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New antioxidant mixture for long term stability of serotonin, dopamine and their metabolites in automated microbore liquid chromatography with dual electrochemical detection

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Abstract

An automated microbore liquid chromatographic assay with dual electrochemical detection is described for the determination of serotonin, dopamine and their metabolites, 5-hydroxyindoleacetic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid. Due to the chemical instability of the compounds, the addition of an antioxidant is required for automated analysis over a long period of time (e.g., 20 h). Therefore, the time stability of these substances was tested with different antioxidants. The stability for serotonin and 5-hydroxyindoleacetic acid was poor in acidic medium containing Na_2EDTA but could greatly be improved by the addition of L-cysteine and ascorbic acid. Using this assay, the neurotransmitters and their metabolites could easily be determined in microdialysates obtained from different rat brain areas. © 1997 Elsevier Science B.V.

Keywords: Microdialysis; Serotonin; Dopamine; 5-Hydroxyindoleacetic acid; 3,4-Dihydroxyphenylacetic acid; Homovanillic acid

1. Introduction

During the last decade, the microdialysis technique [1–3] has been successfully applied to monitor the extracellular levels of a wide range of neurotransmitters and their metabolites in brain tissue. The method involves the collection of perfusates from a dialysis probe implanted in a certain brain area.

Recently, microbore liquid chromatography with electrochemical detection (LC–EC) has become the method of choice for the determination of sub-picogram amounts of neurotransmitters in microdialysates [4]. Indeed, compared to conventional analytical columns (3–4.6 mm I.D.), microbore columns (0.5–1 mm I.D.) lead to a higher mass sensitivity and further require less sample volume to achieve the same concentration sensitivity.

Until now, most LC assays have been described for the determination of either serotonin [5] or catecholamines [4] and their respective metabolites. Few methods [6–8] exist for the simultaneous measurement of serotonin (5-HT), dopamine (DA) and their metabolites, 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Even fewer methods

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are available for the measurement of these compounds in microdialysates [4].

Microdialysis experiments generally yield a large number of samples, especially in the multiple probe experiment, necessitating the use of automated injection. However, this requires the addition of antioxidants to the dialysates since catechols and indoles are subject to oxidative breakdown. In this study, particular attention was paid to the concurrent time stability of these neurotransmitters and metabolites awaiting for microbore LC analysis. Several antioxidant mixtures at different pH values were tested. The acidic solution with final pH 3.2 (i.e., after mixing with Ringer's solution) and containing Na_2EDTA, ascorbic acid and L-cysteine, prevented oxidative degradation of 5-HT, DA and their metabolites for up to 20 h.

2. Experimental

2.1. Chemicals and reagents

The standards 5-HT and 5-HIAA, sodium 1-octanesulphonate and N,N-dimethylacetamide (more than 99% spectrophotometric grade) were supplied by Janssen Chimica (Beerse, Belgium). DA hydrochloride was purchased from UCB (Brussels, Belgium), DOPAC and L-cysteine from Sigma (St. Louis, MO, USA) and iso-HVA (external standard) and HVA from Fluka (Buchs, Switzerland). Ascorbic acid was purchased from Roche (Brussels, Belgium) and dibutylamine from Aldrich-Chemie (Beerse, Belgium). All the other reagents and solutions were supplied by Belgolabo (Overijse, Belgium). The methanol from Belgolabo was of gradient grade for chromatography.

Modified Ringer's solution was used as perfusion fluid in the experiments and contained 147 m*M* Na^+, 4 m*M* K^+, 2.2 m*M* Ca^{2+} (or 1.1 m*M* for experiments in striatum and substantia nigra pars compacta) and 156 m*M* Cl^-. Stock solutions of the standards (0.47 m*M* 5-HT, 0.53 m*M* DA, 0.55 m*M* HVA, 5.23 m*M* 5-HIAA, 5.95 m*M* DOPAC and 54.89 m*M* iso-HVA; kept at 4°C) were prepared in an antioxidant mixture consisting of 0.01 *M* HCl containing 52.6 m*M* $Na_2S_2O_5$ and 0.27 m*M* Na_2EDTA. The first dilution was made in modified Ringer's solution and the final dilutions were made in a mixture of modified Ringer's solution and the tested antioxidant (4:1). The final concentrations of the different standards varied between 0.2 n*M* and 548.9 n*M*, meaning that the amounts of $Na_2S_2O_5$, HCl and Na_2EDTA, originating from the stock solutions, can be neglected in the standard solutions. Two series of four antioxidant mixtures were tested (see Table 1). In a first series, the acidic mixtures contained 0.5 *M* HCl and Na_2EDTA (0.27 m*M*) and/or L-cysteine (3.3 m*M*) and/or ascorbic acid (0.5 m*M*). The second series of mixtures consisted of the same compounds, except that HCl was replaced by 0.1 *M* acetic acid (HAc) resulting in a higher pH value. The final pH is the one obtained when the antioxidant is mixed with modified Ringer's solution. Stability of 5-HT, DA and their metabolites was tested for ca. 20 h. All solutions were prepared using water purified by a Seralpur pro 90 CN (Belgolabo) (output conductivity 0.05 μS/cm). All aqueous solutions were filtered by a 0.2 μm membrane filter.

2.2. Chromatographic set-up

In the LC system, a vacuum degasser LC 26 (Bioanalytical Systems, West Lafayette, IN, USA) was coupled to a Gilson (Villiers le Bel, France) Model 305 piston pump with a manometric module 805 as a pulse damper. This pump was operating at a flow-rate of 0.7 ml/min with a flow splitter kit for Unijet microbore columns (Bioanalytical Systems, West Lafayette, IN, USA), yielding a flow-rate

Table 1
Composition of two series of four antioxidant mixtures tested

Final pH		NA_2EDTA (0.27 m*M*)	L-Cysteine (3.3 m*M*)	Ascorbic acid (0.5 m*M*)
1.3	a	+	o	o
	b	+	+	o
	c	+	o	+
	d	+	+	+
3.2	e	+	o	o
	f	+	+	o
	g	+	o	+
	h	+	+	+

The acidification of the antioxidant mixtures is obtained by adding 0.5 *M* HCl in the first series and 0.1 *M* HAc in the second series.

through the microbore column of 80 μl/min. The microbore analytical column (150 mm×1 mm I.D., C_{18}, 5 μm ODS) (Bioanalytical Systems) was coupled via a fused-silica capillary tubing (50 μm I.D.) to the injection valve to minimize the dead volume of the system. Injections were carried out using a water-cooled (±10°C) autosampler 465 (Kontron instruments, Milan, Italy). The injection volume was 10 μl out of 25 μl for the standards and the dialysates. The run time was 25 min. The analytical column was directly coupled to the electrochemical cell. A 16 μm gasket was utilized to reduce the cell volume. The dual electrochemical detection was performed using LC-4C and LC-3C amperometric detectors (Bioanalytical Systems) coupled for dual mode. The thin-layer cross-flow amperometric cell with dual glassy carbon working electrode was used in parallel configuration to allow measurement of compounds at different ranges. The operating potential of both detectors was set at 700 mV vs. a Ag/AgCl reference electrode. Filter cut-off frequency was set at 0.08 Hz. The current range of the detector was set at 1 nA full scale for testing the stability of the standards in the different antioxidant mixtures. For the analysis of microdialysates, the range of the first channel was set at 0.1 nA for measuring 5-HT, DA, iso-HVA and/or HVA. The range of the second channel was set at 2 nA (or 5 nA for the striatum if necessary) for measuring DOPAC, 5-HIAA, iso-HVA and/or HVA. Integration of the peak areas in chromatograms was performed on a Kontron Data System 450-MT2 integration computer program (Kontron, Milan, Italy).

2.3. Chromatographic conditions

The mobile phase consisted of an acetate–citrate buffer containing 0.1 *M* sodium acetate, 20 m*M* citric acid monohydrate, 1 m*M* octanesulphonate, 0.1 m*M* Na_2EDTA and 1 m*M* dibutylamine. The pH value was adjusted to 3.1 with phosphoric acid. Then 1.50% (v/v) MeOH and 1.75% (v/v) N,N-dimethylacetamide were added to the buffer.

2.4. Microdialysis experiments

Male albino Wistar rats (250–300 g) were anaesthetized with a mixture of ketamine–diazepam (50 mg/kg–5 mg/kg). The rats were placed on a stereotaxic frame. An intracerebral guide cannula (CMA Microdialysis, Stockholm, Sweden) was implanted just above the hippocampus (coordinates relative to bregma L: −4.6; A: −5.6; V: +4.6), the striatum (L: −2.8; A: +1.2; V: +3.4), the substantia nigra pars compacta (L: −2.0; A: −5.8; V: +6.8) or the substantia nigra pars reticulata (L: −2.2; A: −5.8; V: +6.3) with bregma 1 mm higher than lambda for the substantia nigra. Then a CMA 12 microdialysis probe with a membrane length of 3 mm (or 2 mm for the substantia nigra) (CMA Microdialysis) was inserted via the cannula after removing the guide. The probes were connected to a microdialysis pump (CMA 100) by PTFE tubing (FEP 120 mm I.D.) and the modified Ringer's solution was pumped through the microdialysis probe at a constant flow-rate of 2 μl/min (1 μl/min for the substantia nigra pars reticulata). The rats were allowed to recover from surgery and the sampling procedure started 20 h after inserting the probe. Dialysates (under basal conditions) were collected every 20 min. The small plastic vials, used for the collection, contained 10 μl (or 5 μl for 1 μl/min perfusion rate) of filtered antioxidant mixture containing HAc, Na_2EDTA, L-cysteine and ascorbic acid (see Table 1). Iso-HVA was dissolved in antioxidant mixture resulting in a final concentration (in Ringer/antioxidant) of 500 pg/10 μl or 1000 pg/10 μl for the microdialysates. Microdialysis samples were stored refrigerated (4°C) until the start of the automated analysis.

3. Results and discussion

3.1. Stability of the compounds

The stability of the neurotransmitters and their metabolites is an important factor in automated and simultaneous analysis of these compounds in microdialysates. Until recently, an antioxidant, containing $Na_2S_2O_5$ and Na_2EDTA (i.e., the antioxidant used for stock solutions, see Section 2.1) in acid solution was used in the laboratory to prevent degradation of catecholamines and their metabolites in microdialysates. This antioxidant also provides good stability for stock solutions of catecholamines, indoleamines and their metabolites for at least 3

months (results not shown). However, when the antioxidant was mixed with Ringer's solution degradation of the indoleamines occurred within some hours, so that these compounds could only be measured by direct manual injections of the samples [5,9]. To permit automated injection of a high amount of samples, it was therefore necessary to find an antioxidant which prevented air-oxidation of all the compounds simultaneously and for a longer period of time.

The required antioxidant must remove oxygen, which is dissolved in the solution and also must recycle the oxidized compounds (i.e., quinones) back to their reduced form [10,11]. Ascorbic acid and L-cysteine, are oxidized by oxygen and quinones. Oxidation of ascorbic acid leads to dehydroascorbate which is finally irreversibly hydrated [10]. L-Cysteine is oxidized to L-cystin, which is not reduced by ascorbic acid [12].

The oxidation of catechol is also driven by acid–base equilibria. The deprotonated catechol reacts quickly with oxygen dissolved in the aqueous solution whereas the fully protonated molecule remains unchanged. In flow injection analysis (FIA) studies, we compared the oxidation of catechol at different pH values in an air saturated and in an air free medium (N_2 saturated medium) (Fig. 1). At low pH values (Fig. 1a,b) the rate of electrochemical oxidation was lower compared to higher values (Fig. 1c,d_i). When pH increased above 7 the rate of air oxidation increased. Fig. 1 shows that the degradation of catechol is more obvious in an air saturated medium (Fig. 1d_i,e_i) compared to an air free medium (Fig. 1d_{ii},e_{ii}). The air oxidation of the fully deprotonated catechol was so fast that almost all the catechol was degraded before injection (approx. 1 min) (Fig. 1e_i). In contrast, when oxygen was removed (Fig. 1d_{ii},e_{ii}), oxidative breakdown of the catechol did not occur. However, an experimental set-up in which air oxidation is excluded is difficult to obtain.

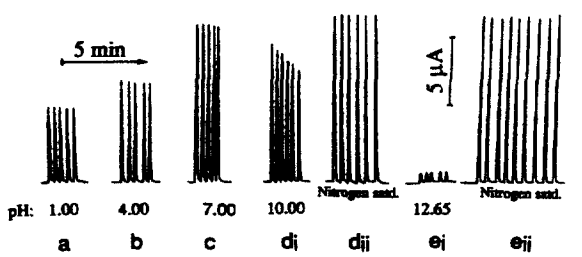

Fig. 1. Flow injection analysis study. The effect of the pH of the catechol solution in an air saturated (i) and deoxygenated medium (ii) on the degradation rate of catechol and the sensitivity of the detector. The pH of the catechol solution was varied between 1.00 and 12.65 (a, b, c, d, e: pH 1.00; 4.00; 7.00; 10.00; 12.65). The following conditions were used: +700 mV vs. Ag/AgCl; flow-rate=0.3 ml/min; injection of 20 µl catechol (100 µ*M*).

The results from this FIA study confirm that catechols are more stable at lower pH. But at very low pH, indoleamines are less stable [10,13]. Moreover, the antioxidant effect of ascorbic acid is lower at lower pH, as it is not protonated and thus can only slowly be oxidized [10]. Therefore, an optimal pH of the antioxidant mixture had to be found to prevent degradation of all the compounds over a long period of time (approx. 20 h).

In this set-up, an autosampler with water-cooling of the samples was used. However, due to the partial evaporation of the samples, iso-HVA was added as an external standard. Two series of antioxidant mixtures, with different pH obtained by adding 0.5 *M* HCl or 0.1 *M* HAc, were tested (Table 1). When mixed with Ringer's solution the final pH was 1.3 and 3.2, respectively. A first antioxidant mixture consisted of an acidic solution containing only Na_2EDTA. Indeed, Na_2EDTA is usually added to eliminate metal traces by complex formation [14]. The stability of those complexes however, is only maximal above pH 5. Verbiese-Genard et al. [15] however, suggested that EDTA could act as a competitive agent, interfering with one of the oxidation steps of the indole derivatives. L-Cysteine showed to be a good antioxidant for 5-HT and 5-HIAA, in agreement with Kalén et al. [16], and for DOPAC. Addition of ascorbic acid to the acidic solution improved the stability of all compounds. Finally an antioxidant mixture containing the four compounds sufficiently protected against the oxidative breakdown, especially when the final pH was 3.2 (see Fig. 2). Coefficients of variation (C.V.) of peak areas (obtained by subsequent injections of standard solutions: every 50 min for ca. 20 h) for 5-HT, DA, 5-HIAA, DOPAC and HVA, using the antioxidant mixture with final pH 1.3 varied between 6.1% and 8.8%. The time stability of the different compounds could be improved using the antioxidant mixture

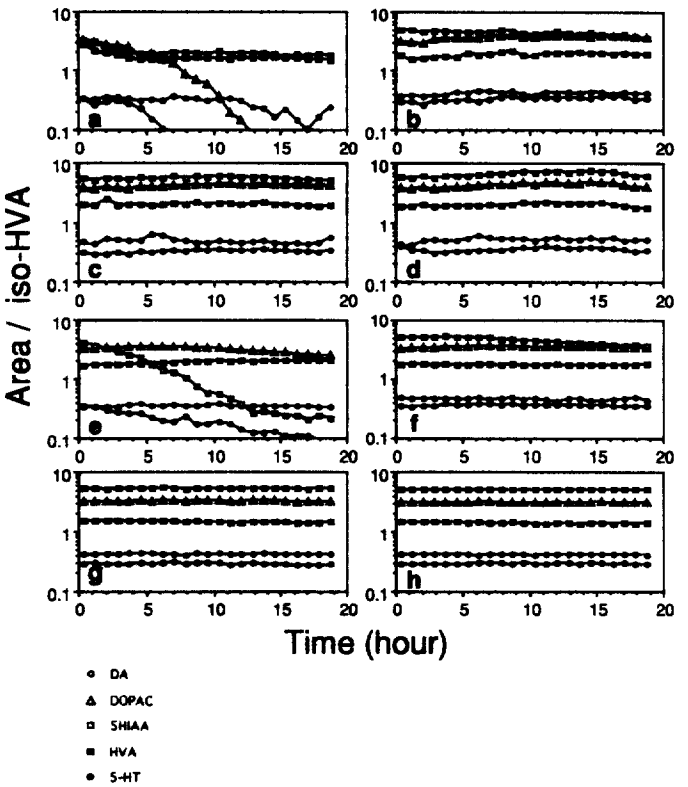

Fig. 2. Effect of different antioxidants on DA, DOPAC, 5-HIAA, HVA and 5-HT. Standards of 10 pg/10 μl 5-HT, DA; 100 pg/10 μl 5-HIAA, DOPAC; 200 pg/10 μl HVA; 500 pg/10 μl iso-HVA were injected. The sensitivity of the detector was set at 1 nA. In the *y*-axis, the logaritmic scale was used to show the behaviour of all the compounds as different concentrations were used for the different compounds. a, b, c, d, e, f, g, h: composition and pH of the antioxidant mixtures according to Table 1.

with final pH 3.2 leading to C.V. between 1.1% and 2.9%.

Several other solutions were also tested but could not be used. β-Mercaptoethanol at 0.02% showed interfering impurity peaks on the chromatogram. Also $HClO_4$ (0.005 *M*) was not able to prevent degradation of all compounds. DOPAC and 5-HIAA degraded even within 1 h.

3.2. Validation of the LC–EC assay

In this study, the LC–EC assay for the simultaneous and automated analysis of 5-HT, DA and their metabolites was validated using the antioxidant mixture containing HAc, Na_2EDTA, L-cysteine and ascorbic acid.

Separation of 5-HT, DA, 5-HIAA, DOPAC, HVA and iso-HVA was obtained using a buffer at pH 3.1. N,N-Dimethylacetamide was added to selectively influence the retention of 5-HT and DA: increasing the amount of this solvent reduces their retention times relative to the other peaks. Methanol was added to the buffer to simultaneously reduce all retention times. In Fig. 3A, the separation of the different compounds is shown.

Registration of hydrodynamic voltammograms for all compounds revealed that electrode potentials of +700 mV for the detectors provided an optimal response. This relatively high potential was necessary to obtain measurable currents of the O-methylated derivatives, such as HVA and iso-HVA.

Calibration curves were made using standard solutions of the compounds containing 0.5 to 10 pg/10 μl for 5-HT and DA, 5 to 100 pg/10 μl for HVA and 100 to 1000 pg/10 μl for 5-HIAA, DOPAC and HVA. Table 2 shows the calibration

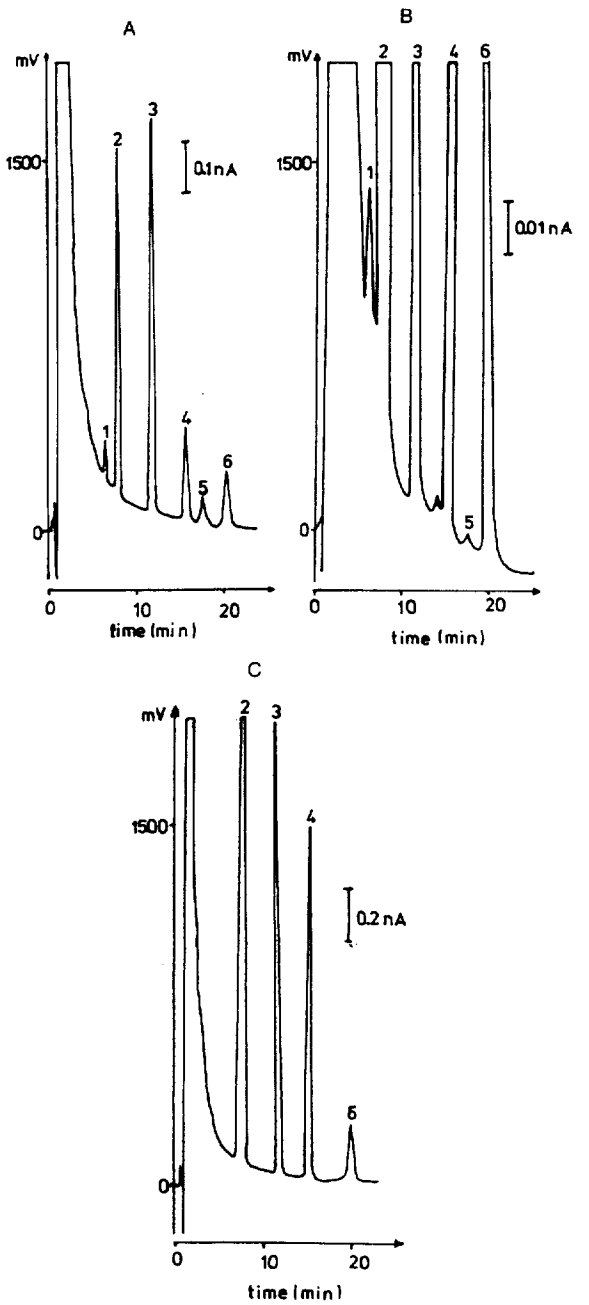

Fig. 3. (A) Chromatogram of a standard containing DA, 5-HT (10 pg/10 μl), DOPAC, 5-HIAA (100 pg/10 μl), HVA (200 pg/10 μl) and iso-HVA (500 pg/10 μl). The sensitivity of the detector was set at 1 nA. (B,C) Chromatograms of a dialysate obtained from the rat striatum. The concentration of iso-HVA was 1000 pg/10 μl. The sensitivity of the two detectors was 0.1 nA (B) and 2 nA (C), respectively. Peaks: 1=DA; 2=DOPAC; 3=5-HIAA; 4=HVA; 5=5-HT; 6=iso-HVA.

Table 2
System suitability test for the various compounds

Compound	C.V. (%)				r^2	
	k'		Area			
	a	b	a	b	*	**
DA	0.280	0.320	3.40	1.64	0.9988	0.9992
DOPAC	0.238	0.263	1.43	1.18	1.0000	0.9980
5-HIAA	0.243	0.276	3.30	1.11	0.9999	0.9988
HVA	0.337	0.191	10.90	2.91	0.9996	0.9995
					0.9997*	0.9995*
5-HT	0.284	0.251	3.80	1.28	0.9966	0.9978
iso-HVA	0.208	0.190	3.37	1.67	–	–

The coefficients of variation (C.V., %) for the capacity factors $(k')=(t_r-t_0)/t_0$, where t_r and t_0 are the elution times of retarded and unretarded solutes, respectively or peak areas are given for a standard solution injected six times containing (a) 2.5 pg/10 μl 5-HT, DA; 5 pg/10 μl HVA; 250 pg/10 μl 5-HIAA, DOPAC; 500 pg/10 μl iso-HVA (sensitivity=0.1 nA for 5-HT, DA, HVA, iso-HVA and 2 nA for 5-HIAA, DOPAC, iso-HVA). (b) 10 pg/10 μl 5-HT, DA; 200 pg/10 μl HVA; 100 pg/10 μl 5-HIAA, DOPAC; 500 pg/10 μl iso-HVA (sensitivity=1 nA on both channels).

The linear relationship is expressed by r^2 in the range 0.5–10 pg/10 μl for 5-HT and DA, 5–100 pg/10 μl for HVA, 100–1000 pg/10 μl for 5-HIAA, DOPAC and HVA (*). Channel 1 of the detector was set at 0.1 nA, channel 2 at 2 nA.

*=Without iso-HVA.

**=Taking iso-HVA into account.

equations, calculated with and without the external standard, iso-HVA. Good linearity was observed for all compounds in the concentration ranges studied.

The LOD of the various compounds were less than 1 fmol on column, except for HVA which was almost 8 fmol on column. They were defined as the amount corresponding to a signal-to-noise ratio of 3. Other assays, measuring DA or/and 5-HT and their respective metabolites [4,5,16,17] showed similar sensitivity.

A system suitability test was carried out by injecting a standard solution (2.5 pg/10 μl 5-HT, DA; 5 pg/10 μl HVA; 250 pg/10 μl 5-HIAA and DOPAC; 500 pg/10 μl iso-HVA) six times into the chromatographic system. The same test was done using a more concentrated standard solution, except for DOPAC and 5-HIAA, (10 pg/10 μl DA, 5-HT; 200 pg/10 μl HVA; 100 pg/10 μl DOPAC, 5-HIAA; 500 pg/10 μl iso-HVA). Coefficients of variation were calculated for the capacity factors and peak areas (Table 2). The precision of the peak of HVA (5

Table 3
Basal dialysate concentrations (mean of three rats) representative for rat hippocampus (H), striatum (S), substantia nigra pars compacta (SN_c) and substantia nigra pars reticulata (SN_r), expressed as fmol/20 μl (DA, 5-HT) or pmol/20 μl[a] dialysate (DOPAC, 5-HIAA, HVA)

Compound	Brain area			
	H	S	SN_c	SN_r
DA	5.3	20.2	6.0	4.9
DOPAC[a]	3.5	21.3	1.3	1.3
5-HIAA[a]	5.2	2.2	4.8	5.9
HVA[a]	0.5	15.8	1.8	0.2
5-HT	12.5	2.3	3.8	4.6

pg/10 μl) was worse than for the other compounds. Indeed, in this assay, at higher sensitivity, a disturbance in the baseline prevented good precision of the HVA peak. This disturbance possibly originates from changes in the equilibrium between the ion-pairing reagent in the mobile phase and the stationary phase when a sample with different ionic composition is injected [18]. However, this problem could not be circumvented by adapting the composition of the sample to that of the mobile phase.

Basal dialysate concentrations of different brain areas are given in Table 3. These values (expressed as fmol or pmol per 20 μl of dialysate) are in agreement with previous studies [4,9,17]. Fig. 3B and Fig. 3C show a dual channel chromatogram of a microdialysate from the striatum illustrating that 5-HT, DA and their respective metabolites can be monitored simultaneously.

4. Conclusions

The excellent stability of the neurotransmitters and their metabolites in the antioxidant mixture containing acetic acid, disodium edetate, L-cysteine and ascorbic acid allows long-term analysis (ca. 20 h) of a high number of microdialysates. The automated microbore LC–EC determination of 5-HT, DA, 5-HIAA, DOPAC and HVA is reliable, reproducible and sensitive. Taking the limits of detection into consideration, dialysate levels of the catechols and indoles can easily be measured in different brain areas.

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