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Highly sensitive assay for the measurement of serotonin in microdialysates using capillary high-performance liquid chromatography with electrochemical detection

Sandrine Parrot [a,*], Laura Lambás-Señas [b], Sabine Sentenac [b], Luc Denoroy [b], Bernard Renaud [a,b,c]

[a] *NEUROCHEM, Institut Fédératif des Neurosciences de Lyon, Plate-forme de Physiologie, Université Lyon 1, France*
[b] *Laboratoire de Neuropharmacologie, EA512, Institut Fédératif des Neurosciences de Lyon, Faculté de Pharmacie, Université Lyon 1, France*
[c] *Service de Neurobiologie, Hôpital Neurologique, Groupement Hospitalier Est, Hospices Civils de Lyon, Bron, France*

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Abstract

A highly sensitive isocratic capillary high-performance liquid chromatographic (HPLC) method with electrochemical detection (ED) for the simultaneous measurement of serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) in microdialysates has been developed using a 0.5 mm i.d. capillary column and a 11-nL detection cell. This method, validated on both pharmacological and analytical bases, can be performed using injection volumes as low as 1 μL. The limits of detection were 5.6×10^{-11} mol/L and 3.0×10^{-9} mol/L for 5-HT and 5-HIAA. Several applications of the present method are given on microdialysates from rodent brain and human spinal cord.

Keywords: Capillary liquid chromatography; Electrochemical detection; Serotonin; Microdialysis

1. Introduction

In the field of neuroscience, *in vivo* microdialysis is a common method for the sampling of chemical substances from the extracellular fluid in targeted brain areas. This technique is classically used to study the regulation of the neurotransmitters and/or the mechanisms of drug action in the central nervous system. The measurement of the compounds present in the microdialysates is allowed by the combination of microdialysis with an analytical technique [1,2].

Serotonin (5-hydroxytryptamine, 5-HT), a major neurotransmitter implicated in the pathology of a variety of psychiatric diseases and neurological disorders [3–5], is currently monitored by microdialysis coupled with high-performance liquid chromatography (HPLC) and numerous studies have reported different procedures to separate and quantify 5-HT present in microdialysates [6–8]. However, as the columns used had internal diameters between 1 and 4.6 mm, the sample volumes required were relatively high (5–20 μL), so that the microdialysates had to be collected with a low sampling rate (5–30 min). In order to monitor fast neuronal events, the microdialysis sampling rate has to be increased; in that case, smaller chromatographic columns have to be employed because they permit to analyse low-volume samples. Recently, Weber's group has reported a HPLC method using 100 μm capillary columns [9], but the detection by photoluminescence following transfer electron requires optical expertise at present.

Electrochemical detection (ED) is the most widespread method coupled to HPLC for the detection of microdialysate 5-HT. Its main inconvenience was the former inability to reduce the size of the detection cells (coulometric or amperometric cells), which had hampered the miniaturization of HPLC-ED, and thus, had prevented from collecting microdialysis samples with a high sampling rate. Recently, Antec Leyden Company has commercialized a new version of its amperometric detector, DECADE II, with a 0.7 mm working electrode, instead of 2 or 3 mm previously, in an 11-nL wall-jet cell. At present, only this

* Corresponding author at: NEUROCHEM, Plate-forme de Physiologie, Faculté de Pharmacie, Université Lyon 1, 8 avenue Rockefeller, 69373 Lyon Cedex 08, France. Fax: +33 4 78 77 72 09.
E-mail address: sparrot@sante.univ-lyon1.fr (S. Parrot).

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amperometric cell is compatible with capillary columns [10], so that microdialysis sampling frequency can be increased to 2–6 min, allowing a better monitoring of changes in neurotransmitter concentrations. However, the set-up described by Lorrain's team shows that a manual injection is required and, consequently, a part of the sample is lost. Moreover, we can point out that the HPLC-ED methods used for serotonin are seldom validated for microdialysates [11,12] and in most cases, lack pharmacological validation.

The aim of the present work was to develop a capillary liquid chromatographic method for the simultaneous determination of 5-HT and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) on small volumes of microdialysates (i.e., 1 μL), using a commercially available automated capillary HPLC instrument combined to the DECADE II amperometric detector. Here, we describe the optimization of the separation using standard solutions and microdialysates. The method has been validated on both analytical and pharmacological bases. Examples of analyses in microdialysates from rodent brain and human spinal cord are given to illustrate the potential applications of the method.

2. Experimental

2.1. Chemicals

Serotonin (5-hydroxytryptamine, 5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), 1-octanesulfonic acid (OSA), and ethylene-diamine-tetra-acetic acid (EDTA) disodium salt were purchased from Sigma (St. Louis, MO, USA), sodium dihydrogen phosphate and methanol from Carlo Erba (Rodano, Italy) and potassium chloride (KCl) was obtained from Fluka (Buchs, Switzerland). Hydrochloric acid 3.6% was purchased from VWR International (Fontenay-sous-Bois, France). Ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Standard solutions of 1 mmol/L 5-HT and 5-HIAA were stored at −20 °C as aliquots in water.

2.2. Capillary high performance liquid chromatography

The capillary HPLC system was composed of an Agilent Model 1100 series equipped with a degasser, a capillary pump equipped with the module for micro-flow rate mode and a flow sensor calibrated to ≤20 μL/min microflow rates, a micro-injector for micro-well-plates thermostated with the ALS therm module and a Decade II electrochemical detector fitted with a μVT03 0.7 mm glass carbon working electrode, an *in situ* Ag/AgCl (ISAAC) reference electrode and a 25 μm spacer (Antec, Leyden, The Netherlands). Separations were performed using a 150 mm × 0.5 mm i.d. C18 5 μm Agilent Zorbax column. The optimised mobile phase was pumped at a microflow rate of 8 μL/min and composed of 0.15 mol/L sodium phosphate, 0.5 mmol/L EDTA, 0.14 mmol/L OSA, 8% methanol, pH adjusted to 3.8 with 3.6% HCl, it was filtered with 0.22 μm cellulose acetate membranes before use. As an ISAAC reference electrode was used, we added 8 mmol/L KCl. The final concentration of chloride ions was around 12 mmol/L, so that eluates were detected at an oxidation potential of 500 mV versus ISAAC reference electrode. The column and the detection cell were housed within the Faraday cage of the electrochemical detector that was set to 35 °C. The electrochemical signal was filtered using the Decade II ADF noise suppression at 0.01 Hz (except when mentioned), the filter setting being determined by the following formula, 1/(2 × peak width). Chromatograms were acquired at a rate of 100 Hz with ChemStation software, the time of acquisition was 15 min. The day of the analysis, 10 μL samples were placed in the autosampler and kept at +4 °C before injection. The injection volume was 1 μL, except when mentioned.

2.3. Microdialysis experiments

The microdialysis probes were constructed from regenerated cellulose dialysis tubing (MWCO 6000 Da, 225 μm o.d., 2 mm active dialysis length, Spectra Por, Spectrum, USA) and fused-silica capillary tubing, the body of the probe being made of a 3 cm 26 G stainless steel tube [13]. After being flushed with water, the probes were perfused at 1 μL/min with artificial cerebrospinal fluid (aCSF, 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.78 mM phosphate buffer, pH 7.4) for the *in vivo* microdialysis experiments. Most experiments were carried out on male Sprague–Dawley rats (300–350 g, Harlan, Gannat, France), which were housed in a light and temperature controlled room (21 °C, light/dark cycle 12 h/12 h, light on 06:00), with food (Harlan Teklad Global 18% Protein Rodent Diet) and water *ad libitum*. The care and the use of laboratory animals were in accordance with the European Communities Council Directives of November 24, 1986 (86/609/EEC). Rats were anaesthetized with urethane (1.4 g/kg/i.p.) and placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA). Body temperature was maintained close to 37.5 °C using a heated underblanket (Harvard Instruments, Murrieta, CA, USA). The skull was exposed and the microdialysis probe was implanted in the right dorsal hippocampus at the following coordinates: posterior 3.8 mm, lateral 1.8 mm, ventral 4.0 mm from bregma and dura according to the atlas of Paxinos and Watson [14]. The collection of samples was initiated 2 h after microdialysis probe implantation. Samples were collected in PCR tubes (ABgene, Epsom, UK) and immediately stored at −40 °C until analysis. At the end of each microdialysis experiment, rats were sacrificed in order to verify the correct placement of the probe.

2.4. Method validation

2.4.1. Pharmacological validation

Two drugs, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), an agonist at 5-HT1A receptors, and citalopram, a selective 5-HT reuptake inhibitor (SSRI) were administered i.p. at 1 mg/kg (injection volume 2 mL/kg). Stock solutions of 2 mmol/L 8-OH-DPAT HBr (Sigma–Aldrich, Saint-Quentin Fallavier, France) and 1 mmol/L citalopram HBr (kindly donated by H. Lundbeck A/S, Copenhagen, DK) were prepared in water and stored at −40 °C. The final solutions were diluted in a 0.9% NaCl solution (Aguettant, Lyon, France).

2.4.2. Quantification validation

Calibration standards for the quantification validation contained 5-HT (range: 3×10^{-11}–10^{-8} mol/L) and 5-HIAA (range: 3×10^{-10}–10^{-6} mol/L). Calibration plots were represented by plotting the peak area versus concentration (number of concentrations of each compound, $n = 6$). Regression equations were calculated by the least-squares linear regression method. The limits of detection and quantification were calculated as the analyte concentration that gives rise to peak areas with a signal-to-noise ratio of 3 and 10, respectively, using the blanks and calibration plots.

Intra-day and inter-day repeatabilities were determined using standard solutions (tested concentrations: 3×10^{-10} or 10^{-9} mol/L for 5-HT and 10^{-7} mol/L for 5-HIAA) and microdialysates containing 5-HT and 5-HIAA in that range of concentrations.

The accuracy of the method was calculated from the analysis of microdialysates spiked with known quantities of standard (in duplicate).

2.5. Data analysis

2.5.1. HPLC data

The retention factor of an analyte was expressed as $k = (t_r - t_m)/t_m$, where t_r is the retention time of the analyte and t_m is the dead time of our chromatographic system. Resolution between two analytes was expressed by the equation $R = 2(t_2 - t_1)/(2\sigma_1 + 2\sigma_2)$, where t_1 and t_2 are the retention times of the two analytes, and $2\sigma_1$ and $2\sigma_2$, their respective widths at 60% of the peak height. The experimental number of plates was determined by the following formula: $N = 5.54\ (t_r/2.35\sigma)^2$, where 2.35σ is the width at 50% of the peak height.

2.5.2. Microdialysis experiments

Data are given as mean ± SEM expressed as a percentage of the mean values preceding drug administration. The comparison between the effect of the drugs and the baseline values was achieved on the mean values using ANOVA with post-hoc Dunnett's test. The level of significance was set at $p < 0.05$ for all comparisons.

3. Results and discussion

3.1. General considerations on the capillary column and the HPLC system

The column used was a 150 × 0.5 mm i.d. C18 5 μm Agilent Zorbax Stable Bond column. The optimal flow rate (FR) was estimated using the following formula: $FR = u_{opt} \times \varepsilon_T \times d_c^2 \times \pi/4$, where u_{opt} is the optimal linear speed for 5 μm particles, i.e. 0.1 cm/s, ε_T represents the extra- and intra-particular spaces estimated to 60–80%, and, d_c the internal diameter of the column. Optimal FR was estimated to be 8 μL/min. In order to limit the external phenomena of dispersion induced by the HPLC system itself, the connecting tubings between the Agilent 1100 series capillary HPLC system and the electrochemical detector were carefully chosen to be compatible to the capillary column (50 μm diameter before the column, 75 μm between the column and the detector micro-cell).

Practically, the flow rate (8 μL/min) is delivered by the Agilent capillary pump using its precise micro-flow mode, i.e. after a sensor-controlled splitting from a flow of 200 μL/min. In that case, the wasted mobile phase is recycled in order to limit its consumption. If the oxidized mobile phase is also recycled after the detection outlet, a batch of 100 mL mobile phase is sufficient for one month and a half of routine analysis.

3.2. Analytical developments

We started using one mobile phase previously reported and containing OSA, an ion-pairing agent, as commonly employed for the measurement of both 5-HT and 5-HIAA in microdialysates [15]. At their initial conditions (0.15 mol/L NaH_2PO_4, 0.5 mmol/L EDTA, 0.025 mmol/L OSA, 12.5% methanol, pH adjusted to 3.8), we added KCl for an optimal use of the ISAAC reference electrode and we choose 35 °C as running temperature as recommended by the detector supplier. Under these HPLC conditions, standard 5-HT and 5-HIAA were well separated in 3.8 and 6.7 min, respectively ($k = 0.6$ and 2, respectively) as expected, but microdialysate 5-HT was not separated from other compounds (data not shown). In order to increase the retention factor ($k \geq 1$) for a better separation, the concentration of OSA was increased to 0.14 mmol/L. In that case (Fig. 1A, black line), standard 5-HT and 5-HIAA peaked at 5.6 and 6.9 min, respectively ($k = 1.5$ and 2, respectively), but microdialysate 5-HT was not detectable because of a high background noise (Fig. 1A, dotted line versus black line). When the OSA concentration was increased to 0.25 mmol/L (data not shown), 5-HT and 5-HIAA co-migrated at 6.7 min ($k = 2$). As the migration of the 5-HIAA peak was not sensitive to the variations of the OSA concentration, we tested the influence of the percentage of the organic modifier. The concentration of methanol was lowered

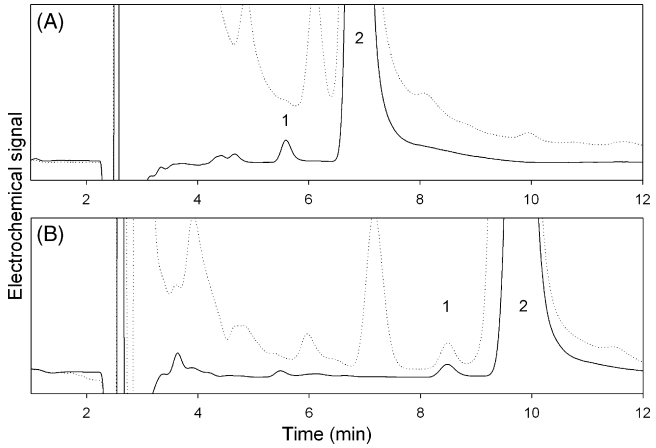

Fig. 1. Optimization of methanol concentration on the separation of 5-HT (analyte 1) and 5-HIAA (analyte 2). Chromatograms were obtained from standard solutions (black lines) and hippocampal microdialysates (dotted lines). Mobile phase contained 12.5% (A) or 8% (B) methanol. For (A), the electrochemical signal was filtered at 0.02 Hz; for (B), at 0.01 Hz. The injected volume was 1 μL. See other details in Section 2.2. Note, in (B) the lack of interference induced by endogenous compounds present in the microdialysates.

Table 1
Qualitative parameters for the separation of 5-HT and 5-HIAA in hippocampal microdialysates

Parameters	5-HT	5-HIAA
Retention time (min)[a]	8.250 ± 0.005	9.517 ± 0.006
Peak width (min)[a,b]	0.263 ± 0.003	0.399 ± 0.001
Number of plates[c]	≈4000	≈2300
Resolution	1.91 ± 0.01	

[a] Ten replicates.
[b] Taken as 2σ (60% of the peak height).
[c] Calculated as 5.54 × (retention time/2.35σ)^2.

to 8% and 0.14 mmol/L OSA was used. As expected (Fig. 1B, black line), the retention of 5-HT and its metabolite 5-HIAA increased and the compounds peaked at 8.4 and 9.7 min, respectively (k = 2.7 and 3.3). In these new conditions, no molecule seemed to interfere with 5-HT and 5-HIAA peaks in the microdialysates (Fig. 1B, dotted line versus black line).

Thus, the final conditions chosen for the mobile phase were: 0.15 mol/L NaH_2PO_4, 0.5 mmol/L EDTA, 0.14 mmol/L OSA, 8% methanol, pH adjusted to 3.8 with HCl, 8 mmol/L KCl being added for a final concentration of 12 mmol/L chloride ions.

Qualitative parameters of the separation—variability of retention time, peak width, number of plates and resolution—were then evaluated, especially in microdialysates (Table 1). The variability of retention times for 5-HT and its metabolite was inferior to 0.07% and the variability of peak widths was 1.1% and 0.25% for 5-HT and 5-HIAA, respectively. The experimental number of plates was determined for standard solutions and microdialysates, 4000–5000 for 5-HT and 2000–3600 for 5-HIAA. The value is quite satisfactory for 5-HT even if lower than the expected one (theoretical N ≈ 7500). We first hypothesized that lowered efficiency was due to a peak dispersion generated by the injection of 1 μL samples, instead of 0.1 or 0.2 μL as recommended by the column supplier. However, we demonstrated that smaller injection volumes (0.8, 0.5, 0.2 and 0.1 μL) did not enhance at all the experimental number of plates for 5-HT (data not shown). The number of plates is relatively low for 5-HIAA, and can be easily explained by (i) the presence of the acidic moiety for 5-HIAA instead of the primary amine group for 5-HT, and (ii) the use of separation conditions (column, mobile phase with ion-pairing agent) favorable to amines. However, under our HPLC conditions, the resolution between 5-HT and 5-HIAA was superior to 1.5 in all the biological samples tested (for instance, rodent microdialysates from prefrontal cortex and hippocampus) as well as standard solutions, showing that 5-HT and 5-HIAA were well-separated.

3.3. Validations

3.3.1. In vivo pharmacological validation

A primary identification of the peaks present in microdialysates was made by comparing their retention times to those of standards. However, as such a criterion is poorly informative, a pharmacological validation was undertaken to determine whether 5-HT in microdialysates really corresponded to the analyte of interest. Administration of a 5-HT$_{1A}$ agonist, 8-OH-DPAT (1 mg/kg i.p.) induced a significant −80% decrease of the area of the peak present in hippocampal microdialysates and exhibiting the same migration time as 5-HT standard (Fig. 2). The area of the same peak was increased by +80 to 90% as compared to baseline after the administration of the selective serotonin reuptake inhibitor citalopram (1 mg/kg i.p., data not shown). These data are qualitatively and quantitatively similar to literature data on the effects of these drugs on 5-HT concentration in microdialysates analyzed by HPLC using classical columns [16,17]. Consequently, it can be concluded that the microdialysate peak exhibiting the same migration time as standard 5-HT really corresponds to endogenous 5-HT.

3.3.2. Storage and preservation of samples

Before beginning the quantitative validation of the method, we tested the conservation of our samples in our HPLC system (4 °C) and at storage temperature (−40 °C) before analysis. Standard solutions of 5-HT prepared in aCSF cannot be kept without degradation more than 1 h at +4 °C whereas 5-HT contained in microdialysate is extremely stable in the same conditions. In order to guarantee further quantitative validation, we tested the conservation of standards and samples at −40 °C. We found that standard 5-HT is degraded whereas microdialysate 5-HT can be kept at least fifteen days without changes in its concentrations. This study showed that standard 5-HT solutions could be prepared using a pool of dialysates instead of aCSF and stored at −40 °C. Practically, the pool of dialysates could be collected just after probe implantation, i.e. during the 2 h stabilization period. The inconvenience of those samples is that the concentrations are much larger than those measured in the stabilized microdialysates. Thus, we have finally chosen to prepare standard 5-HT solutions in aCSF extemporaneously for each

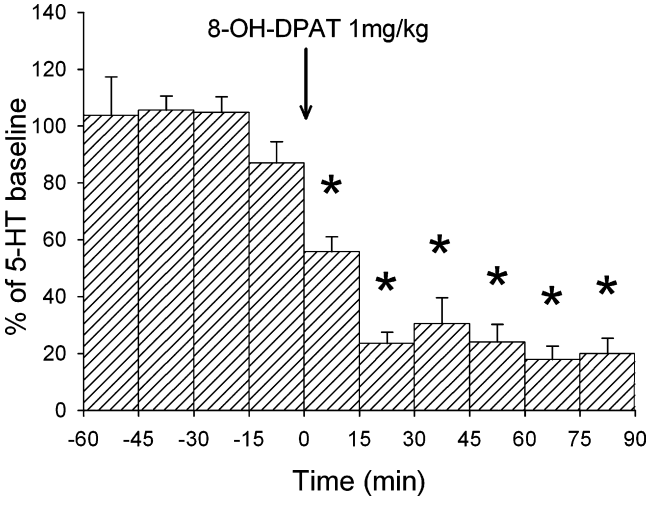

Fig. 2. Pharmacological validation of the separation of 5-HT. A 5-HT$_{1A}$ agonist (8-OH-DPAT, 1 mg/kg i.p.) was administered *in vivo* (black arrow). 5-HT level was monitored in rat hippocampal microdialysates collected every 15 min. Data are expressed as percent (mean ± SEM) of the baseline values preceding drug administration. The basal concentration of 5-HT in microdialysates was 0.16 ± 0.05 nmol/L (n = 3).

Table 2
Quantitative parameters for the analysis of 5-HT and 5-HIAA in standard solutions and hippocampal microdialysates

	5-HT	5-HIAA
Standards		
Calibration range (mol/L)	3×10^{-11}–10^{-8}	3×10^{-10}–10^{-6}
Regression coefficient of the calibration (r^2)	0.9992	0.9999
Inter-day repeatability (%RSD)[a]	9.2	6.3
Limit of detection (mol/L)	8.4×10^{-11}	4.5×10^{-10}
Limit of quantification (mol/L)	10^{-10}	10^{-9}
Microdialysates		
Calibration range (mol/L)	3×10^{-11}–10^{-8}	3×10^{-9}–10^{-6}
Regression coefficient of the calibration (r^2)	0.9999	0.9976
Intra-day repeatability (%RSD)[b]	2.3–2.9	1–1.5
Inter-day repeatability (%RSD)[c]	3.0	4.0
Accuracy (%)[d]	3.6	7.1

[a] Eight days, tested concentration: 10^{-9} mol/L 5-HT and 10^{-7} mol/L 5-HIAA each day.
[b] Ten replicates.
[c] Eight days (except for microdialysate 5-HIAA: six days), three replicates each day.
[d] Spiked microdialysates (the added volume represents 10% of the microdialysate volume), tested concentration: 3×10^{-10} mol/L 5-HT and 10^{-7} mol/L 5-HIAA, in duplicates.

batch of analysis, at a range including the expected dialysate concentrations.

3.3.3. Validation of quantification

The following validation parameters such as linearity, intra-day repeatability, inter-day repeatability, accuracy, limits of detection and quantification were determined for 5-HT and 5-HIAA contained in standard solutions as well as in pooled brain microdialysates. The results obtained with the final conditions are shown in Table 2. Several considerations can be given. First, as the standard solutions degraded rapidly at +4 °C, intra-day repeatability was not evaluated for standard solutions and linearity was determined using only one point per concentration. In contrast, intra-day repeatability for microdialysate 5-HT and 5-HIAA showed good results and the linearity was estimated with duplicates. Second, the results obtained for inter-day repeatability showed that a daily calibration of the system was necessary. Third, the regression coefficient of the calibration obtained with standard solutions and microdialysates spiked with known quantities of standard showed a good linearity and lead us to use in routine only two points of the calibration curve. Finally, the limits of detection (LOD) and quantification (LOQ) for 5-HT and 5-HIAA were lower than the concentrations measured in the microdialysis samples. In terms of concentrations, our LOD is generally 4–10-fold lower than those previously reported for HPLC-ED [11,15,18]. Moreover, when comparing the mass detected with a classical HPLC-ED method exhibiting the same LOD as ours [12] or even twice smaller [19], our system allows to detect only 84 amol of 5-HT in 1 μL, instead of 750–1000 amol in 15–20 μL (Table 3), which proves that the miniaturization is not associated with a loss in the electrochemical signal, provided that an appropriate HPLC-ED system is used.

3.4. Potential applications

Our HPLC-ED procedure has been fully validated on dorsal hippocampal microdialysates from rats. It was also applied to several types of rodent and human dialysates (Fig. 3) collected from areas of the central nervous system having serotonergic afferences: prefrontal cortex and dorsal hippocampus from rats, ventral hippocampus from mice, and, for the first time, 5-HT is detected in spinal cord dorsal horn from an patient suffering from spasticity. The method was also tested on a extract of fish diencephalon (Fig. 4, right); in that case, the sample was acidic because of the extraction procedure used, but the separation obtained was similar to the one observed for neutral samples.

Eventually, the major potentiality of capillary HPLC-ED is the determination of indolamine contents on small volume samples as illustrated in Fig. 4 (left), for which 5-HT and its metabolite were detected and quantified in a 2 μL rat hippocampal microdialysate. One can point out that monitoring variations of neurotransmitters by microdialysis on a short-time scale (1–2 min) is now possible using a commercially available capillary HPLC system. Up to now, to our knowledge, only one

Table 3
Comparison of quantitative parameters for the analysis of 5-HT obtained with typical ED-HPLC methods applied to the field of neuroscience

ED-HPLC method	Sample volume (μL)	Injected volume (μL)	Mass detection limit (S/N = 3) (amol)	Linearity	Retention time (min)
[18]	6	0.5	13[a]	n.r.	≈7
[15]	10	n.r.	4760	n.r.	≈3.5
[11]	30	n.r.	3000–6000	>0.998	≈21[b]
[12]	15	n.r.	1000	n.r.	≈25[b]
[19]	45	20	750	n.r.	n.r.
This work	2–10	1	84	>0.9992	≈8

n.r., not reported.
[a] Value of S/N chosen not reported.
[b] Longer retention times because the method is devoted to both indolamine and catecholamine contents.

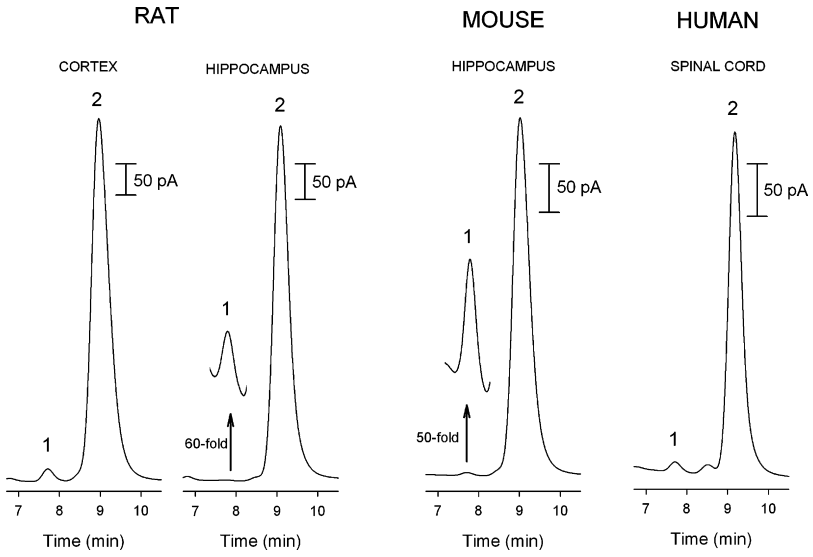

Fig. 3. Typical chromatograms of microdialysates collected from rat cortex, rat dorsal hippocampus, mouse ventral hippocampus and human spinal cord dorsal horn. 1: 5-HT; 2: 5-HIAA. The injected volume was 1 μL. The concentrations for 5-HT were 8.9, 0.4, 1.3, and 3.7 nmol/L, respectively. The concentrations for 5-HIAA were 364, 217, 194, and 136 nmol/L, respectively.

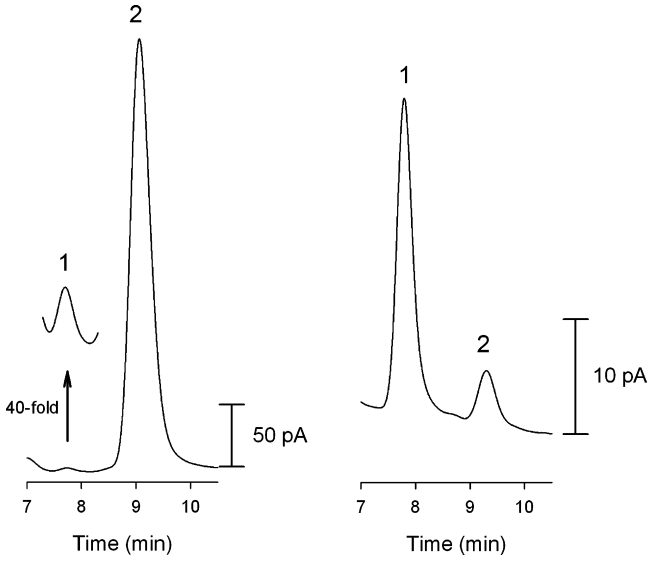

Fig. 4. Typical chromatograms of a 2 μL microdialysate from rat dorsal hippocampus (left) and an extract of bass diencephalon (right). 1: 5-HT; 2: 5-HIAA. The injected volume was 1 μL and 0.1 μL for the dialysate and the extract, respectively. The concentrations for 5-HT were 0.9 and 104 nmol/L, respectively. The concentrations for 5-HIAA were 163 and 0.30 nmol/L, respectively.

team has succeeded in performing such a microdialysis with high temporal resolution using classical HPLC [20], whereas the groups using capillary HPLC have carried out microdialysis experiments with 3 or 5 min temporal resolutions [21–23].

4. Conclusion

In conclusion, the present work shows that the miniaturization, the optimization and the validation of a highly sensitive HPLC-ED method can be easily carried out with a commercial automated capillary HPLC system, a 5 μm 150 mm × 0.5 mm i.d. capillary column, capillary connections and a 0.7 mm working electrode in a 11-nL electrochemical cell. As smaller columns exist, we can also hypothesize that the limits of detection and the speed of analysis can be improved, especially by using columns with 1.9 or 3 μm particles.

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