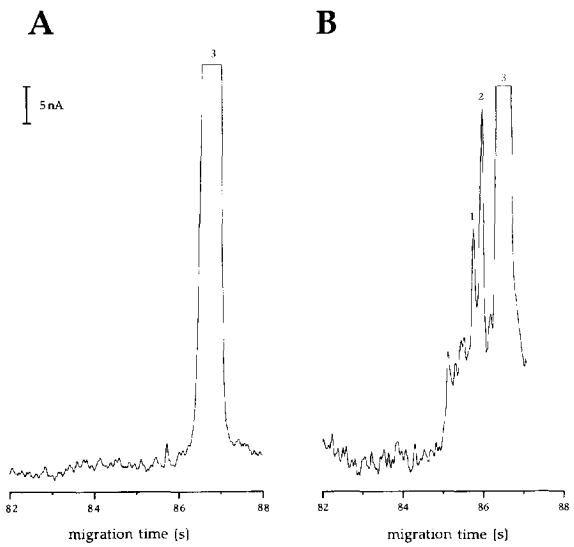

Fig. 3. Electropherogams of (A) 7.4 n*M* DHBA–CBI (3) and (B) 0.33 n*M* DA–CBI (1), NA–CBI (2) and 7.4 n*M* DHBA–CBI (3) corresponding to 836 zmol detected for DA and NA, injected using a sample stacking procedure. Separation conditions: see text and Fig. 2. Note that the signal-to-noise ratios were 11:1 and 6:1 for 0.33 n*M* NA–CBI and DA–CBI, respectively.

225 zmol detected), respectively. The present concentration detection limits for catecholamines are in the range of those we already obtained [21,29], and are lower than those previously reported by other authors using CE with LIFD [30,31] or electrochemical detection [32–34]. Moreover, the present CE–LIFD method has a limit of detection similar to the best values reported for the conventional analytical techniques used for the analysis of catecholamines in microdialysis samples, i.e., HPLC with electrochemical detection (0.15–0.3 n*M*) [2,35,36], HPLC with fluorimetric detection (0.5 n*M*) [4] or radio-enzymatic assay (0.1 n*M*) [5].

3.1.3. Optimization of amino acids separation conditions

The CE–LIFD analysis of Glu and Asp has previously been reported using NDA as a fluorescent reagent [17,22,37]. Since, NDA reacts with primary amines of amino acids [38], the catecholamines derivatization procedure was used in order to determine also catecholamines together with excitatory amino acids in a single sample. In the present work, the separation buffer described by Ueda and coworkers [37] (borate buffer 100 m*M*, pH 9) was selected. Fig. 4A shows a typical electropherogram obtained in these conditions: 77 n*M* of Glu-, Asp- and AAd–CBI (internal standard) were separated from each other with a short run time (2.5 min). The use of a sample stacking system (H_3PO_4 10 m*M*, 100 pl) improved the separation of these compounds (Fig. 4B) through (1) a better peak symetry and resolution, (2) an increase in the number of theoretical plates (11- and 2.5-fold for Glu– and Asp–CBI, respectively) reaching $0.25 \cdot 10^6$ for Glu–CBI, (3) an increase in peak height (1.8- and 4.1-fold). Extrapolations based on the fluorescent signal from the electropherogram of the Fig. 4B showed that the detection limits ($S/N=3$) for Glu and Asp was 4.2 n*M* (i.e., 326 zmol detected) and 7.9 n*M* (i.e., 612 zmol detected), respectively. The sensitivity of the determination was sufficient for microdialysis experiments because typical concentrations of Glu and Asp in brain microdialysis samples are in the range of 0.1–10 μ*M* [7,10,18,39].

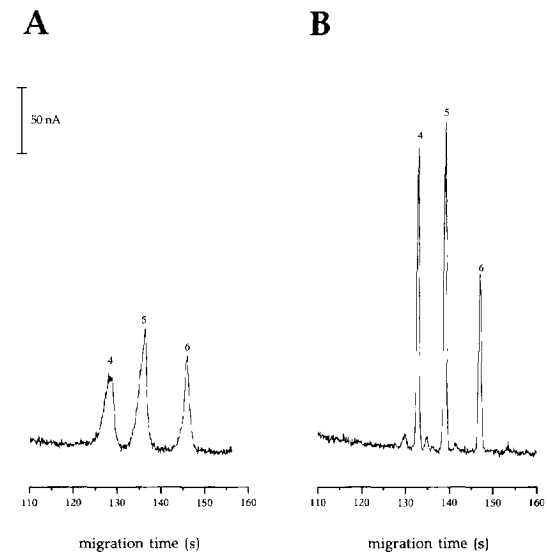

Fig. 4. Electropherograms of 77 n*M* AAd–CBI (4), Glu–CBI (5) and Asp–CBI (6) using (A) a simple hydrodynamic injection of sample (100 pl) and (B) a stacking procedure: the injection of 100 pl of sample followed by of 100 pl H_3PO_4 (10 m*M*). Separation conditions: 100 m*M* borate buffer, pH 9.20, as the separation buffer; separation current of ~16 μA; capillary, 43 cm (effective length 23 cm)×25 μm I.D.; applied voltage, 25kV; PMT applied voltage=570 V. Note that the use of a sample stacking procedure induces an increase in peak heights and a decrease in peak widths.

3.1.4. Development of separation conditions for routine use

These two protocols of analysis allowed the determination of catecholamines and excitatory amino acids in a single sample (1) with a very short analysis time (the total run time for the two analyses was less than 5 min), (2) with detection limits lower than the estimated concentrations in the microdialysis samples, and (3) with very small injection volumes (2.6 nl are sufficient) for the two analyses.

In spite of the high performances obtained with the 25 μm I.D. capillary, they were unfortunately more prone to clogging than larger capillaries and have been found to be too less reliable for a routine use. Catecholamines and excitatory amino acids separations with the sample stacking system described above were thus adapted on a 50 μm I.D. capillary, the other characteristics being identical. Fig. 5A and 5B show typical electropherograms obtained on a 50 μm I.D. capillary. An injection of 1.1 nl of sample followed by 1.1 nl of H_3PO_4 (10 m*M*) was found to be optimal for the separation of catecholamines using a 110 m*M* phosphate buffer as separation buffer and 12 kV as applied voltage. The decrease of the applied voltage and of the ionic strength of the catecholamines separation buffer allowed to minimize Joule heating, separation currents of ~80 μA being then obtained. As a consequence, the migration times of catecholamine were increased (~1.5 min) as compared to those obtained with the 25 μm I.D. capillary. The separation efficiency for catecholamines–CBI was kept high with $2.8 \cdot 10^6$ theoretical plates for DA- and NA–CBI (Fig. 5B). Moreover, we have shown that DA, NA and DHBA were separated not only from each other but also from amino acids and primary amines potentially present in microdialysis samples [21,23] [29].

For the amino acids separation, the optimal stacking system was composed by the injection of 550 pl of sample followed by 550 pl of H_3PO_4 (20 m*M*) using a 100 m*M* borate buffer (pH 9.2) as separation buffer and 25 kV as applied voltage. The separation of amino acids–CBI was performed within 2 min with theoretical plates up to $0.25 \cdot 10^6$ and excellent resolution (Fig. 5C and Fig. 5D).

The 50 μm I.D. capillary was finally chosen for

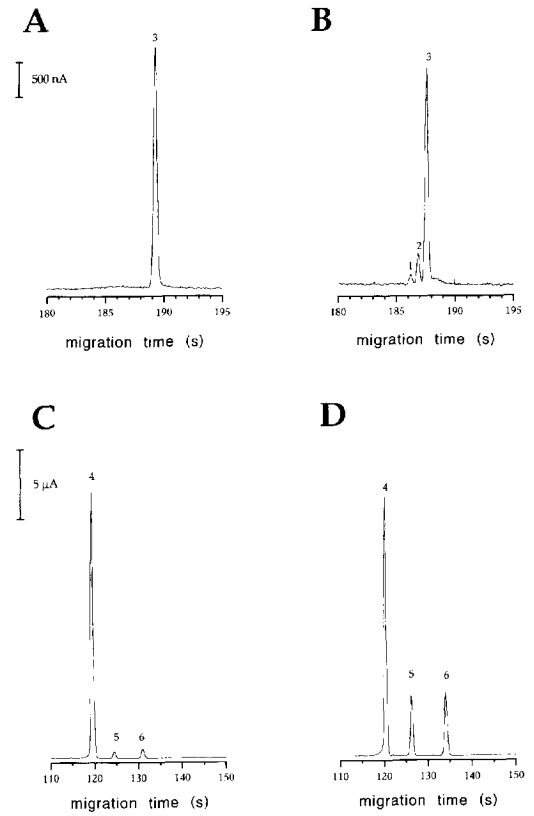

Fig. 5. Electropherograms of two derivatized 30-s fractions obtained from a microdialysis probe placed: (A, C) in aCSF and (B, D) in a standard solution containing DA (1) and NA (2) at 10 n*M*, and Glu (5) and Asp (6) at 1 μ*M*. Each derivatized sample was first analysed in catecholamines separation conditions (A, B) and finally in amino acids separation conditions (C, D). The concentrations before derivatization were estimated with the in vitro recovery of the probe: 3.4 n*M*, 4.5 n*M*, 0.33 μ*M* and 0.4 μ*M* for NA, DA, Glu and Asp, respectively. The concentrations of the internal standards were 12.5 n*M* and 1 μ*M* for DHBA (3) and AAd (4), respectively. Separations conditions: capillary, 43 cm (effective length, 23 cm)× 50 μm I.D.; hydrodynamic injection (depression, 175 mmHg). Catecholamines: 110 m*M* phosphate buffer (pH 7.06) as separation buffer, separation current of ~80 μA; injection of 1.1 nl of sample and then of 1.1 nl of phosphoric acid (10 m*M*); applied voltage, 12 kV; PMT applied voltage, 700 V. Amino acids: 100 m*M* borate buffer (pH 9.2) as separation buffer, separation current of ~80 μA; injection of 545 pl of sample, and then of 545 pl of phosphoric acid (20 m*M*); applied voltage, 25 kV; PMT applied voltage, 550 V. Note the derivatization of low concentrations of catecholamines and excitatory amino acids gives peaks with good signal-to-noise ratios despite the presence of small amounts of Glu- and Asp–CBI as blank contaminants (C).

optimal reliability, short separation times and good separation efficiencies, and used for all subsequent experiments.

3.2. CE–LIFD with on-line derivatization

In order to accurately monitor rapid fluctuations in the concentrations of neurotransmitters in the brain, it is necessary to sample the extracellular fluid at short intervals. One way to achieve a fast sampling rate is to increase the perfusate flow-rate and to collect the same volume of perfusate in a shorter period of time. In this case, artificial concentration gradients within the extracellular fluid become large and may alter neuronal activity [24]. An alternative approach for increasing the sampling rate is to collect lower sample volume. Using perfusate flow-rates of less than 1 μl/min, the sample volumes will be in the submicroliter range for a 1-min sampling rate. As a consequence, a derivatization reaction performed on nanoliter volumes is needed to detect the molecules of interest. As the manual derivatization described above could not be performed with a good reproducibility on sample volumes inferior to 3 μl (data not shown), another derivatization procedure should be considered. Continuous flow derivatization (on-line derivatization) on nanoliter volumes of microdialysates samples has been recently reported by Lunte and co-workers [22] for the determination of Glu and Asp concentrations. This procedure has the interest of continuously adding very small volumes of reagents to the perfusate sample with good reproducibility while preventing evaporation and sample loss. Based on this work, we developed an on-line derivatization system with new mixing chambers and reagents flow-rates, adjusted for our derivatization procedure (Fig. 1). Two further off-line analyses of on-line derivatized microdialysis samples were then performed for the determination of catecholamines and amino acids.

3.2.1. Reproducibility

Interassay reproducibility of the on-line derivatization system was tested by collecting successive derivatized samples at different flow-rates and collection times (Table 1). The relative standard deviations of the peak areas corrected by the appropriate internal standard were similar to those obtained on higher volumes (1 ml) [21] and can be considered as satisfactory for biological studies. The difference of reproducibility between catecholamines and excitatory amino acids analysis can be due to the differences in the concentrations used. Nevertheless, the interassay reproducibility is not decreased by the reduction in the perfusate flow-rates or collection times, the limiting parameter being the total sample volume. In this way, a 500-nl microdialysis sample was found to be the minimum sample volume to be derivatized in the present conditions. Moreover, several injections can be performed on the same microdialysis sample since only 1.1 nl is needed out of a total volume of 750 nl after derivatization: in this case, the intra-assay reproducibility is satisfactory since four successive injections produce a relative standard deviation of only 2.8%. Stored at −20°C, the derivatized samples of 750 nl were stable for at least one week.

Even if the system was found to be reliable at

Table 1
Interassay reproducibility ($n = 10$) of the on-line derivatization procedure at different flow-rates and collection times

Collection time (min)	Perfusate flow-rate (μl/min)	Sample volume (nl)	Relative standard deviation (%) of corrected peak area			
			NA	DA	Glu	Asp
2	1	2000	11.4	n.d.[a]	6.3	9.9
2	0.25	500	10.6	13	6.3	20.5
1	1	1000	13.2	n.d.	4.6	12.9
1	0.5	500	10.8	12	3.5	8.4
0.5	1	500	10.7	16.3	1.1	4.4

The catecholamines were derivatized at 10 n*M* and their peak areas corrected by the area of the internal standard of catecholamines, DHBA (50 n*M*). The excitatory amino acids were derivatized at 1 μ*M* and corrected by the area of the internal standard of amino acids, AAd (1 μ*M*). The sample volume corresponds to the volume of microdialysis sample before derivatization. Separation techniques: see Fig. 5.
[a] n.d.: not determined.

slower perfusate flow-rate, we selected a 1 μl/min perfusate flow-rate for all subsequent experiments in order to have a short collection time (30-s fractions). Such high sampling rates have been previously reported for DA monitoring using microbore HPLC–ED (1-min fractions) [40], for ascorbic and/or lactic acid monitoring using CE with UV detection (injections every 50, 85 s) [19,20], for Glu and Asp monitoring using CE–LIFD (injections every 2-min) [22] and for NA and Glu monitoring using CE–LIFD (2-min fractions) [23]. Since only ~0.2% of the total microdialysate volume was used for the two determinations, subsequent analysis to determine other neurotransmitter concentrations would thus be possible without hampering the microdialysis temporal resolution.

3.2.2. Linearity and detection limits

The linearity was tested in the conditions to be used for in vivo microdialysis experiments: a 4-mm probe was placed in a solution prepared in aCSF with concentrations ranging from 1 n*M* to 400 n*M* for NA, from 4 n*M* to 400 n*M* for DA and from 0.4 μ*M* to 40 μ*M* for excitatory amino acids. Corrected by the in vitro recovery of the probe, the concentrations before derivatization ranged from 0.34 n*M* to 140 n*M* for NA, 1.8 n*M* to 180 n*M* for DA, 130 n*M* to 13 μ*M* for Glu and 160 n*M* to 16 μ*M* for Asp. Linear relationships between the NA–, DA–, Glu– and Asp–CBI concentrations and the peak areas corrected by the appropriate internal standard were obtained with regression coefficients not inferior to 0.997 (Table 2).

The minimum derivatized concentration of both catecholamines (Table 2) corresponding to the detection limits ($S/N=3$) were 0.34 n*M* (i.e., 0.17 fmol derivatized) and 1.8 n*M* (i.e., 0.9 fmol derivatized) for NA and DA, respectively, without interference from the blank (Fig. 5A). The estimated injected volume being 1.1 nl, the corresponding detected amounts were 1.3 amol and 250 zmol for DA–CBI and NA–CBI, respectively (Table 2). The use of such an on-line derivatization system allows the handling of sub-μl volume of sample and femtomole amount of compounds without any loss in sensitivity: detection limits are similar to those obtained using a 25 μm I.D. capillary with derivatization on larger volumes (see above).

For the amino acid analysis, the extrapolations based on the fluorescent signal from the electropherogram presented in Fig. 5D showed that the theoretical detection limits ($S/N=3$) for Glu–CBI and Asp–CBI could be 2.3 n*M* (i.e., 840 zmol detected) and 2.6 n*M* (i.e., 952 zmol), respectively. However, in spite of the great care taken in the preparation of aCSF, reagents and separation buffers, the analysis of blank derivatized samples showed the presence of small amounts of Glu–CBI and Asp–CBI: 57±6 n*M* and 0.10±0.02 μ*M* ($n=6$), respectively (Fig. 5C). These contaminations explain why a

Table 2
Linear relationships between the concentration of NA, DA, Glu and Asp and the peak areas corrected by the corresponding internal standards (DHBA for catecholamines and AAd for amino acids) for solutions derivatized at concentrations ranging from 0.34 n*M* to 1.6 μ*M*, depending on the molecule tested

	NA	DA	Glu	Asp
y-intercept	0.055	0.033	−0.114	−0.082
Slope	29.3×10^{6}	2.83×10^{6}	$9.35-10^{3}$	624×10^{3}
Corr. coeff.	0.998	0.997	0.999	0.999
Number of points	20	16	15	15
Min. conc. derivatized (n*M*)	0.34	1.8	130	160
Min. amount derivatized (fmol)	0.17	0.9	65	80
Min amount detected (amol)	0.25	1.3	47	58
Max. conc. derivatized (μ*M*)	0.14	0.18	13	16
Max. amount derivatized (fmol)	70	90	6500	8000
Max. amount detected (amol)	102	131	4728	5819

The concentrations are corrected by the in vitro recovery of a 4-mm dialysis probe. Derivatization conditions: on-line system; perfusate flow rate: 1 μl/min; 30-s collection time (i.e., 750 nl derivatized samples). Separation conditions: see Fig. 5.

deviation from the linearity was found for concentrations below 0.1 μ*M*; taken this blank contamination into account, the minimal quantifiable concentrations were about 0.1 and 0.2 μ*M* for Glu and Asp, respectively. Indeed, this contamination is likely to be due to the presence of amino acids in the different reagents or glassware used, even if this possibilty has not yet been demonstrated. Such a contamination has been reported in previous studies, mainly for Asp, using HPLC with fluorimetric detection with orthophtalaldehyde derivatization [41,42].

These results show that the simultaneous determination of very small amounts of catecholamines and amino acids (Table 2) can be performed on 30-s microdialysis samples with short analysis times (less than 8 min for the two determinations) and very low injection volumes (1.65 nl for the two determinations). Such performances are better than those of the other analytical methods like HPLC where both total elution times and injected volumes are higher (25–70 min and 30–60 μl) [7–10]. The concentration and mass detection limit obtained shows that the simultaneous determination of the in vivo extracellular concentrations of DA, NA, Glu and Asp in 30-s fractions will be possible because the extracellular concentrations are commonly in the nanomolar range for catecholamines and in the micromolar range for amino acids [2,7–10,18,39,40].

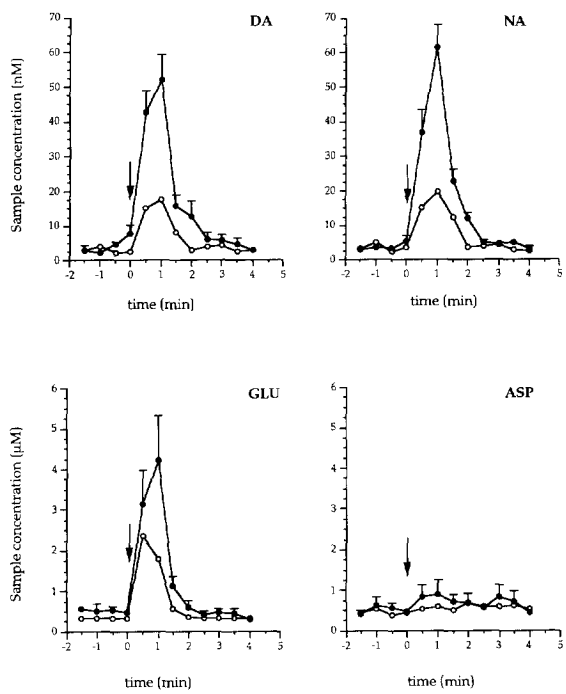

Fig. 6. In vitro microdialysis response to step changes of 5 s (–•–, $n=3$) and 1 s (–○–, 1 trial) duration in the external concentration. The arrow indicates the time when a 4-mm probe was transferred from a 10 n*M* DA and NA, and 1 μ*M* Glu and Asp solution, to a 1 μ*M* DA, NA and Asp and 100 μ*M* Glu solution (i.e., no change for Asp). On-line derivatized samples were collected every 30-s (perfusate flow-rate of 1 μl/min) and analysed for catecholamines and amino acids with CE–LIFD. Separation conditions: see Fig. 5.

3.3. In vitro response

In order to test the ability of the microdialysis–CE–LIFD process to follow changes that occur in the external medium in which the microdialysis probe is placed, the in vitro response to rapid step changes was studied. The microdialysis probe was placed in a basal external medium containing DA, NA, Glu and Asp in a range of concentrations similar to the basal values expected for in vivo studies. The observed responses for step changes of varying durations (5 and 1 s) are shown in Fig. 6. The probe was manually placed in a concentrated external medium where the concentrations were then 100-fold higher than the basal values for catecholamines and Glu reaching 1 μ*M* and 100 μ*M*, respectively, while the concentrations of Asp remained unchanged as a control. For the 5-s step changes, the data represent the means±SEM of three step changes obtained with three different probes. Corrected with the appropriate travel time (dead volume) of each probe, the maximum response was observed in the first and second dialysate fractions following the step change. Increases from +700 to 1700% for the 5-s step change and from +300 to 600% for the 1-s step change were observed for DA, NA and Glu. As expected, no significant variation was observed for the Asp concentration. These results indicate that rapid fluctuations (less than 5 s) of the external medium produced significant responses of the probe. A quantitative and specific determination of rapid variation in the external medium could thus be achieved in the present experimental conditions.

4. Conclusion

The present paper shows that CE–LIFD allows a rapid, sensitive and specific quantification of DA, NA, Glu and Asp on the same derivatized sample. With an on-line derivatization system and a 50 μm I.D. separation capillary, these determinations could be achieved on very low sample volume (500 nl) with good reproducibility and linearity and used for a routine quantification of these four neurotransmitters in 30-s microdialysis samples.

Since a large number of compounds present in the extracellular medium could be collected and derivatized at a high sampling rate, off-line CE–LIFD analysis of on-line derivatized microdialysates could be a powerful method to investigate interactions between neurotransmitters in the brain. Further developments will be made to adapt this technique to the simultaneous determination of DA, NA, Glu and Asp in in vivo microdialysis samples and to the determination of other neurotransmitters, such as other amino acids or neuropeptides.

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