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Monitoring of dopamine and its metabolites in brain microdialysates: Method combining freeze-drying with liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive assay method was developed for a parallel, rapid and precise determination of dopamine and its metabolites, homovanillic acid, 3-methoxytyramine and 3,4-dihydroxyphenylacetic acid, from brain microdialysates. The method consisted of a pre-treatment step, freeze-drying (lyophilization), to concentrate dopamine and its metabolites from the microdialysates, and a detection step using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). In particular, the reaction monitoring mode was selected for its extremely high degree of selectivity and the stable-isotope-dilution assay for its high precision of quantification. The developed method was characterized by the following parameters: the precision of the developed method was determined as ≥88.6% for dopamine, ≥89.9% for homovanillic acid, ≥86.1% for 3-methoxytyramine and ≥88.1% for 3,4-dihydroxyphenylacetic acid; the mean accuracy was determined as ≥88.2% for dopamine, ≥88.3% for homovanillic acid, ≥85.9% for 3-methoxytyramine and ≥88.6% for 3,4-dihydroxyphenylacetic acid. The developed method was compared to (1) other combinations of pre-treatment methods (solid phase extraction and nitrogen stripping) with LC–MS and (2) another detection method, liquid chromatography, with electrochemical detection. The novel developed method using combination of lyophilization with LC–ESI-MS/MS was tested on real samples obtained from the *nucleus accumbens* of rat pups after an acute methamphetamine administration. It was proven that the developed assay could be applied to both a simultaneous analysis of all four substrates (dopamine, homovanillic acid, 3-methoxytyramine and 3,4-dihydroxyphenylacetic acid) in microdialysis samples acquired from the rat brain and the monitoring of their slight concentration changes on a picogram level over time following methamphetamine stimulus.

1. Introduction

During the last decades it has become apparent from experimental studies that catecholamine neurotransmission is a focal point of research in neuroscience. In particular, dopamine (DA) is a major modulatory catecholamine in the brain involved in motor functions, mood, learning and reward. Furthermore, it plays an important role in several psychiatric disorders such as addiction, depression, schizophrenia and Parkinson's disease. The action of DA at the synapse is terminated by two main mechanisms: (1) DA is drawn back into the pre-synaptic neuron (reuptake) and recycled; (2) DA is sequentially transformed into metabolites in two metabolic phases. In the phase I of the biotransformation DA is metabolized enzymatically by catechol-*O*-methyltransferase (COMT) and/or by monoamine oxidase (MAO) to homovanillic acid (HVA), 3-methoxytyramine (3-MT) and 3,4-dihydroxyphenylacetic acid (DOPAC) (Fig. 1) [1]. The levels of DA metabolites produced in the phase I directly reflect the behavior of the dopaminergic system and provide vital information about the enzyme action and abnormalities [2]. Then, the metabolic phase II produces hydrophilic conjugates (glucuronides and sulfates) of DA and its phase I metabolites [3].

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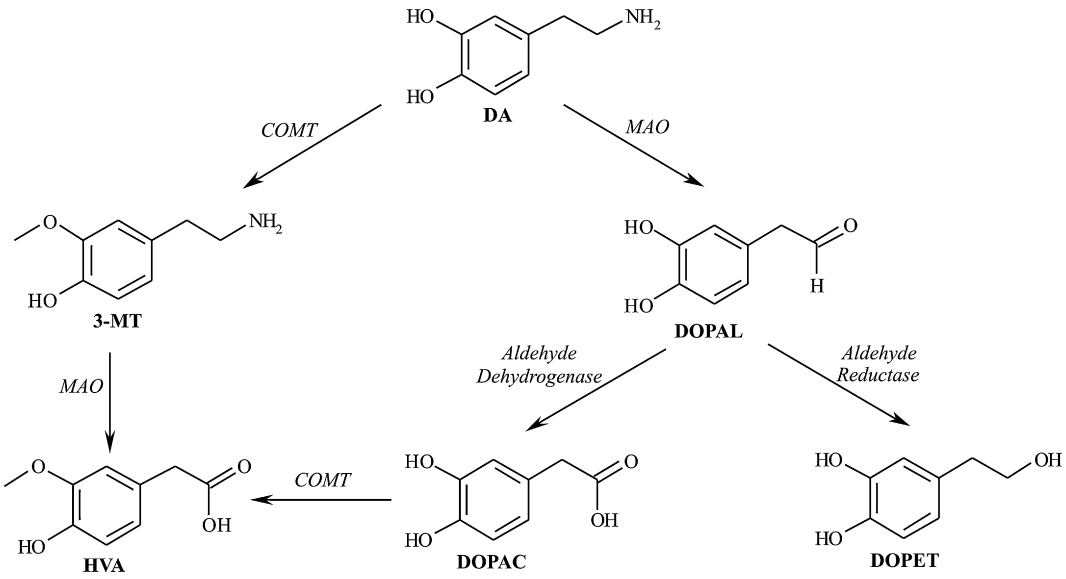

Fig. 1. Enzymes catalyzed metabolic pathways of dopamine: COMT = catechol-*O*-methyl transferase; MAO = mono amine oxidize; DA = dopamine; 3-MT = 3-methoxy-4-hydroxyphenethylamine; HVA = 3-methoxy-4-hydroxyphenylacetic acid; DOPAL = 3,4-dihyroxyphenylacetaldehyde; DOPAC = 3,4-dihydroxyphenylacetic acid; DOPET = 3,4-dihydroxyphenylethanol.

An acute administration of methamphetamine (MA) elevates extracellular levels of DA and 3-MT and decreases those of DOPAC and HVA [4,5]. Nevertheless, increased HVA levels after high MA doses have been also reported and thus the results are rather contradictory [6,7]. Some studies revealed that exposure to MA during pregnancy could impair the development of the neonatal central nervous system in rat pups [8].

The metabolism of neurotransmitters in the brain can be studied by microdialysis, where physiological perfusion fluid is pumped through a dialysis membrane that is surgically implanted into an animal brain region of interest [9]. The brain extracellular fluid contains synaptically released neurotransmitters and their metabolites, as well as compounds from non-synaptic sources [10]. These low molecular weight compounds in the extracellular fluid are extracted to the perfusion fluid by passive diffusion. The recovery of these compounds is dependent on many variables including temperature, molecular weight and charge, flow rate of the perfusion fluid and the surface area of the dialysis membrane [11].

Different analytical techniques have been employed to monitor DA and its metabolites (HVA, 3-MT, DOPAC) in biological samples, such as chromatography with fluorescence (FL), chemiluminescence (CL), ultraviolet (UV), electrochemical (EC) and mass spectrometry (MS) detection [10–14]. The most popular and successful technique widespread in the neurosciences has been high-pressure liquid chromatography (HPLC) coupled with electrochemical detection (ECD). Although this has long been the method of choice, it has disadvantages in the lengthy equilibration required and time-consuming analysis. Furthermore, analytes can only be identified by their retention times as is common with chromatographic methods. HPLC with mass spectrometry (HPLC–MS) is an alternative approach to clearly identify an analyte. The separation of analytes has commonly been achieved by reversed-phase liquid chromatography, utilizing an ion-pairing reagent. The derivatization of neurotransmitters has also been used to improve their separation and ensure sensitive detection by HPLC-FL as the concentrations of neurotransmitters in the brain microdialysates were extremely low (picomole and femtomole concentrations) [15]. Reversed-phase chromatography using volatile eluents has usually been used for HPLC of DA and its metabolites [16–18]. Since these polar analytes were only weakly retained on C18, a pentafluorophenylpropyl stationary phase was utilized to enhance the retention and achieve an adequate separation of the analytes from the inorganic salts of the artificial cerebrospinal fluid used in the microdialysis [3].

The present work deals with the development of a highly selective and precise analytical method for the simultaneous determination of DA and its metabolites (HVA, 3-MT and DOPAC) in brain microdialysis samples. The method consists of the pre-concentration step, lyophilization and LC–ESI-MS/MS detection. During the method development, three different preconcentrations were tested (lyophilization, solid phase extraction (SPE), nitrogen stripping) and compared. The methods were validated and compared with LC-ECD, the most frequently used method for the detection and quantification of neurotransmitters and their metabolites. The developed method (lyophilization and LC–ESI-MS/MS) was utilized for the analyses of *in vivo* samples from the *nucleus accumbens* collected after an acute MA administration. It was unambiguously demonstrated that the assay could be applied to the simultaneous analysis of all four analytes (DA, HVA, 3-MT and DOPAC) in microdialysis samples drawn from the rat brain and that it was sensitive enough for monitoring small changes in concentration over time.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of commercial origin: 3-hydroxytyramine hydrochloride (=dopamine hydrochloride; =DA·HCl, ≥99.0%; Sigma–Aldrich, USA); homovanillic acid (=HVA, ≥99.0%; Sigma–Aldrich, USA); 3,4-dihydroxyphenylacetic acid (=DOPAC, ≥99.0%; Sigma–Aldrich, USA); hydrochloric acid (37%; Sigma–Aldrich, USA); sodium chloride (99.5%; Sigma–Aldrich, USA); magnesium chloride (purum; Sigma–Aldrich, USA); calcium chloride (anhydrous, 93%; Sigma–Aldrich, USA); potassium chloride (purum; Sigma–Aldrich, USA); citric acid (≥99.5%; Sigma–Aldrich, USA); ethylenediaminetetraacetic acid (=EDTA, 99.99%; Sigma–Aldrich, USA); sodium acetate (anhydrous, ≥99.0%; Sigma–Aldrich, USA); sodium hydroxide (99.99%; Sigma–Aldrich, USA); [1,1′,2,2′^{2}H$_4$] dopamine hydrochloride (=DA·HCl-d_4, ≥98.0%; Cambridge Isotope Laboratories Inc., USA); 3-methoxytyramine hydrochloride (=3-MT·HCl, ≥99.0%; Fluka, Switzerland), water

(LC–MS grade; Fluka, Switzerland); methanol (LC–MS grade; Fluka, Switzerland); acetic acid (99.9%; Fluka, Switzerland); trifluoroacetic acid (=TFA, ≥99.0%; Fluka, Switzerland); sodium 1-octanesulfonate monohydrate (≥99.0%; Fluka, Switzerland); ketamine hydrochloride (Narkamon® 5%; Vétoquinol, France); xylazine hydrochloride (Rometar® 2%; Bioveta, Czech Republic); halothane (Narcotan® 0.01%; Zentiva, Czech Republic).

D-Methamphetamine hydrochloride (=MA·HCl, 99.8%) used in the animal study was prepared by the Faculty of Pharmacy of Charles University in Hradec Králové (Czech Republic).

The artificial cerebrospinal fluid, used for the microdialysis and dilution of the standards, contained 147.0 mM sodium chloride, 1.3 mM calcium chloride, 0.9 mM magnesium chloride and 4.0 mM potassium chloride in water (pH 6.5–7.0).

2.2. Microdialysis experiment

Guide cannulae were implanted on the 90th postnatal day under ketamine (2 mg/kg, *i.p.*) and xylazine (0.5 mg/kg, *i.p.*) anesthesia. The MAB 4.15 IC (Agn Tho's AB, Sweden) guide cannulae were implanted 2 mm above the *nucleus accumbens* at the stereotaxic coordinates AP-1.6, ML ± 1.5, DV-7.3 according to the atlas of Paxinos and Watson [19]. The rats were allowed to recover for seven days before the experiments commenced.

The microdialysis experiments were carried out on anesthetized rats using halothane. Microdialysis probes MAB 4.15.2.Cu (Agn Tho's AB, Sweden), (cuprophane 0.2 mm membrane; cut off 6 kDa) were inserted through the guide cannula into the *nucleus accumbens*. The probes were perfused by the ACSF solution at a flow rate of 4 μl/min (60 min) using a microinjection pump (Univentor 864, Agn Tho's AB, Sweden). Then, the dialysates were collected every 20 min into plastic vials containing hydrochloric acid (20 μl of 0.1 M). Three microdialysis samples were collected prior to the drug administration, and the sampling continued for 180 min after the drug was administered, i.e. a total of 12 samples were collected (each containing 80 μl of the dialysate and 20 μl of hydrochloric acid). Each sample was spiked immediately after the collection with an internal standard (=IS; 10 pg DA-d_4) and frozen at −80 °C for a period not exceeding 1 month. The locations of the microdialysis probes were verified histologically at the end of the study.

2.3. Pre-treatment methods

2.3.1. Freeze-drying (lyophilization)

The frozen microdialysis sample (−80 °C, 80 μl of the dialysate and 20 μl of hydrochloric acid) together with the IS (DA-d_4 10 pg) was inserted into a freeze dryer (Labconco Free Zone, USA) for 2 h. The freeze dryer condenser coil was cooled to −47 °C and the pressure in the device had stabilized to 9 kPa. The lyophilization residue was dissolved in methanol (10 μl) and vortexed (2 min). Suspension of precipitated salts was centrifuged (2 min; 700 g) and the supernatant was immediately analyzed by LC–ESI-MS/MS. The sample was kept in the dark throughout the processing to prevent any light-induced changes.

2.3.2. Solid phase extraction

The non-polar SPE PerfectPure C18 tip (Eppendorf, Germany) was preconditioned with methanol (250 μl) and with an aqueous solution of trifluoroacetic acid (2.5% TFA in water, 40 μl). The sample, spiked with the IS (DA-d_4, 10 pg), was soaked onto the tip adsorbent and washed with water (40 μl). The substances anchored onto the adsorbent of the SPE tip were eluted with methanol (50 μl). The solvent was stripped off with nitrogen and the sample was re-suspended in methanol (10 μl). The sample was immediately analyzed by LC–ESI-MS/MS.

2.3.3. Nitrogen stripping

The stripping needle with flowing nitrogen was inserted into the microdialysis sample (80 μl of the dialysate and 20 μl of hydrochloric acid with the IS – DA-d_4, 10 pg) for a period sufficient for the complete drying of the sample. The residue was re-suspended in methanol (10 μl). The sample was immediately analyzed by LC–ESI-MS/MS.

2.3.4. LC-ESI-MS/MS – analysis apparatus and conditions

A Varian ProStar HPLC system, consisting of a ProStar 210 dual pump, a degasser, Varian 410 autosampler (Varian, USA) equipped with Gemini 5 μ C18 110 Å 150 mm × 2 mm column with a Gemini pre-column (Phenomenex, USA), was used. The mobile phase (solvent A: aqueous solution of acetic acid (pH 2); solvent B: methanol) was used for a gradient elution at a flow rate of 150 μl/min. The HPLC elution program was as follows: 5% B (3 min) → 30% B (linear increase in 2 min) → 30% B (10 min) → 5% B (linear decrease in 1 min) → 5% B (4 min). The column temperature was maintained at 25 °C. The injection volume was 5 μl.

The HPLC system was directly coupled to a Varian 1200L triple quadrupole mass spectrometer (Varian, USA) equipped with an electrospray ion source operating in the positive ion mode ESI for the measurement of DA with a deuterium labelled IS DA-d_4, 3-MT and in the negative ion mode ESI for the measurement of HVA and DOPAC. The selective reaction monitoring (SRM) mode was used. The product ion fragments selected corresponded to the maximum intensities for the analyte and the deuterated IS, ensuring maximum sensitivity. The scan monitoring reactions (precursor ion → fragment ion) used for the analyses and their collision induced dissociated (CID) energy were as follows: m/z 137 → m/z 120 (precursor ion → fragment ion) (CID = −17.5 eV) for DA; m/z 141 → m/z 123 (CID = −17.5 eV) for DA-d_4; m/z 181 → m/z 122 (CID = 17.0 eV) for HVA; m/z 168 → m/z 151 (CID = −11.5 eV) for 3-MT and m/z 167 → m/z 122 (CID = 8.5 eV) for DOPAC. The dwell time for each analyte was set up to 0.05 s. The MS measurement was divided into two segments; the first was operated in positive ion ESI mode (0.0–6.0 min), whereas the second was in negative ion ESI mode (6.1–20.0 min). CID was performed under argon at a pressure of 2.2 mTorr with a capillary voltage of 65 V (positive ion ESI) and −70 V (negative ion ESI). A needle voltage of 5000 V (positive ion ESI) or −4500 V (negative ion ESI) was used. The temperature of the ESI ion source was 50 °C. Air (Siad, Czech Republic) was used as the nebulizing gas in negative ion ESI mode (50 psi), whereas nitrogen (Siad, Czech Republic) was used as the nebulizing gas in positive ion ESI mode (50 psi) and as the drying gas (17 psi, temperature 250 °C). Data were acquired and evaluated using ProStar version 6.91 (Varian, USA).

2.4. LC-ECD analysis, apparatus and conditions

The HPLC system consisted of an ESA 582 pump and ESA CoulArray 5600A multi-electrode array detector with one Model 6210 cell with four working electrodes (ESA Biosciences Inc., USA). Separation was achieved on a Gemini 5 μ C18 110 Å 150 mm × 2 mm column with a Gemini pre-column maintained at 33 °C. The water mobile phase contained 45 mM sodium acetate, 30 mM citric acid, 1.5 mM sodium 1-octanesulfonate, 1.5 mM EDTA and 6% methanol at pH 4.5 (adjusted by sodium hydroxide solution). The flow rate was 150 μl/min and the injection volume was 50 μl. The analytical cells were set at +100, +200, +300 and +400 mV. The current response was recorded and the data evaluated using CoulArray Data Station 3.0 (ESA Biosciences Inc., USA).

2.5. Validation of assay procedure

2.5.1. Standard preparation and calibration procedure

The stock solution (concentration of 1 mg/ml) was freshly prepared daily by dissolving DA·HCl, HVA, 3-MT·HCl and DOPAC in water. It was used for the preparation of other solutions with the following concentration levels: 0.5; 1; 5; 10; 25; 50; 100; 200; 300; 400 and 500 pg/10 μl. The prepared calibration solutions were spiked with the IS (DA-d_4; 10 pg/10 μl). The peak area of DA, HVA, 3-MT or DOPAC with the assigned IS was used as the function to quantify concentrations.

2.5.2. Precision and accuracy

The precision and accuracy of the developed method were determined by the analysis of five concentration levels of DA (0.026, 0.529, 2.645, 5.290 and 13.224 nM), HVA (0.027, 0.549, 2.745, 5.489 and 13.723 nM), 3-MT (0.025, 0.493, 2.467, 4.934 and 12.336 nM) and DOPAC (0.030, 0.595, 2.974, 5.947 and 14.868 nM) with a constant level of IS DA-d_4 (0.518 nM). Each concentration was replicated five times ($n = 5$). The samples were submitted to the pre-treatment procedure (lyophilization, SPE or stripping by nitrogen) followed by LC–ESI-MS/MS or analyzed by LC-ECD. Subsequently, the mean of each set of concentrations, the standard deviation (SD) and the relative standard deviation (RSD) were calculated and the value of the precision parameter was determined. The accuracy of the method was calculated as the difference between the standards (corresponding concentration level of a particular substrate was dissolved in water and analyzed by LC–ESI-MS/MS), and the samples prepared by the pre-treatment procedure. The relative error (RE) and the recovery were calculated on this basis.

2.5.3. Limit of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined using the assay of the blank matrix (mixture of 80 μl of ACSF contained 10 pg of DA-d_4 and 20 μl of hydrochloric acid was pre-treated with LC–ESI-MS/MS detection or analyzed by LC-ECD; $n = 5$). The values of LOD and LOQ were calculated as a mean value of blank matrix treble signals or ten times the amount of the standard deviation respectively.

2.6. Stability experiments

2.6.1. Stability testing conditions

The stability of DA, HVA, 3-MT and DOPAC was tested in water at 25 °C under normal light conditions for two days. In parallel, the stability of substances in methanol under identical temperature and light conditions was tested. For long term storage of samples, the stability of DA, HVA, 3-MT and DOPAC was monitored in ACSF solution at −80 °C for one month.

2.6.2. Stability test instrumentation

NMR spectra were recorded on Bruker AVANCE III 600 MHz (600.23 MHz for ^{1}H, Bruker BioSpin GmbH, Rheinstetten, Germany) in D_2O at 25 °C. The ^{1}H NMR spectrum was measured using manufacturers' standard software. It was zero filled to fourfold data points and a line broadening (0.3 Hz) was applied to improve the signal to noise ratio.

2.7. Animals

Female Wistar rats (Anlab farms, Czech Republic) weighing between 250 and 300 g and aged 4 months were used for the experiment. The rats were fed by food and water *ad libitum*. The animals were housed in pairs in plexiglass cages at ambient temperature (24 °C) with a 12 h light–dark cycle (start of light cycle at 7:00 am).

Female rats were randomly assigned to a MA exposed group and a control group (saline treated). MA rats were injected daily subcutaneously (*s.c.*) with a dose of 5 mg/kg from the first to the last day of gestation. Then the pups were divided into a MA prenatally exposed group ($n = 6$) and a control group ($n = 6$). The experiments were performed in conformity with the Animal Protection Laws of the Czech Republic and complied in full with the European Communities Council requirements relating to the use of experimental animals (Directive 86/609/EEC).

2.8. Statistical analysis

The acquired data were statistically analyzed using Statistica version 6.0. The statistical analyses were performed using Student's *t*-test, where *P* values were considered significant if $P < 0.05$.

3. Results and discussion

3.1. Stability of analytes

The stability of analytes in biological matrices is a critical consideration in the selection of an appropriate method to prepare the samples for analysis (pre-treatment method) and in the validation of an analytical method. The problems resulting from the stability of the substance can be minimised by performing replicate analyses, i.e. assuring that all samples are treated alike and measured at the same time. However, this process is not feasible for biological samples that are usually collected at different times and/or require storage before analysis.

The monitored substances, especially DA in microdialysis samples, were typically unstable in samples stored under normal laboratory conditions. Dissolved oxygen, daylight and temperature are reported as the main causes of sample degradation [20,21]. Stability tests were performed in both an aqueous solution simulating the ACSF and a methanolic solution used as the solvent for LC–ESI-MS/MS analysis. The stability of DA, HVA, 3-MT and DOPAC was monitored in water and methanol at 25 °C under standard laboratory light conditions for two days. Moreover, the long term storage of samples was experimentally evaluated (−80 °C, absence of daylight and an ACSF solution were selected as the environmental parameters). In contrast, DA·HCl exhibited higher stability. Therefore, the stabilities of DA and 3-MT were also determined in their hydrochloride form.

The stability tests were carried out using Fourier Transform ^{1}H NMR. The standard of a particular substance was dissolved in D_2O and immediately measured. Fig. 2 shows the stability measurements of DA and DA·HCl where the regions of aromatic signals are depicted. The signals of satellites, due to the natural abundance of ^{13}C isotopes, are marked with asterisks. The obtained spectrum of DA (Fig. 2A) served as a reference for possible changes. The kinetics of the DA decomposition was followed by NMR (Fig. 2B). Changes in the ^{1}H NMR spectrum and the rate of decomposition were observed and evaluated. DA was decomposed at a rate of 0.108%/h, corresponding to about 5.2% within two days. The spectrum of DA·HCl (Fig. 2C) served as a reference for possible changes during the storage of DA in the hydrochloride form. The solution had been left under daylight for two days. The spectrum after this period exhibited only minor changes and thus we did not expect any substantial decomposition of DA·HCl in our samples (Fig. 2D). The only exception was the change in the colour of the solution that occurred during the measurement period (the colour became grey over time) but the decrease in concentration was determined as less than 1.0% in 48 h. All the other analytes under the test range of parameters (methanolic and aqueous solutions, temperatures of 5, 20 and 35 °C, presence and absence of daylight) showed high stabil-

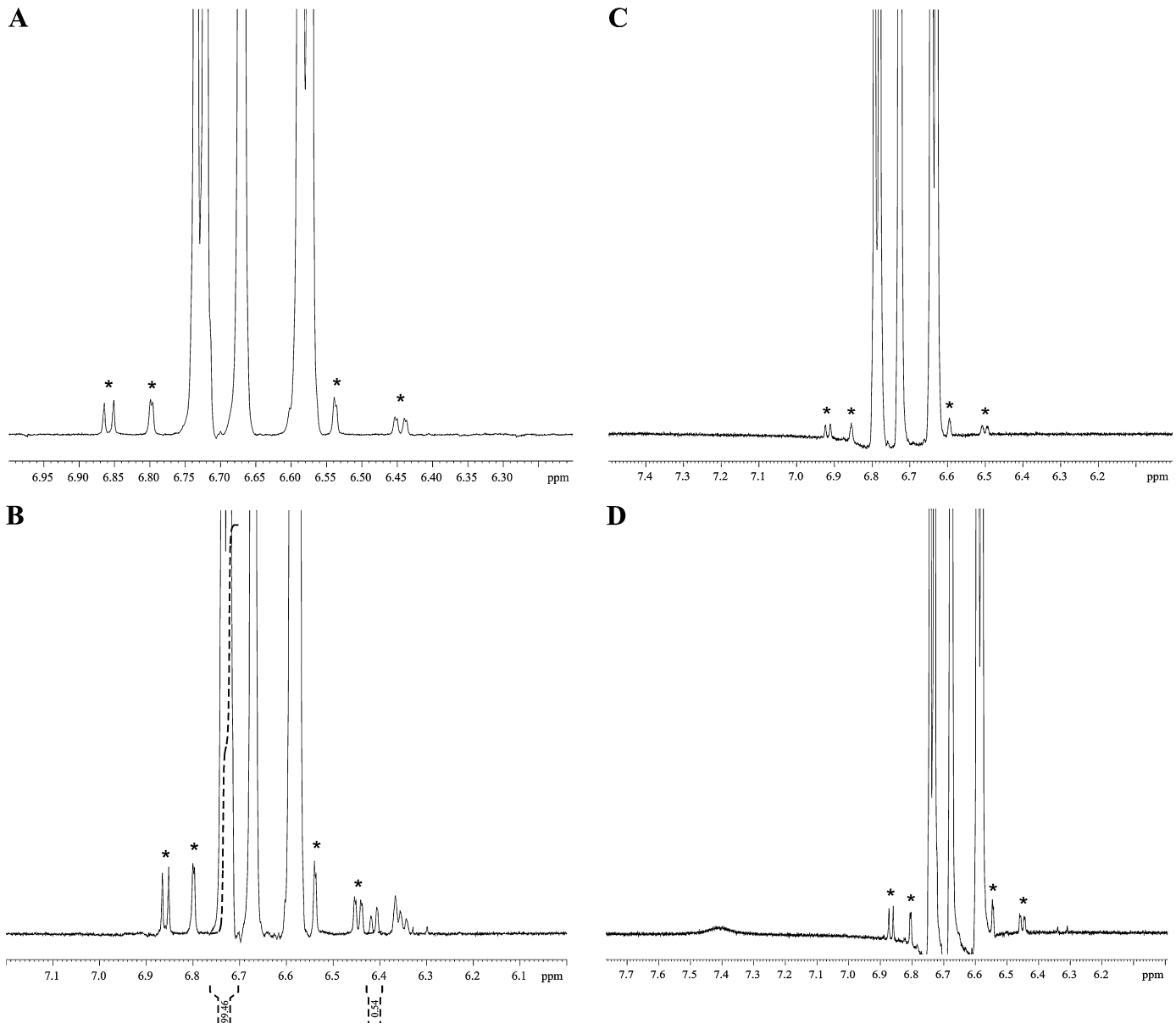

Fig. 2. Dopamine stability determined by ^{1}H NMR spectra (the region of aromatic signals). (A) DA in D_2O; received immediately after dilution. (B) DA in D_2O; received after 5 h. (C) DA·HCl in D_2O; received immediately after dilution. (D) DA·HCl in D_2O; received after 2 days.

ity and also an absence of colour changes. Long-term stability was tested in the ACSF for one month at −80 °C in the absence of daylight (storage conditions). During this period, the degradation of DA and its metabolites had been monitored, while the concentration level of DA had decreased by 2.8%, HVA by 2.1%, 3-MT by 2.3% and DOPAC by 1.9%. Due to the lower stability of DA in its free form, microdialysis samples (80 μl) were collected in Eppendorf flasks containing hydrochloric acid (20 μl), which guaranteed the immediate transformation of DA to its hydrochloride. Additionally, an isotope-labelled standard (DA·HCl-d_4) of the monitored substances (DA·HCl, HVA, 3-MT·HCl and DOPAC) was added to the microdialysis samples immediately after their collection to minimize the influence of sample storage on the processing of the results.

3.2. Pre-concentration method – freeze-drying (lyophilization)

Lyophilization is an excellent method for concentrating non-volatile (or *semi*-volatile) substances dissolved in water and highly suitable for heat-labile or light-labile substances, was used as the pre-concentration method for DA, HVA, 3-MT and DOPAC. During freeze-drying of the microdialysis samples, no temperature-, light- or air-stress on DA and its metabolites occurred, which would have led to the preference for lyophilization over other potential pre-treatment methods (e.g. SPE, nitrogen stripping etc.).

Pre-frozen microdialysis samples (−80 °C; microdialysate 80 μl; hydrochloric acid 20 μl and 10 pg of isotope-labelled standard DA·HCl-d_4) were introduced into a lyophilizer for 2 h. The lyophilized residue was dissolved in methanol (10 μl) and vortexed. Suspension of precipitated salts was centrifuged (2 min; 700 g) and the supernatant was immediately analyzed by LC–ESI-MS/MS (the addition of methanol and vortexing caused precipitation of the salts and dissolution of DA·HCl and its metabolites).

3.3. LC-ESI-MS/MS optimization

Liquid chromatography separation was performed on a Gemini C18 column, where the mobile phase consisted of water with pH adjustment by acetic acid to 2 (eluent A) and methanol (elu-

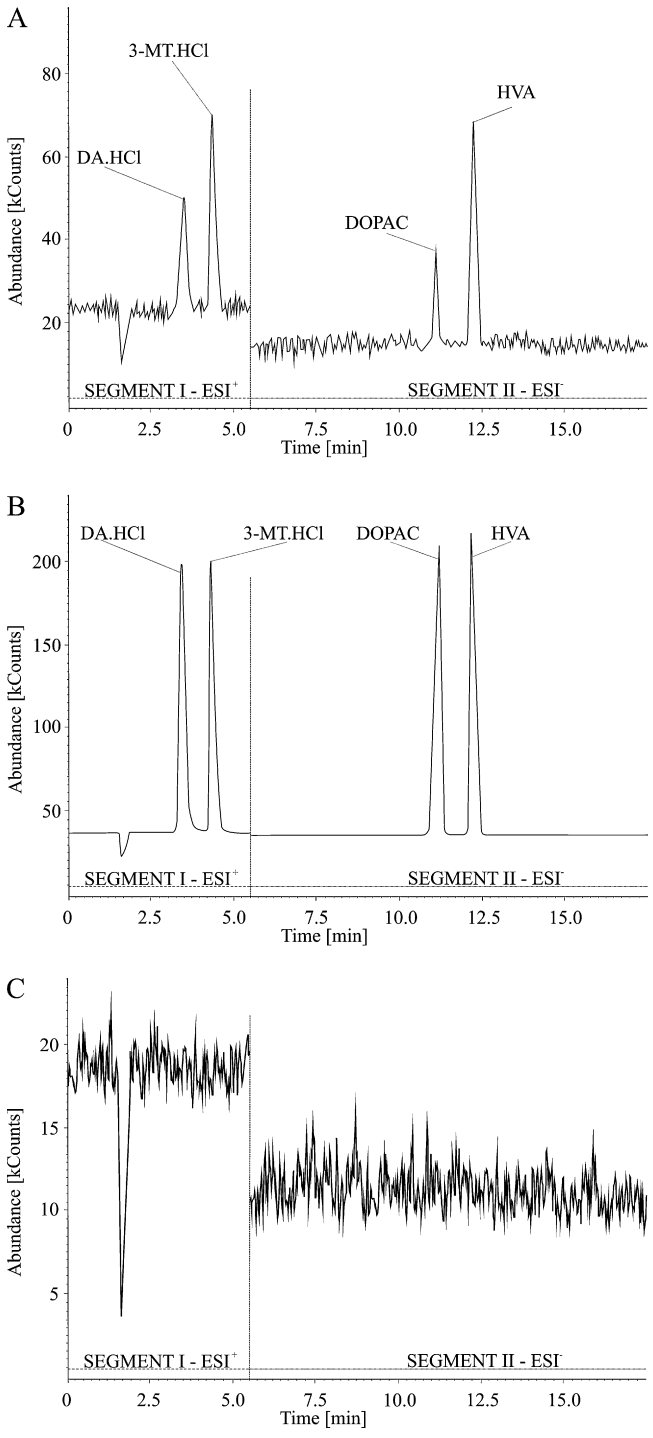

Fig. 3. LC–ESI-MS/MS chromatograms with marked ionization modes (positive ion ESI and negative ion ESI segment). (A) Chromatogram of a microdialysate sample (after lyophilization). (B) Chromatogram of a microdialysate sample spiked with DA·HCl and its metabolites (50 pg of each) (after lyophilization). (C) Chromatogram of a blank ASCF (after lyophilization).

ent B) was used for a gradient elution at a flow rate of 150 μl/min. The LC conditions allowed the retention of the eluted substance of interest from the solvent front, thus avoiding a signal suppression effect during the mass spectrometry ionization due to salts from the ACSF co-elution (the dead time of the column was 0.8 min). Fig. 3 shows the chromatogram obtained under optimal analytical conditions, demonstrating the successful separation of all the analytes (DA, HVA, 3-MT and DOPAC; retention time of DA-d_4 was equal to DA). LC conditions were optimized not only with respect to optimal chromatographic separation, but also to achieve maximum sensitivity of the mass spectrometry detection.

ESI-MS/MS in SRM mode represents a highly sensitive and selective tool for analyses of neurotransmitters and their metabolites in biological matrixes, where the particular neurotransmitter or its metabolites are present in very low concentrations (femto- or picograms per milliliter). The LC–MS method, used for the determination and quantification of DA, HVA, 3-MT and DOPAC, was divided into two segments differing in the mode of electrospray ionization, as shown in Fig. 3. DA, DA-d_4 and 3-MT were eluted in segment I (0–6.0 min), and detected in positive ion ESI. The analysis of DA in negative ion ESI mode was less sensitive, as demonstrated by the higher values for the limit of detection and limit of quantification (LOD = 1.43 nM and LOQ = 2.88 nM). Equivalent behavior with regards to both LOD and LOQ was observed for 3-MT, therefore the positive ionization mode was used as well (Fig. 4B). The analysis of DOPAC and HVA was performed in negative ion ESI in segment II (6.1–20.0 min). Fig. 4C and D shows the negative ion ESI-MS spectra of HVA and DOPAC. The application of negative ion ESI for HVA and DOPAC as the optimal method can be explained by their acidic characters.

The product ion mass spectra were recorded after the collision induced dissociation (CID) in the second quadrupole (Q2) of the deprotonated or protonated molecule isolated in the first quadrupole (Q1) as $[M-H]^-$ of HVA and DOPAC, $[M+H]^+$ of 3-MT and the deaminated protonated molecule of DA ($[M-NH_3+H]^+$) in the first quadrupole (Q1), and the final ion scanning in the third quadrupole (Q3) (Fig. 5). The protonated molecule $[M+H]^+$ of DA with m/z = 154 Da was not used as the precursor ion because of its lower abundance compared with the deaminated fragment ion with m/z = 137 when an acceptably low LOD and LOQ were received (see Section 3.4). The most intense product ions were used for the SRM mode, ensuring maximum sensitivity. The effects of instrument parameters (capillary voltage, needle voltage and temperature of ESI ion source) on the sensitivity were investigated. Maximum intensities of the product ions were obtained under the analytical conditions for LC mentioned above with the ion source parameters described in the experimental section as a result of a series of optimization experiments. As Fig. 3 shows, the results were not influenced by other substances potentially present in the microdialysate samples.

3.4. Validation of the method

The accuracy and precision parameters were assessed by analyzing four different concentration levels of the particular substances (DA, HVA, 3-MT and DOPAC). The precision (RSD) was within the interval of 8.0–11.4% for DA, 7.6–10.1% for HVA, 8.5–13.9% for 3-MT and 8.7–11.9% for DOPAC. The accuracy (RE) varied from −4.4 to −11.8% for DA, −4.8 to −11.7% for HVA, −5.7 to −14.1% for 3-MT and −7.1 to −11.4% for DOPAC. The developed method enabled the use of the following criteria: an acceptable value of RSD for the concentration interval of LOQ-0.6 nM was lower than 20% and for the concentration interval of 0.6–15 nM, it was lower than 10%; an acceptable value of RE for the concentration interval of LOQ-0.6 nM lay within the interval ±20% and for the concentration interval of 0.6–15 nM, it lay within the interval ±10%. The values of the precision and accuracy parameters for each particular substrate are summarized in Table 1. Calibration graphs were constructed as the dependence of the ratio of a particular substrate peak area to the deuterium-labelled IS peak area (*x*-axis) *versus* the substrate concentration (*y*-axis). A least squares regression analysis was applied. The Pearson's regression correlation coefficients (R^2) were higher than 0.998 for all the analytes calibration curves (the acceptable value of R^2 for each calibration curve was $R^2 \geq 0.99$). The back-

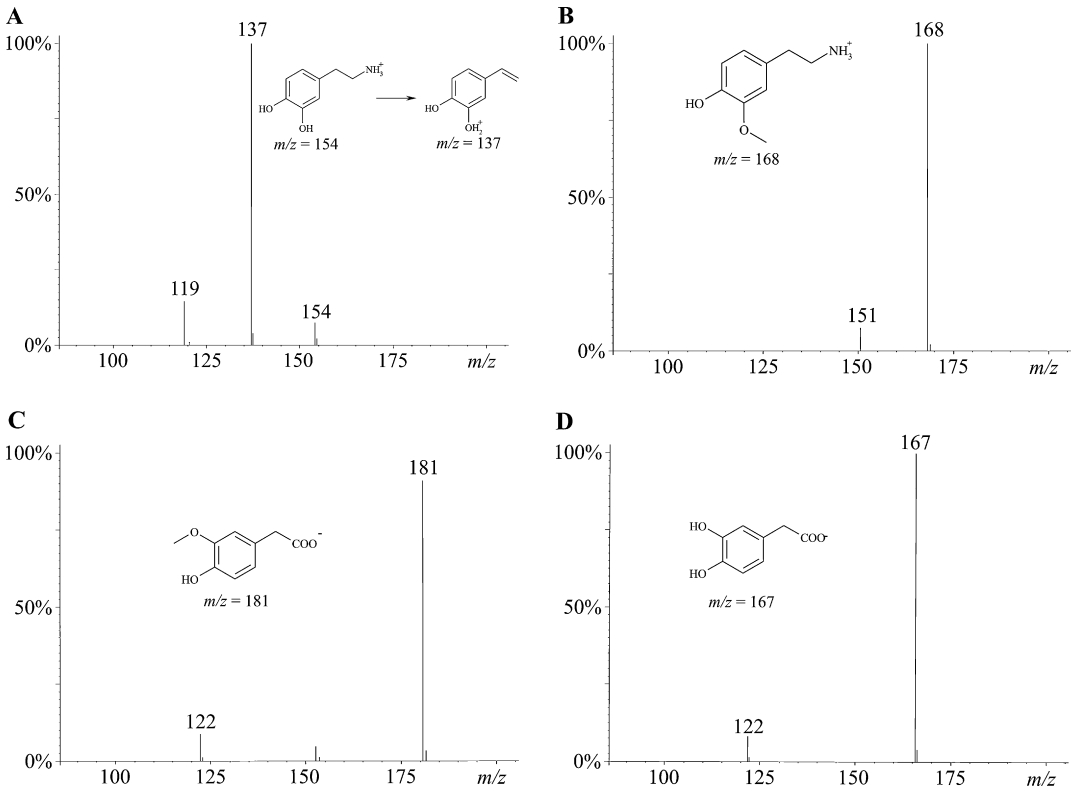

Fig. 4. (A) MS spectrum of DA in positive ion ESI mode. (B) MS spectrum of 3-MT in positive ion ESI mode. (C) MS spectrum of HVA in negative ion ESI mode. (D) MS spectrum of DOPAC in negative ion ESI mode.

calculated values of the calibration points were in good agreement with the theoretical concentrations with relative standard deviations between 2.9 and 9.7% of the nominal concentrations. Table 2 depicts variations in the calibration curves prepared in the course of the study (inter-day variance), characterized by the standard deviation and the relative standard deviation ($n = 10$). The calibration graphs were constructed for the ACSF matrix as well. The calibrations for particular substances in water compared to ACSF exhibited no substantial differences in the values of correlation coefficients, slopes and intercepts of the calibration curves (Table 2). Based on the results obtained in ACSF and water solutions any matrix effect was excluded.

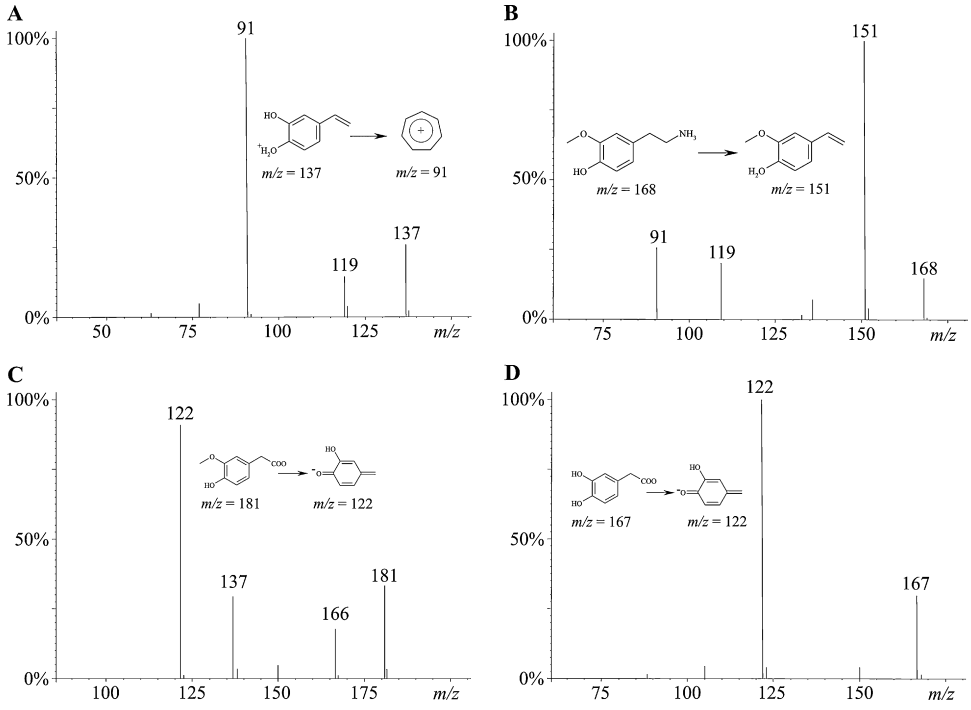

Fig. 5. ESI-MS/MS spectra of DA (A), 3-MT (B), HVA (C) and DOPAC (D).

Table 1
Method (lyophilization – LC–ESI-MS/MS) validation: accuracy, precision and recovery parameters for different concentrations in ACSF samples.

Added amount (nM)	Deter. am. (mean) (nM)	SD (nM)	Precision RSD (%)	Accuracy RE (%)	Recovery (%)
DA					
0.026	0.023	2.64×10^{-3}	11.4	−11.8	88.2
0.529	0.493	4.81×10^{-2}	9.2	−6.8	93.2
2.645	2.497	2.29×10^{-1}	9.1	−5.6	94.4
5.290	5.010	4.07×10^{-1}	8.1	−5.3	94.7
13.224	12.648	1.07	8.2	−4.4	95.6
HVA					
0.027	0.024	2.20×10^{-3}	10.1	−11.7	88.5
0.549	0.511	4.50×10^{-2}	8.8	−6.9	93.1
2.745	2.614	2.04×10^{-1}	7.8	−4.8	95.2
5.489	5.242	3.99×10^{-1}	7.6	−4.5	95.4
13.723	12.747	1.02	8.0	−7.2	92.8
3-MT					
0.025	0.021	2.96×10^{-3}	13.9	−14.1	86.1
0.493	0.453	5.03×10^{-2}	11.1	−8.2	91.8
2.467	2.290	2.37×10^{-2}	10.4	−7.2	92.8
4.934	4.611	4.07×10^{-1}	8.8	−6.4	93.5
12.336	11.636	9.95	8.5	−5.7	94.3
DOPAC					
0.030	0.026	2.97×10^{-3}	11.9	−11.4	88.6
0.595	0.543	5.47×10^{-2}	10.1	−8.7	91.3
2.974	2.746	2.52×10^{-1}	9.2	−7.6	92.3
5.947	5.515	4.85×10^{-1}	8.8	−7.3	92.7
14.868	13.805	1.20	8.7	−7.1	92.9

3.5. Method comparison

The presented method encompasses a pre-treatment lyophilization, allowing a rapid and effective concentration of DA and its metabolites (HVA, 3-MT, DOPAC) from microdialysis samples, combined with a highly selective and sensitive detection by LC-ESI-MS/MS. The developed method was compared with other pre-treatment methods combined with LC-ESI-MS/MS detection as well as with HPLC coupled with ECD, still the most popular and widespread technique in neurosciences. The other methods used in combination with MS detection were the pre-concentration and the pre-separation method, SPE performed on non-polar C18 tips and nitrogen stripping used for pre-concentrating the sample. The comparison of the methods is shown in Table 3 where the values of precision, accuracy, recovery, LOD and LOQ parameters are presented.

The analytical methods, where LC–ESI-MS/MS was used for the detection, demonstrated significantly lower limits of detection and quantification in comparison with LC-EDC (LOD – 3.71×10^{-3}–4.76×10^{-3} nM and LOQ – 4.24×10^{-3}–5.42×10^{-3} nM for LC–ESI-MS/MS; LOD – 4.72×10^{-1}–7.85×10^{-1} nM and LOQ – 5.32×10^{-1}–1.05 nM for LC-ECD). The advantage of LC-EDC is the absence of pre-treatment stage in the method; however, its disadvantage is the absence of qualitative information in comparison with MS methods. Moreover, it requires a lengthy equilibration and time-consuming analysis.

Table 2
Linear regression analysis of calibration curve ($n = 10$).

Substance	Slope	Intercept	SD	RSD [%]	R^2
Matrix: water					
DA	9.82	0.14	0.115	1.711	≥0.9994
HVA	10.38	0.11	0.189	1.821	≥0.9991
3-MT	14.86	0.18	0.267	1.796	≥0.9992
DOPAC	12.69	0.20	0.238	1.875	≥0.9989
Matrix: ACSF					
DA	9.95	0.10	0.182	1.827	≥0.9986
HVA	10.46	0.04	0.203	1.945	≥0.9983
3-MT	14.73	0.14	0.265	1.802	≥0.9984
DOPAC	12.76	0.18	0.244	1.914	≥0.9978

The pre-treatment methods connected with LC–ESI-MS/MS detection produced different values for the validation parameters (precision, accuracy and recovery). The SPE method demonstrated a significantly lower recovery and accuracy (recovery > 77.7% and RE < 21.3%) compared to the other pre-treatment methods. Lyophilization had identical recovery and accuracy values to stripping with nitrogen (RE < 14.1% for lyophilization; RE < 18.3% for nitrogen stripping and recovery > 86% for lyophilization; recovery > 83.7% for nitrogen stripping), but differed dramatically in terms of accuracy. With respect to nitrogen stripping, a significantly lower precision was achieved (RSD < 19.4%) compared to lyophilization (RSD < 13.9%).

3.6. Animal study

The developed analytical method was used for an experimental study determining the changes in concentration levels of DA, HVA, 3-MT and DOPAC with time in samples from brain microdialysis after acute MA exposure. The microdialysis samples were collected continuously from the *nucleus accumbens* of experimental rat pups, while the third sampling was followed by the MA application (1 mg/kg, *s.c.*). The levels of DA and its metabolites immediately responded to this stimulus by showing a change in concentration. The sampling continued for 180 min after the MA was administered. Each sample was immediately spiked after collection with an IS (10 pg DA-d_4) and frozen at −80 °C. Then, the sample was subjected to lyophilization and analyzed by LC–ESI-MS/MS. The typical time dependence of the concentration of DA and its metabolites before and after the MA administration is depicted in Fig. 6.

Two groups differing in MA exposure history were introduced to the experimental study. The experimental female rats were randomly assigned to an MA exposed group and a control group (saline treated). The MA exposed rats were injected daily with MA (5 mg/kg, *s.c.*) from the first to the last day of gestation. Then, the pups were divided into an MA prenatally exposed group ($n = 6$) and a control group ($n = 6$). The two groups were used in the experi-

Table 3
Validation of the methods used for DA and its metabolites (HVA, 3-MT and DOPAC) quantification.

Substance	Precision RE RSD (%)	Accuracy (%)	Recovery (%)	LOD (nM)	LOQ (nM)
Lyophilization – LC–ESI-MS/MS					
DA	11.4	−11.8	88.2	3.71×10^{-3}	4.24×10^{-3}
HVA	10.1	−11.7	88.5	4.39×10^{-3}	4.94×10^{-3}
3-MT	13.9	−14.1	86.1	4.44×10^{-3}	5.42×10^{-3}
DOPAC	11.9	−11.4	88.6	4.76×10^{-3}	5.35×10^{-3}
SPE – LC–ESI-MS/MS					
DA	13.6	−18.3	80.6	5.30×10^{-3}	6.36×10^{-3}
HVA	13.5	−20.6	79.2	8.78×10^{-3}	9.33×10^{-3}
3-MT	15.3	−16.8	82.5	5.42×10^{-3}	6.41×10^{-3}
DOPAC	14.6	−21.3	77.7	1.07×10^{-2}	1.13×10^{-2}
Nitrogen stripping – LC–ESI-MS/MS					
DA	17.6	−13.4	85.3	4.24×10^{-3}	4.77×10^{-3}
HVA	15.5	−13.0	87.8	4.94×10^{-3}	5.49×10^{-3}
3-MT	18.3	−16.9	83.7	5.42×10^{-3}	6.41×10^{-3}
DOPAC	19.4	−12.4	88.0	5.35×10^{-3}	5.95×10^{-3}
LC-ECD					
DA	10.2	−7.3	94.7	4.72×10^{-1}	5.32×10^{-1}
HVA	12.6	−6.2	95.8	7.63×10^{-1}	9.99×10^{-1}
3-MT	9.3	−5.5	96.0	6.32×10^{-1}	8.04×10^{-1}
DOPAC	9.9	−6.6	95.1	7.85×10^{-1}	1.05

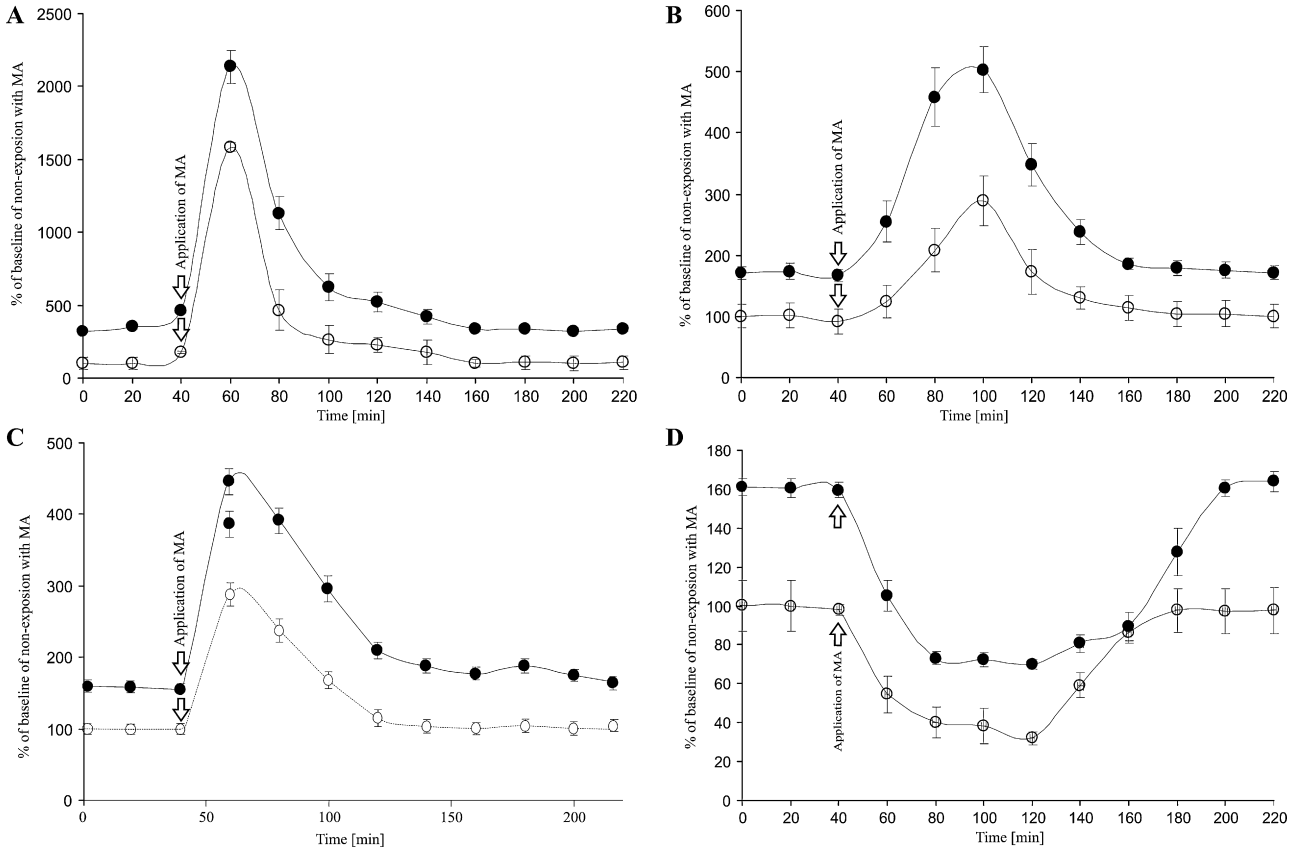

Fig. 6. Typical time dependences of DA (A), HVA (B), 3-MT (C) and DOPAC (D) concentrations in the course MA test. (●) = MA prenatally exposed group; (○) = control group.

mental study. A higher basal concentration of DA, HVA, 3-MT and DOPAC were found in the prenatally MA exposed group (Fig. 6). The levels of HVA, 3-MT and DOPAC changed over the same time period as that of DA in accordance with the mechanism of action of MA. Details of the study were provided in another publication [22].

4. Conclusion

An analytical method was developed for a parallel, rapid and precise detection and quantification of DA and its most prominent metabolites (HVA, 3-MT and DOPAC) in microdialysis samples. The assay incorporated a pre-treatment step for a rapid and effective pre-concentration (lyophilization) of low concentrations of DA and its metabolites in microdialysates. This step fulfilled the requirements of a rapid and mild isolation prior to a subsequent analysis that was compatible with the limited stability of DA. LC–ESI-MS/MS was used as the detection method, where the SRM mode was used for its extremely high degree of sensitivity and selectivity, and the isotope IS assay for its high precision in quantification, which has been established as the technique of a choice for trace analyses of various compounds present in complex biological matrixes. The combination of mass spectrometry detection with liquid chro-

matography separation was selected to retain the analytes from the solvent front as well as to avoid the co-elution of salts and endogenous matrix components that could suppress the ionization of an analyte during the ionization (ESI). The analytical procedure was optimized and validated. Precision values for DA and the particular metabolites contained in the microdialysates ranged in an interval from 86.1 to 92.4%, the recovery values ranged in an interval from 86.1 to 95.6%, LOD values were 3.71×10^{-3} to 4.76×10^{-3} nM and LOQ was determined in levels from 4.24×10^{-3} to 5.42×10^{-3} nM.

The developed assay method for DA, HVA, 3-MT and DOPAC potentially expands the diagnostic/treatment potential regarding neuropsychiatric and neurological disorders where the concentration levels of DA and its metabolites are altered in comparison to normal physiological levels. The method may contribute to a better understanding of the pathophysiology and pathogenesis of many neuropsychiatric disorders (drug addiction, schizophrenia, Parkinsonism, Alzheimer dementia) and to pharmaceutical research into new drugs to treat neurological diseases.

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