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Biogenic amines and their metabolites in mouse brain tissue: Development, optimization and validation of an analytical HPLC method

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ABSTRACT

A simple and fast HPLC method based on an isocratic, reversed-phased ion-pair with amperometric end-point detection for simultaneous measurement of noradrenergic (MHPG/NA and A), dopaminergic (DOPAC, HVA/DA) and serotonergic (5-HIAA/5-HT) compounds in mouse brain tissue was developed. In order to improve the chromatographic resolution (Rs) with an acceptable total analysis time, experimental designs for multivariate optimization of the experimental conditions were applied. The optimal conditions for the separation of the eight neurotransmitters and metabolites, as well as two internal standards, i.e., DHBA and 5-HMT, were obtained using a mixture of methanol–phosphate–citric buffer (pH 3.2, 50 mM) (9:91, v/v) containing 2 mM OSA as mobile phase at 32 °C on a microbore ALF-115 column (150 mm × 1.0 mm, 3 μm particle size) filled with porous C_{18} silica stationary phase. In this study, a two-level fractional factorial experimental design (½ 2^K) was employed to optimize the separation and capacity factor (k') of each molecule, leading to a good separation of all biogenic amines and their metabolites in brain tissue. A simple method for the preparation of different bio-analytical samples in phosphate–citric buffer was also developed. Results show that all molecules of interest were stabilized for at least 24 h in the matrix conditions without any antioxidants. The method was fully validated according to the requirements of SFSTP (Société Française des Sciences et Techniques Pharmaceutiques). The acceptance limits were set at ±15% of the nominal concentration. The method was found accurate over a concentration range of 4–2000 ng/ml for MHPG, 1–450 ng/ml for NA, 1–700 ng/ml for A, 1–300 ng/ml for DOPAC, 1–300 ng/ml for 5-HIAA, 1–700 ng/ml for DA, 4–2800 ng/ml for HVA and 1–350 ng/ml for 5-HT. The assay limits of detection for MHPG, NA, A, DOPAC, 5-HIAA, DA, HVA and 5-HT were 2.6, 2.8, 4.1, 0.7, 0.6, 0.8, 4.2 and 1.4 pg, respectively. It was found that the mean inter- and intra-assay relative standard deviations (RSDs) over the range of standard curve were less than 3%, the absolute and the relative recoveries were around 100%, demonstrating the high precision and accuracy, and reliability of the analytical method described to apply in routine analysis of biogenic amines and their metabolites in brain tissue.

1. Introduction

In the brain, biogenic amines play important roles as major neurotransmitters, being involved in the control and regulation of principal functions and behaviors [1]. It has been repeatedly demonstrated that the alterations of biogenic amines and their metabolites in (nor)adrenergic, serotonergic and dopaminergic systems, such as (nor)adrenaline (NA, A) and 3-methoxy-4-hydroxyphenylglycol (MHPG); serotonin (5-HT) and 5-hydroxy-3-indolacetic acid (5-HIAA); dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), contribute to mood, anxiety, aggression and various neuropsychiatric disorders [2–16]. These biogenic amines and their metabolites were also highlighted as molecular targets involved in treatments of multiple neuropsychiatric disorders, for example in treatment of Parkinson's and Alzheimer's disease [17–31].

The determination of these biogenic amines and their metabolites in brain tissue requires a highly, sensitive and selective method. In the past ten years, a number of reliable HPLC methods with online sensors, e.g., mass spectrometry (MS), fluorescence (FD) or electrochemical detection (ECD), has been developed to measure these endogenous molecules [17–19]. HPLC with FD or MS is more sophisticated and difficult to apply in a routine setting. Therefore, many applications using ion-exchange reversed-phase HPLC with ECD (HPLC–ECD) have been developed for the determination of the endogenous molecules. The recent use of electrochemical detectors with wall thick jet, thin layer cell, horizontal

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flow and multiple-working electrodes has significantly improved the sensitivity and selectivity of the analytical method. Also, a miniaturized LC system (column size: 150 mm × 1 mm, C_{18}, 3 μm, flow rate: 0.05 ml/min) has replaced the conventional system (column size: 250 mm × 4.6 mm; C_{18}, 5 or 10 μm, flow rate: 1 ml/min), yielding higher mass sensibility, requiring a small injection volume, significantly reducing mobile phase consumption rendering HPLC–ECD one of the most suitable methods for analysis of biogenic amines and metabolites [26].

Simultaneous determination of biogenic amines and their metabolites in brain tissue using HPLC–ECD, thought straightforward and feasible, seemed poorly achievable and limited to 2–5 compounds in previous studies [27–35]. This is most likely due to the difficulty in association of very early eluting of MHPG peak and very late eluting peak of 5-HT in the same chromatogram. To overcome this dilemma, Cao and Hoshino in 1996 suggested using a HPLC with dual pump systems and diode-array electrodes [36]. Later, Antec Leyden Inc. developed a dual chromatographic system containing two pumps, two injection loops, two columns (50 and 150 mm × 1 mm, C_{18}, 3 μm) and two glassy carbon working electrodes positioned in parallel for the simultaneous determination of the eight molecules. DA and 5-HT (group 1) are separated on the short column (50 mm) using a mixture of methanol–phosphate buffer (pH 6.0, 50 mM) (12.5:87.5, v/v); meanwhile, MHPG, NA, A, DOPAC, 5-HIAA and HVA (group 2) are separated on the long column (150 mm) using a mixture of methanol–phosphate–citric buffer (pH 3.5, 50 mM) (1:9, v/v) as mobile phases. Both mobile phases contain 2.33 mM OSA as ion counter and 0.1 mM Na_2EDTA. The two columns are maintained at 35 °C; flow rates are set at 0.05 ml/min; and detectors at 300 and 590 mV for the determination of the two groups (1 and 2), respectively. Samples are prepared in 10 mM acetic acid and injected into HPLC by an automatic injection system at 4 °C. Integration of chromatograms is performed with a dual-channel integration Clarity computer program.

In the beginning, our analyses were run on this system. However, peaks were tailing, no separation between A/DOPAC was achieved, and MHPG was eluted in association with the solvent peak. Sample extraction in acetic acid solution was not selective enough and did not stabilize the endogenous molecules. To increase the efficiency of chromatographic separation and resolution, we used Design Of Experiment (DOE) to optimize the HPLC method for simultaneous determination of the eight molecules of interest in an acceptable time of analysis using only a conventional HPLC system (one pump, one injection, one column and one detector). The combination of multivariate techniques, including selecting parameters most influential on chromatographic behavior, designing and performing a series of screening experiments, integrating and interpolating the most significant variables influencing the analytical method (e.g., capacity factor k' and separation factor α) led to successful identification of optimal values for analytical variables (temperature of the column, concentration of the modifier, of ion-pair counter, pH of the buffer and voltage of detection). To our best knowledge, the presented chemometric study is the first successful attempt to design and optimize a reversed-phase HPLC model for simultaneous separation and determination of MHPG, NA, A, DOPAC, 5-HIAA, DA, HVA, 5-HT and two internal standards DHBA and 5-MHT.

Since the inbred C57BL/6 mouse has been widely used as a background strain for knockout and transgenic animal models of complex neuropsychiatric disorders with important findings in neurochemical, biochemical, pharmacological, anatomical, and behavioral areas [37–40], we report herein an easy and suitable method to extract and stabilize the eight endogenous compounds in mouse brain tissue. Finally, to ensure that the analytical method can fulfill its objectives, the validation method has been performed on different parts of mouse brain tissue for between-run precision, recovery of standard additions and stability of the compounds of interest.

2. Experimental

2.1. Animals

Male C57BL/6J mice were bred within our research facility. The mice were housed in standard mouse cages under conventional laboratory conditions: food and water *ad libitum*, constant room temperature and humidity and a 12/12 h light/dark cycle. All experiments were carried out in compliance to the European Communities Council Directive (86/609/EEC).

2.2. Sample collection

Mice were killed by means of cervical dislocation. The brain was quickly removed from the skull, and the cerebellum, left and right hemisphere were separated and immediately fixated and frozen in 2-methylbutane kept at −40 °C over dry ice in order to avoid degradation of the biogenic amines and metabolites. The tissues were stored at −80 °C until analysis.

2.3. Chemicals

Phosphoric acid, citric acid, octan-1-sulfonic acid sodium salt, acetic acid glacial 100%, sodium acetate, *di*-potassium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide were of analytical grade and purchased from Merck (Darmstadt, Germany), Na_2EDTA from GibcoBRL (Life Technologies, Paisley, UK) and methanol (HPLC grade) from Biosolve (Valkenswaard, The Netherlands).

Standards and internal standards, MHPG (as potassium sulfate salt), NA (as hydrochloride), A (as free base), DA (as hydrochloride), DOPAC, 5-HIAA, HVA (free acid), 5-HT (as hydrochloride), DHBA (dihydroxybenzylamine hydrochloride, an internal standard), 5-HMT (5-hydroxy-N-methyl tryptamine oxalate, an internal standard), with a minimum purity of 99% were purchased from Sigma–Aldrich (Bornem, Belgium).

Ultrapure water from a Milli Q apparatus by Millipore (Milford, MA, USA) was used.

2.4. HPLC conditions

2.4.1. Instrumentation

An Alexys monoamines analyzer HPLC system (Antec Leyden, The Netherlands) consisted of a LC 110 pump operating at a flow rate of 0.05 ml/min. The separations were achieved on a micro-column (ALF-105, 150 mm × 1 mm, C_{18}, 3 μm). The Decade II electrochemical detector was equipped with a thin layer electrochemical cell fitted with a glassy carbon working electrode, which was set at various voltages ranging from 300 to 900 mV and an Ag/AgCl reference electrode. Injection of 5 μl was done by an automated sample injector AS 100 Alexys. Integration of the chromatogram was performed with channel integration M018/EN25B Clarity software (DataApex Ltd., Prague, The Czech Republic).

2.4.2. Chromatographic conditions

2.4.2.1. Development of analytical method. The mobile phase consisted of phosphoric–citric buffer (8 mM KCl, 50 mM H_3PO_4, 50 mM citric acid, 2.33 mM octan-1-sulfonic acid sodium salt (OSA), 0.1 mM Na_2EDTA, adjusted to pH 3.25 with 50% NaOH) and 10% methanol. Flow rate was 0.05 ml/min. The working electrode of the detector was set at 590 mV and column temperature was set at 35 °C. The standard solutions contained MHPG, NA, A, DOPAC, DHBA, 5-HIAA, DA, HVA, 5-HT and 5-HMT in different running

Table 1
Experimental design: experiments were carried out in order from 1 to 20; Pattern showed randomization of the experimental selection around (+/−) or at center point (0); [MeOH], [OSA] indicate the concentration of MeOH and OSA in the mobile phase, temperature was set for the column in °C; Detector was set in mV, and pH indicates the pH buffer value.

Experiment	Pattern	[MeOH]	pH	Temperature	[OSA]	Detector
1	−−−−+	9	3	30	2	650
2	+++−−	11	3.5	40	2	530
3	−+++−	9	3.5	40	2.66	530
4	0	10	3.25	35	2.33	590
5	+−−++	11	3	30	2.66	650
6	0	10	3.25	35	2.33	590
7	−+−−−	9	3.5	30	2	530
8	++−−+	11	3.5	30	2	650
9	+++++	11	3.5	40	2.66	650
10	−−−+−	9	3	30	2.66	530
11	−+−++	9	3.5	30	2.66	650
12	0	10	3.25	35	2.33	590
13	++−+−	11	3.5	30	2.66	530
14	+−−−−	11	3	30	2	530
15	−++−+	9	3.5	40	2	650
16	+−+−+	11	3	40	2	650
17	+−++−	11	3	40	2.66	530
18	0	10	3.25	35	2.33	590
19	−−+++	9	3	40	2.66	650
20	−−+−−	9	3	40	2	530

buffers, e.g., acetic acid (#1), acetate (#2), phosphate (#3–4–5) and phosphate-citric (#10) (see preparation of buffers) in a concentration of 34, 6.4, 7.3, 8.3, 5.9, 4.5, 5.6, 28, 7.5 and 7.7 ng/ml, respectively.

Samples were prepared in 10 mM acetic acid or in running phosphate–citric buffer (#10). Briefly, brain tissue (100 mg) was homogenized (30 s) in 4 ml buffer at 4 °C using an Ultra-Turrax TR50 Crusher (Ika, Staufen, Germany). A part of the mixture was taken to measure pH; the rest was transferred into 1-ml eppendorf tubes and promptly centrifuged at 14,000 rpm and at 4 °C during 15 min. The supernatant was then filtrated through a 0.2-μm Millipore filter (Millipore, Ireland). Further elimination of proteins was accomplished with a spin 10-kDa protein cut-off filter (Millipore, Ireland). Time of centrifugation was 20 min at 10,000 rpm, and the samples were maintained at 4 °C. For left and right hemisphere, the filtrate was diluted 2, 4, 6 and 8 times with buffers, while no dilution was needed for cerebellum extraction.

2.4.2.2. Optimization of analytical method. Twenty mobile phases based on phosphoric–citric buffers (#6–25), each containing 8 mM KCl, 50 mM H_3PO_4, 50 mM citric acid, 0.1 mM Na_2EDTA, a modified quantity of OSA and methanol are described in Table 1. The buffer pH was adjusted with 50% NaOH solution. The detector was set at 530, 590 or 650 mV and the temperature of the column was maintained at 30, 35 or 40 °C (Table 1).

Standard solutions contained either each standard alone or a mixture of ten molecules (MHPG, NA, A, DOPAC, DHBA, 5-HIAA, DA, HVA, 5-HT and 5-HMT) in running buffers (#6–25) in a concentration of 34, 6.4, 7.3, 8.3, 5.9, 4.5, 5.6, 28, 7.5 and 7.7 ng/ml, respectively. Injecting standards separately helped to identify the retention time of standards in the mixture in each running experiment. Integrating and interpolating of the results from 20 screening experiments allowed identification of the optimal chromatographic conditions for analysis of the ten molecules. The optimal mobile phase consisted of phosphoric–citric buffer (8 mM KCl, 50 mM H_3PO_4, 50 mM citric acid, 2.0 mM octan-1-sulfonic acid sodium salt (OSA), 0.1 mM Na_2EDTA, adjusted to pH 3.2 with 50% NaOH) and 9% methanol. Flow rate was 0.05 ml/min. The working electrode of the detector was set at 650 mV and the column was kept at 32 °C.

Samples were prepared in the buffer which was used to prepare the optimal mobile phase. The buffer was additionally filtrated through a 0.2-μM Millipore filter before use for sample preparation. The homogenization, centrifugation, filtration and protein-cut off elimination were carried out in the same manner as used for the development of the method. Analyses of biogenic amines and their metabolites in three sections of mouse brain (right hemisphere, left hemisphere and cerebellum) were done using the optimal chromatographic system.

2.4.2.3. Validation of analytical method. The optimal chromatographic conditions were applied for the complete validation process. Four standard solutions in running buffer containing MHPG (10.9, 17.8, 26.1, 34 ng/ml), NA (2, 3.3, 4.9, 6.4 ng/ml), A (2.3, 3.8, 5.6, 7.3 ng/ml), DOPAC (2.6, 4.3, 6.4, 8.3 ng/ml), DHBA (1.9, 3, 4.5, 5.9 ng/ml), 5-HIAA (1.4, 2.3, 3.5, 4.5 ng/ml), DA (1.8, 3, 4.3, 5.6 ng/ml), HVA (8.9, 14.7, 21.5, 28 ng/ml), 5-HT (2.4, 3.9, 5.7, 7.5 ng/ml) and 5-HMT (2.5, 4, 5.9, 7.7 ng/ml) were prepared for calibration curves. The calibration standard curve for each molecule was established on the basis of the concentrations [nM] vs. peak area [nA s]. Linear curve fit was used and correlation coefficiency (r^2) was found to be higher than 0.995. The two internal standards (DHBA and 5-HMT) were chosen based on structural similarity with the molecules of interest, respectively, for catecholamines and serotonergic molecules. The run-to-run variability is eliminated giving more precise results.

Sample preparation was done as described in the optimization process. Validation was demonstrated in the three mouse brain regions studied.

2.4.3. Preparation of mobile phases

Preparation of mobile phases contained two steps: (1) Buffer preparation: KCl, H_3PO_4, citric acid, OSA and Na_2EDTA were dissolved in an appropriate volume of water, and pH was adjusted with 50% NaOH. Water was supplemented to guarantee the correct concentration of each component in the solution if required. Buffer predestined for sample preparation, was filtrated through a 0.2-μm Millipore filter and kept at 4 °C during sample preparation. (2) Mobile phase preparation: Buffer was mixed with MeOH with concentration listed in Table 1, and immediately filtrated through a 0.2-μm Millipore filter. The mobile phase was degassed for 15 min in an ultrasonic bath (Branson 3510, CT, USA) before use.

2.4.4. Preparation of buffers

A total of 25 different buffers was prepared and evaluated for their buffer capacity in biological samples. Buffer #1 contained 10 mM acetic acid in distilled water (pH 3.48). Acetate buffer (#2, pH 4.75) was made by adding sodium acetate to 10 mM acetic acid. 50 mM or 100 mM phosphate buffers (#3–4, pH 4.75) were obtained by adding *di*-potassium hydrogen phosphate to 50 mM or 100 mM potassium dihydrogen phosphate solution. Phosphate buffer (#5, pH 3.25) was prepared by adjusting the pH of 50 mM *di*-potassium hydrogen phosphate solution with phosphoric acid. Phosphate–citric buffers (#6–25) contained 8 mM KCl, 50 mM phosphoric acid, 50 mM citric acid, 0.1 mM Na_2EDTA, a modified OSA concentration ranging from 2.0 to 2.66 mM (listed in Table 1), adjusted with 50% NaOH to the pH calculated (from 3.0 to 3.5) (see Table 1). All buffers were filtrated through a 0.2-μM Millipore filter and degassed prior to use.

2.5. Optimization of analytical method

2.5.1. Experimental design

Starting with modifying one factor (e.g., MeOH concentration, pH or OSA concentration, flow rate, range of detector), preliminary experiments were run to assess the level of influence of these factors on the separation of the ten molecules. Two-level fractional factorial experimental design ($½\ 2^K$) was employed to observe the effect of these factors on a selected suitability parameter.

2.5.2. Selection of factors and levels

Five quantitative factors significantly influencing chromatographic behavior were selected to design the experiments: modifier methanol concentration [MeOH], pH of the mobile phase (pH), temperature of the column (t°), ion-pair counter concentration [OSA] and voltage of detector (Detector). Three levels of each factor were selected symmetrically around the nominal value (−1, nominal and +1) of the corresponding factor found in the method development. A total of 20 experiments (Table 1) were obtained with experiment design by JMP® 8.0.1 software (SAS Institute Inc.) where the different levels of the five factors were statistically combined.

2.5.3. Performance of the screening experiments

The 20 screening experiments were run in strict order from #1 to 20 to avoid systematic errors (Table 1). Experiments #4, 6, 12 and 18 reflected the central points and showed identical conditions of the method development. The experiments were blocked according to the columns and the instruments. Standard solutions were prepared in citric–phosphate buffer as described above and injected into the HPLC system.

Samples were prepared as described above. The identification of the peaks was on the basis of similarity of the retention time and the voltammogram of each identified substance in the sample and in the standard solutions. Sample measurements were based on two injections from individual solutions.

2.5.4. Running JMP program

The capacity factor (k') and separation factor (α) were calculated and pasted into the JMP program in which the conditions of the twenty screening experiments were described. The main effect of each parameter, i.e., [MeOH], pH, t°, [OSA], Detector, and two-way interactions of these parameters to the capacity factor of each substance (k') and to the separation factors (α) were analyzed by the effect screening and least squared fit. These parameters were separately chosen as variables, and linearity models were run. Two prediction profiles were then obtained to demonstrate if two-factor interactions were either confounded with main effects, higher-order interactions and/or with each other. Manual modification of each factors and/or interactions was carried out and finally, the optimal conditions for the separation of biogenic amines and their metabolites in both standard and sample solutions were achieved.

2.6. Validation of analytical method

2.6.1. Stock standard solution

Stock standard solution of a mixture of MHPG, NA, A, DOPAC, DHBA, 5-HIAA, DA, HVA, 5-HT and 5-HMT in phosphate–citric running buffer (at 4 °C) in a concentration of 7, 1.3, 1.2, 1.4, 1.6, 4.7, 1.5 and 1.9 mg/ml was prepared. The stock solution was then kept in 1-ml cryo-tubes and stored immediately at −80 °C. Upon use, stock solution was thawed on ice and diluted with phosphate–citric buffer at 4 °C.

2.6.2. Stability of standard and test solutions

The stability of MHPG, NA, A, DOPAC, 5-HIAA, DA, HVA and 5-HT was studied in both matrix and nonmatrix conditions at 4 °C. Standards in phosphate–citric buffer (i.e., nonmatrix conditions) were injected every 5 h over three days to evaluate long-term stability in nonmatrix conditions. The concentration of standards was back-calculated and compared with the appropriate concentration from the first injection. If the reduction was more than 10%, products were considered not stable at the time of injection.

Stability of the eight molecules in matrix conditions (i.e., endogenous molecules in phosphate–citric buffer) was examined by repeatedly injecting sample solutions every 5 h into HPLC for three days. The recovery of the products was calculated as percentage of the response at measurement time to that at time 0.

2.6.3. Specificity

The specificity of the method was studied in the presence and absence of the matrix to learn the susceptibility of substances to the interference. The following solutions were prepared and injected according to the analysis method obtained in the optimization: citric–phosphate buffer, standards or samples in citric–phosphate buffer, standard solutions spiked with the suitable quality of sample solutions, whether or not containing the degradation products (the degradation products were generated by sampling at ambient temperature and 24 h after preparation).

2.6.4. Response function

About 24 different levels of standard solutions in citric–phosphate buffer were prepared and injected into HPLC. The responses vs. concentrations were graphed and a nonlinear fit curve for each analyte (response function) was predicted by least squares method and its stability was checked with every sample analyzed.

2.6.5. Linearity

Fifteen different concentrations of standard solutions in citric–phosphate buffer were added to a sample solution. The analysis was performed in duplicate and the results were plotted on a calibration curve for each substance obtaining by linear regression models.

2.6.6. Precision (repeatability and intermediate precision)

Both instrumental and method precision were studied to verify the repeatability of the system and the proposed method.

2.6.6.1. Instrumental system precision. The standard solutions were each injected 10 times according to the optimal analytical method to study the repeatability of the instrumental system. Further, the immediate precision of the system was studied, evaluating the variability of the responses between two different days.

2.6.6.2. Method precision and intermediate precision. Brain tissue was collected and homogenized in citric–phosphate buffer. This solution was divided into six aliquots, centrifuged and filtered separately through a 0.2-μm Millipore filter and a 10-kDa-protein cut-off filter. The filtrates were diluted prior to injection into the HPLC system (see above). Method precision was evaluated by relative standard deviations (RSD) which obtained from the results of six injections in the same day, and intermediate precision was evaluated by RSD which obtained from injections in different days.

2.6.7. Accuracy (recovery study)

The absolute recovery and the relative recovery were studied to verify the accuracy of the proposed method.

2.6.7.1. The absolute recovery (extraction recovery). The absolute recovery studies the retrieval of standards added to a biological sample, which has run through all steps of extraction and filtration. In this study, the concentration of eight molecules was determined by repeatability measurement, and then three levels of standards corresponding to 30, 50 and 70% of the concentration of each molecule in the sample were added to brain tissue. Samples containing added standards were subjected to all steps of samples preparation to assure adequate mixing of endogenous and added biogenic amines (i.e., homogenization, centrifugation, Millipore & protein cut-off filters) and the quantity of each substance recovered in relation to the added amount was calculated.

2.6.7.2. The relative recovery. Standard solutions, containing an equivalent concentration of the active substances in the sample, were treated following the same method as for biological sample. This procedure allowed determining a relative recovery of each substance by the developed method.

2.6.8. Effect of dilution

Sample solution was prepared as described above and then diluted 2, 4, or 6 times in citric–phosphate buffer. Five μl of each solution was injected in hexaplicate into HPLC and the concentration of each substance was calculated on the basis of response and dilution. Comparison of the average results in each dilution was done using a one-way ANOVA (Tukey-HSD analysis, SPSS version 17.0, Chicago, USA).

2.6.9. Limit of detection (LOD), limit of quantitation (LOQ)

Given the similarity of the signal-to-noise ratio in standard and sample chromatograms (Fig. 3), the LOD was determined by injecting 5 levels of standard solution and the levels in which the response was 3× higher than the threshold was taken as LOD. The LOQ for each substance was identified from the LOD as follows: LOQ = (10 × LOD)/3.

3. Results and discussion

3.1. Method development for sample preparation

Biogenic amines and their metabolites are unstable and can be rapidly oxidized especially in neutral, alkaline or in strong acidic medium [41,42]. In 1973, Kissinger et al. first introduced an HPLC–ECD technique with ion-pair reversed-phase for the measurement of these molecules [43]. This has stimulated the development of a highly sensitive method to assay the endogenous molecules at low concentration in biological samples. However, it also brought some difficulty to stabilize these substances during sample preparation, injection and preservation.

To ensure the reliability of the assay, most researchers carried out the experiments in the presence of protective agents. Ascorbic acid in acidic condition proved to efficiently stabilize biogenic amines and their metabolites in plasma [44]. Addition of oxalic acid could efficiently prevent the degradation of DA, 5-HT, DOPAC, HVA and 5-HIAA during 24 h incubation at room temperature [45]. Nitrophenylboronic acid/H_3BO_3; acetic acid; L-cysteine; $Na_2S_2O_5$, $NaHSO_3$ were also used to increase the long-term stability of the analytes [28,32,35,36,40,46–48]. Nevertheless, the use of most antioxidants always causes a large t_0 peak, thereby vigorously affecting the separation of MHPG, which, in many cases, becomes unidentifiable. Most researchers, therefore, neglect the determination of this molecule in biological samples; even understanding that knowing the level of this molecule in brain tissue and/or the metabolite rate to form this molecule may explain the neurochemical imbalance in a neurological disorder.

A series of stability experiments was initiated by testing the interference of standards in different buffers with the chromatographic behavior. A total of 25 different buffers was prepared and filtrated before use. In 0.1 mM acetic acid buffer, standards were very stable, but samples were not. The pH of the diluted sample solutions (1×, 3×, 5× and 8×) changed dramatically from 4.25 (1×) to 3.75 (8×) and this affected not only the retention time of the analytes [28,32], but also the response as shown in our measurements, meaning that the concentration of each analyte found by calculation from the same sample was not identical. This may be due to the low "buffer capacity" of acetic acid. In 10 mM acetate buffers or in 50 and in 100 mM phosphate buffers, the analytes were more stable, but the separation was unsatisfactory; the peaks were tailing and broad (in particular for 5-HT, DA and HVA). The use of phosphate–citric buffers for sample preparation was finally chosen, which brought us two advantages: no t_0 peak was observed in the chromatogram of standards, while the t_0 peak in the chromatogram of samples was spectacularly reduced, and, therefore, a very nice separation of MHPG from the t_0 peak was achieved. In addition, the decreases in peak heights of the molecules at interest were generally less than 10% after one day of incubation at 4 °C, indicating that all biogenic amines and their metabolites were stable in phosphate–citric buffer at 4 °C in the absence of antioxidants.

Biogenic amines and their metabolites are also sensitive to light and temperature [33]. They are easily oxidized in the presence of transition-metal cations such as Fe^{2+} [32], hence our assays were always carried out in dark, at low temperature (4 °C) and with the use of the chelating agent Na_2EDTA in the mobile phase. The use of 10-kDa-protein cut-off filters to eliminate mainly enzymes involved in the degradation of the biogenic amines and their metabolites (e.g., MAO enzymes), has clearly increased the stability of these molecules and reduced the time of analysis. Our proposed method for sample preparation allowed direct injection of a clean biological sample without employing the complex extraction techniques, such as liquid/liquid extraction [49–52], adoption on aluminum oxide [28,29,53–58], or solid phase extraction using ion-exchange [58–61], which previously have been used to extract trace amounts of the endogenous molecules in brain tissues. Consequently, our proposed method reduces laborious work for sample preparation, increases the reliability of the results, is simple, quick, inexpensive and satisfies all criteria for a good biological sample preparation.

3.2. Optimization of HPLC conditions

The goal of this study was to identify the influence of HPLC conditions on the separation and the capacity factor of each analyte, and based on experimental data analysis to find out the best conditions for simultaneous determination of the analytes of interest and two internal standards (DHBA and 5-HMT) in biological samples in an acceptable time.

Two-level fractional factorial experimental design (½ 2^K) involving maximum five factors was used in screening experiments requiring to study the joined effect of these factors on the response. Based on the preliminary data, the effect of pH, detector, [MeOH], [OSA] and t° on the chromatographic behavior was studied. Experiments were set up by random combination of the maximum and minimum values of the five factors. In order to evaluate the pure experimental error, four experiments were performed in the center of the experiment domain (pH 3.25, detector at 590 mV, 10% MeOH, 2 mM OSA and temperature 35 °C) in a total of 20 screening experiments which were constructed and performed randomly (Table 1).

The separations between A and DOPAC, between DOPAC/DHBA, and 5-HIAA/DA were critical in obtaining a successful analysis because the separation factors (α_3, α_5, α_7) were ~1.00. The chromatograms obtained from 20 screening experiments clearly showed that if all separation factors were increased to a minimum value of 1.15, a good chromatogram could be achieved. From the analytical point of view, the most important separation to optimize was certainly between A and DOPAC because in many experiments, these two peaks overlapped. The k'-value of MHPG was small (<1.00) leading to a difficult separation between MHPG and the solvent peak. The aim of the design was to obtain a good separation between MHPG and the solvent peak, thus the idea was to achieve the maximum k'-value of MHPG in an acceptable time of analysis (max. of 30 min) for the 10 analytes. Increasing the k'-value of MHPG, unluckily, usually led to an increased k'-value of 5-HMT and with the k'-value of 5-HMT ≥ 13, the time of analysis ranged from 31 to 40 min (experiments #1, 7, 10, 11).

A cross-effect between the five factors (pH, t°, voltage of detector, [OSA] and [MeOH]), and the k'-values of all analytes and the separation factors were then analyzed using a "standard least squares" model. [OSA] had a very strong negative effect to the separation factor of A/DOPAC (α_3/Fig. 1), suggesting that the minimum [OSA] (2 mM) gave the best separation of A/DOPAC. [MeOH] also exhibited a small negative effect on the k'-value of MHPG (Fig. 2) and on all separation factors (Fig. 1), suggesting that the minimum [MeOH] (9%) lead to the best separation of all analytes. Temperature (t°) had a negative effect on the k'-values of all molecules (Fig. 2), suggesting that at the minimum temperature (30 °C) the highest k'-values of MHPG (0.79) and 5-HMT (15.09) could be obtained. In order to reduce the time of analysis to maximum 30 min, temperature was manually increased until reaching a k'-value of 5-HMT < 13. Taking into account that temperature had a negative effect on the separation of A and DOPAC (effect of α_3/t°) (Fig. 1), it was decided that the optimal temperature for the experiment was 32 °C. At this temperature, all analytes as well as the two internal standards were stable. In contrast to the temperature, pH had a small positive effect on k'-values of all analytes (Fig. 2). Increasing pH thus led to an increased k'-value of MHPG. However, pH had a negative effect to the separation of A/DOPAC (crossing effect α_3/pH in Fig. 1). Besides, during performing experiments, it was noticed that at pH 3.0, A and NA were less stable, while at pH 3.5, 5-HIAA and HMPG were less stable. A mobile phase at pH 3.2 was then finally selected as the optimal condition. The potential of the detector had no influence on the separation of A/DOPAC (cross effect of α_3/detector in Fig. 1) but it had a small negative effect on the k'-value of MHPG (Fig. 2). However, when the detector was set at 590 mV, the peak of MHPG was small. The voltammogram of each substance was analyzed and it clearly showed that at 650 mV, MHPG gave a superior response. Thus, in order to increase the precision of the method as well as the response of MHPG, the detector was set at 650 mV.

The optimal conditions were applied to separate eight biogenic amines and their metabolites, and two internal standards, and as expected, all substances were completely separated from each other. All separation factors were calculated and showed values ≥1.15, hence, an optimal chromatographic method was obtained (Fig. 3a). The k'-values were in the range of our prediction, meaning that retention time of the last separation component was less than 30 min (t_R of 5-HMT = 28.35 min). The retention time of MHPG was found also to be sufficiently high (t_R = 3.43 min) allowing complete separation of MHPG from the solvent peak in biological samples (Fig. 3b and c). Different HPLC parameters were studied and the results showed that all peaks were symmetric (tailing factors ≈ 1.0); the maximal resolution was obtained to guarantee a good separation between peaks; also the maximal number of theoretical plates was reached (data not shown), suggesting a good chromatographic method was achieved. To our knowledge, this is the first report of a one-run analytical HPLC method with a good separation of MHPG from a mixture of eight biogenic amines and their metabolites (including two internal standards) in (mouse) brain tissue.

3.3. Validation of analytical method

Development and optimization are important and essential steps when developing new analytical procedures. To this end, many validation procedures have been proposed by, e.g., FDA, ICH and SFSTP with the aim to validate novel bio-analytical methods and bring it to the routine work setting [62–66]. We report the validation process of our proposed analytical method in compliance with SFSTP guidelines.

3.3.1. Selectivity

Citric–phosphate buffer naturally did not show any peaks with retention time corresponding to these shown by the standards. Samples in citric–phosphate buffer showed peaks superimposed on the ones found in the spiked solution. Moreover, peaks of interest were totally separated from degradation products as shown in our experiment (data not shown), thereby proving method selectivity.

3.3.2. Linearity, LOD and LOQ

The linearity of an analytical method is its ability to obtain the response directly proportional to the concentration of the analyte in the sample within a definite range. For all series, a regression line was fitted on the calculated concentrations vs. the introduced concentration by applying the linear regression model $y = ax + b$. The equation obtained for each analyte with their correlation coefficient ($r^2 \approx 1.000$) of determination and the residues (≤0.5 nA s) (see Table 2) demonstrated the good linearity according with different specifications.

LOD is defined as the smallest quantity of the molecule of interest that can be detected, but not necessary qualified as an exact value, whereas LOQ is the smallest quantity of the molecule of interest in sample that can be quantified with a well-defined precision and accuracy [67]. LOD and LOQ were estimated using the signal-to-noise method [64]. Therefore, the noise value was calculated from 10 independent injections of the blank (citric–phosphate buffer) and the relative standard deviation (RSD) for the 10 blank measurements was 32%, thereby approximating values reported in the literature [68]. Five standard solutions, which contained ten analytes at LOD or LOQ concentrations, were injected 10 times to determine the precision expressed as RSD. The RSDs for the ten measurements for ten molecules at LOD ranged from 10 to 18% and were smaller than 8% at LOQ. This procedure is generally accepted to determine LOD and LOQ [69,70]. Signal-to-noise is frequently used to estimate the LOD and LOQ in HPLC [71,72] as it is easy to implement. However, its accuracy depends critically on the analyst's interpretation of the noise amplitude.

The results of the LOD and LOQ are listed in Table 2, showing that the ECD is very sensitive (pg) and that the developed method

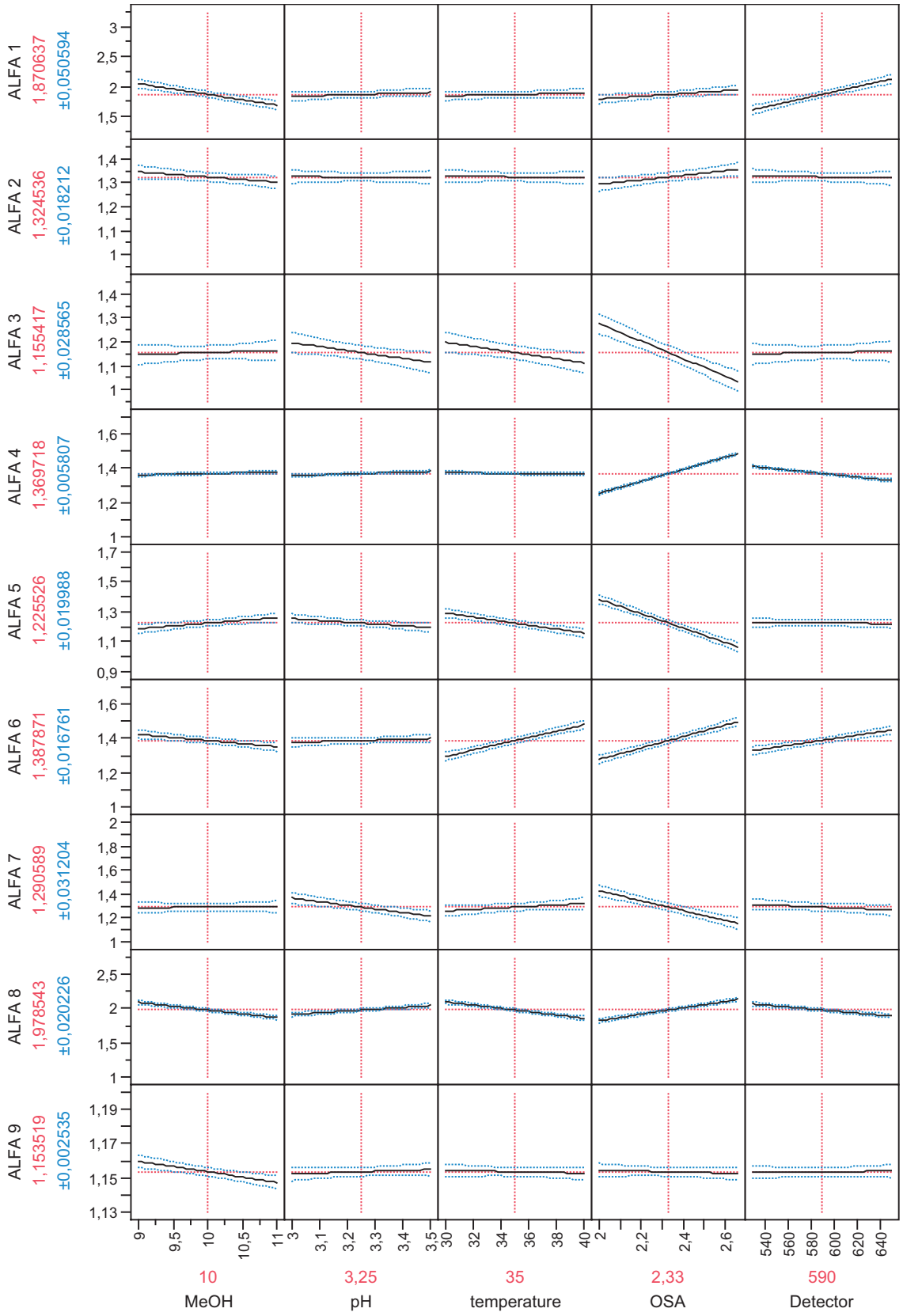

Fig. 1. Prediction profile showing the cross-effect of the five factors (pH, t°, detector setting, [MeOH], [OSA]) to the separation of 8 biogenic amines and metabolites and 2 internal standards. The separation factor was calculated: $\alpha = K'_1/K'_2$ in which K_2 and K_1 were capacity factors of two separated substances. Legend: [MeOH], methanol; OSA, octan-1-sulfonic acid sodium salt.

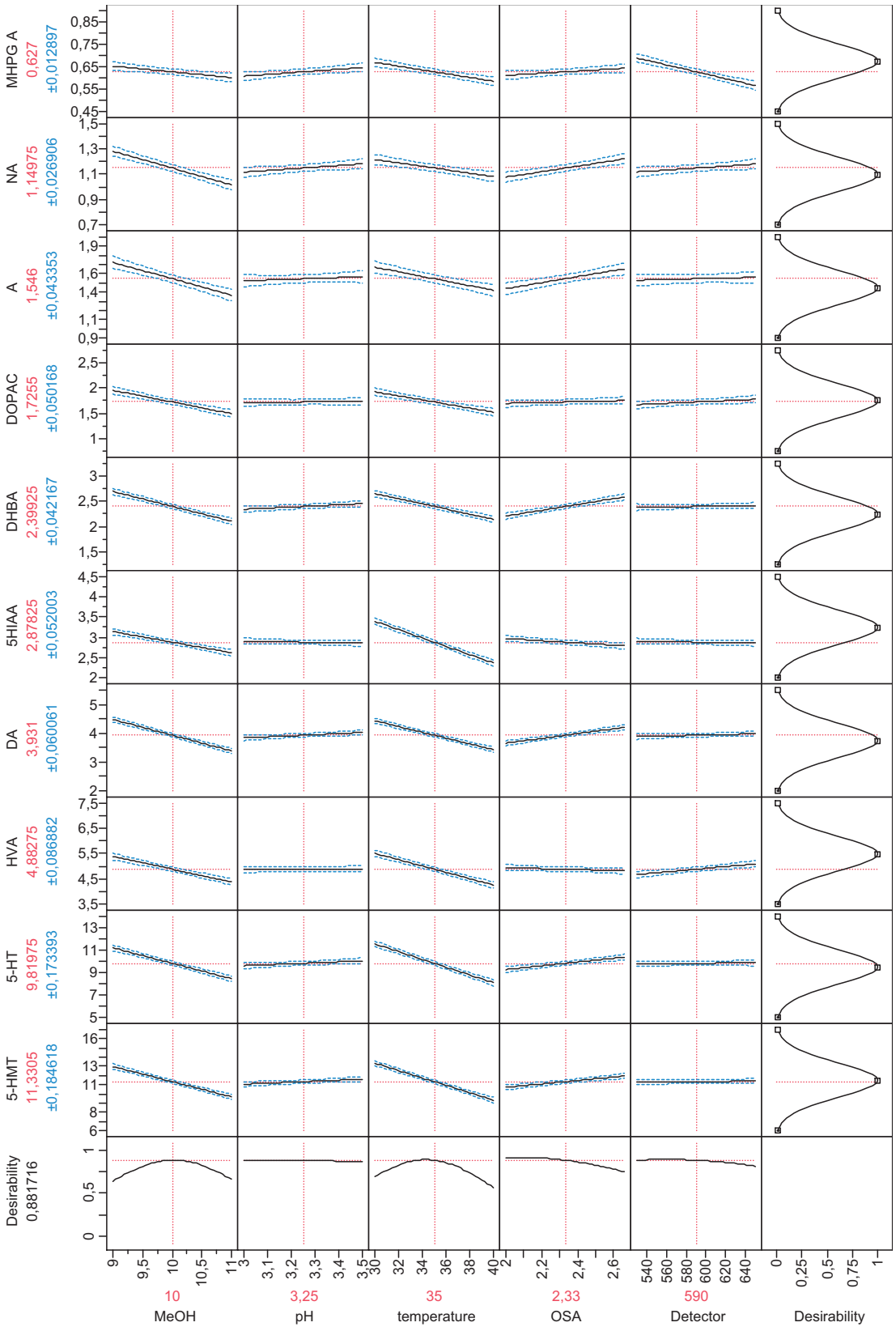

Fig. 2. Prediction profile showing cross-effects of capacity factors of the substances at interest (MHPG, NA, A, DOPAC, DHBA, 5HIAA, DA, HVA, 5-HT, 5-HMT) and five factors (pH, t°, detector setting, [MeOH], [OSA]) obtained by analysis of the results from 20 screening experiments run on an Antec Leyden HPLC system. The separations were achieved on an ALF-115 column (150 mm × 1.0 mm, 3 μm, C_{18}) at different temperatures; mobile phases were mixtures of citric–phosphate buffer and methanol containing KCl, EDTA and octan-1-sulfonic acid sodium salt (OSA) with a flow rate of 0.05 ml/min, pH adjusted to different values with 50% sodium hydroxide; electrode potentials (from 530 to 650 mV) vs. Ag/AgCl reference electrode. Capacity factor was calculated as: $K' = (t_R - t_0)/t_0$; t_R and t_0: retention times of substance and solvent peak, respectively. Legend: [MeOH], methanol; OSA, octan-1-sulfonic acid sodium salt.

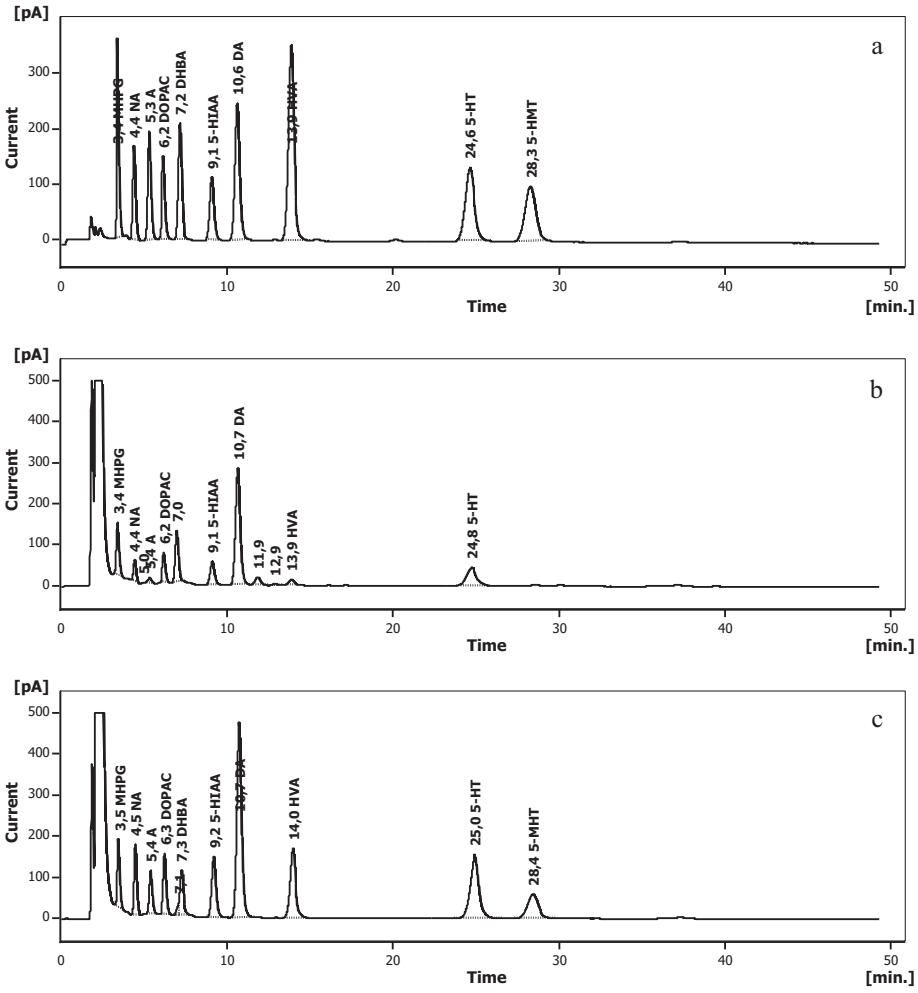

Fig. 3. Representative chromatogram of (a) the standards; (b) right hemisphere; (c) right hemisphere with standards added obtained with the optimized chromatographic conditions: citric–phosphate buffer–methanol (91:9) containing 2.0 mM OSA (pH 3.2) with flow rate 0.05 ml/min as mobile phase, an ALF-115 column (150 mm × 1.0 mm, 3 μm, C_{18}) maintained at 32 °C as stationary phase and detector setting at 650 mV. Peaks: MHPG (t_R = 3.45 min), NA (4.42 min), A (5.37 min), DOPAC (6.23 min), DHBA (7.20 min), 5-HIAA (9.15 min), DA (10.61 min), HVA (13.90 min), 5-HT (24.66 min) and 5-MHT (28.35 min).

Table 2
Validation results of biogenic amines and their metabolites in right-hemisphere of mouse brain tissues.

Validation criterion	MHPG	NA	A	DOPAC	5-HIAA	DA	HVA	5-HT
Precision (RSD %)								
Instrumental precision (k = 3, n = 10)	1.9/2.0	0.9/2.6	1.4/2.9	0.9/2.2	1.2/3.8	1.4/1.4	0.5/2.3	0.5/2.6
Repeatability (m = 3, n = 6)	1.5/2.2	2.2/5.0	1.4/1.7	1.7/2.4	2.7/4.1	2.2/2.6	3.3/4.3	1.3/3.4
Immediate precision (d = 3, n = 6)	2.3/2.2	5.7/2.2	1.5/1.9	2.4/1.8	4.1/2.7	2.6/2.2	4.3/2.4	3.4/1.4
Accuracy (k = 3) (% recovery)								
Absolute recovery	99.3 ± 1.0	96.6 ± 0.8	94.2 ± 2.3	94.2 ± 2.2	100.1 ± 0.52	97.2 ± 1.7	99.0 ± 2.0	100.7 ± 1.1
Relative recovery	96.7 ± 1.2	96.0 ± 0.5	94.0 ± 1.2	95.6 ± 0.8	93.7 ± 1.2	92.8 ± 0.7	96.0 ± 0.3	82.9 ± 2.3
Linearity (ng/ml)								
Calibration range	[4–2000]	[1–450]	[1–700]	[1–300]	[1–300]	[1–700]	[4–2800]	[1–350]
Slope	0.02629	0.1148	0.0955	0.2796	0.6082	0.2960	0.0978	0.5666
Intercept	0.57038	0.4618	0.6021	0.0508	−0.3695	3.0540	0.6724	0.7144
Correlation coefficient (r^2)	0.9997	0.9997	0.9997	r^2 = 0.9997	r^2 = 0.9999	r^2 = 0.9999	r^2 = 0.9999	r^2 = 0.9999
Residium (nA s)	0.4048	0.3853	0.4709	0.4943	0.5951	0.50372	0.7373	0.6372
LOD (pg)	2.6	2.8	4.1	0.7	0.6	0.8	4.2	1.4
LOQ (pg)	8.8	9.2	13.5	2.5	1.9	2.7	14	4.5
Effect of dilution (ng/g of tissue ± SEM)								
2×	1355.8 ± 16.6	461.7 ± 4.5	114.2 ± 2.7	184.7 ± 2.0	380.8 ± 2.3	2055.4 ± 19.6	407.9 ± 10.3	748.1 ± 3.2
4×	1395.1 ± 16.0	432.4 ± 4.3	106.9 ± 2.9	189.7 ± 2.5	392.5 ± 2.4	2098.8 ± 16.0	410.3 ± 6.3	741.5 ± 3.2
6×	1470.8 ± 18.6	456.8 ± 3.7	114.2 ± 2.7	193.5 ± 1.8	403.3 ± 3.1	2110.3 ± 12.1	411.1 ± 6.5	756.1 ± 5.3

Standard or sample solutions were prepared in different days (d = 3). Different sample concentrations (k = 3) were prepared in the same day. Standard or sample solutions were analyzed six times (n = 6). Results were calculated from 3 injections (m = 3) for each sample solution. Concentrations of biogenic amines and their metabolites (ng/g of tissue) were calculated from hexaplicate injections of each diluted sample solution (2, 4 and 6 times) and expressed as mean ± SEM.

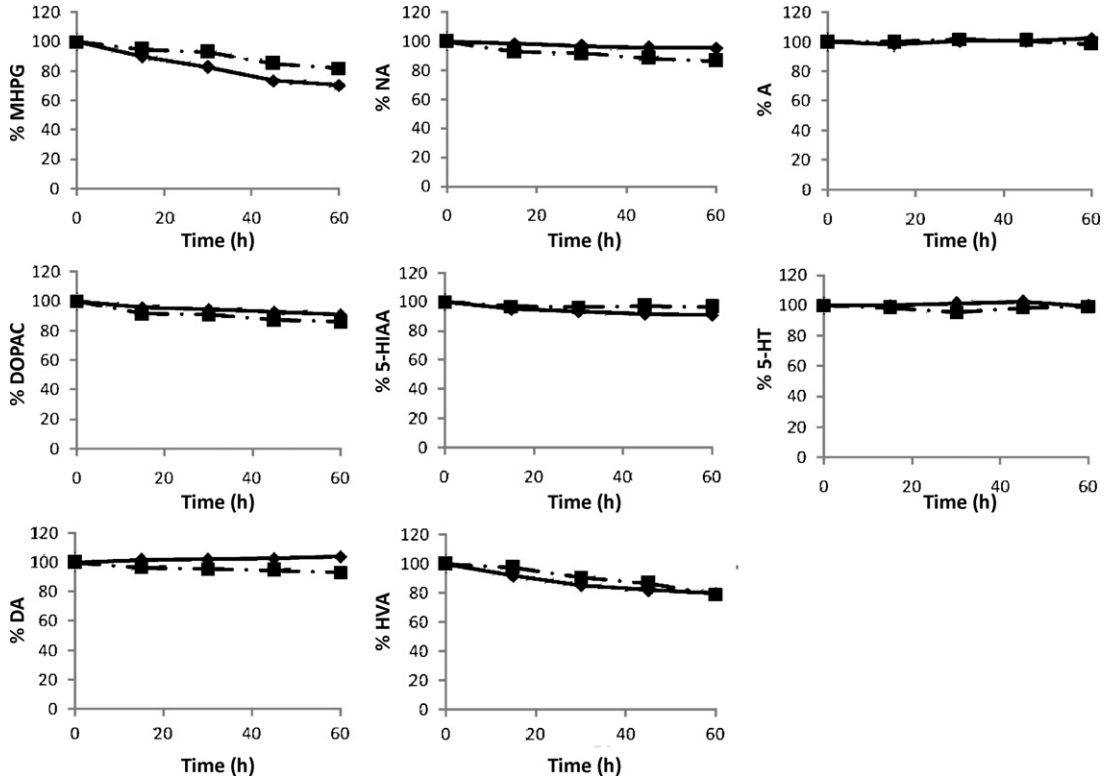

Fig. 4. Stability studies of eight biogenic amines and their metabolites, MHPG, NA, A; DOPAC, HVA, DA; 5-HIAA, 5-HT in nonmatrix conditions (full lines), and in matrix conditions (broken lines) (see conditions in the text). The recovery of each substance over time was calculated as percentage of its response to its starting response.

is accurate at very low concentrations; about 10 pg for MHPG, NA, A and HVA and less than 4 pg for DOPAC, 5-HIAA, DA and 5-HT.

3.3.3. Precision

The variability of the responses of each component is calculated and expressed as RSD (%) in Table 2. The results show a good repeatability of response of HPLC system to different runs of the same solution (RSD ≤ 3%).

The precision of the developed method was estimated by determination of the RSD for repeatability and time-different intermediate precision at three concentration levels used in the validation method for different compounds [73]. The RSD values presented in Table 2 were relatively low (≤5%) showing a good precision of the developed method.

3.3.4. Accuracy

The recovery of eight analytes was around 100%, MHPG, DA, 5-HIAA, HVA and 5-HT and for A, DOPAC (94.2, 94.2%) and NA (96.6%) indicating an excellent accuracy of the developed analytical method (Table 2).

The relative recovery of analytes in nonbiological matrix conditions after undergoing every extraction step refers to the accuracy of the analytical method. The relative recovery of the eight molecules was studied in citric–phosphate buffer and found to be around 95% (Table 2). In addition, the average of standard error mean (SEM) calculated was small (≤1.2) indicating a good accuracy of the developed analytical method. The percentage of recovery of 5-HT in nonmatrix conditions was lower than observed in matrix conditions. Due to the fact that the eight standard molecules were dissolved in phosphate–citric buffer (i.e., nonmatrix conditions) and then filtrated through a protein-cut-off filter, some of the more hydrophobic compounds (e.g., 5-HIAA, DA, HVA, and 5-HT) may have been retained on the filter, causing lower recovery of these molecules. The viscosity is higher in matrix vs. nonmatrix conditions, which may facilitate the filtration process, and hence, recovery of compounds.

3.3.5. Stability of stock, standard and tested solutions

While standards in citric–phosphate buffer were used to study the stability of biogenic amines and their metabolites in nonmatrix conditions, biogenic amines and their metabolites in biological samples were extracted in citric–phosphate buffer and used to study their stability in matrix conditions. All preparations and injections were carried out at 4 °C to reduce the degradation of the products [44]. The recovery of each substance over time was calculated as the percentage of its response to its starting response. Fig. 4 shows clearly that most molecules were stable in both matrix and nonmatrix conditions for at least 15 h. 5-HT in particular, was stabilized in both conditions for 60 h with about 100% recovery (99.6 and 99.8% in nonmatrix and in matrix conditions, respectively). NA, DOPAC, DA and 5-HIAA were stable in both conditions but after 30 h, some degradation of NA and DOPAC could be observed. MHPG and HVA were found to be less stable molecules. The recovery of these molecules dropped to 70.6–82% for MHPG and 79.1–79.2% for HVA after 60 h in both conditions. The stability of these molecules is higher in matrix conditions compared with citric–phosphate conditions. Recapitulating, it is advised to prepare and run samples within 1 day and standards within 15 h.

3.3.6. Effect of dilution

The levels of biogenic amines and their metabolites are not identical in different parts of the brain tissue [40], thus, different dilutions of the samples might be applied to obtain suitable concentrations for the analysis. As reported above (Section 3.1), dilution of brain sample in 10 mM acetic acid had a strong effect on the pH of the solution, thereby affecting the retention time and response

(signals) of the molecules of interest, leading to potential misinterpretation of the levels of biogenic amines and their metabolites in different parts of the brain.

Dilution of the brain extract in citric–phosphate buffer (2, 4 and 6 times) gave identical and stable pH solutions. The retention time of the eight analytes was identical in hexaplicate injections of diluted solutions; no significant difference between the means of each analyte was found in the diluted samples (Table 2), suggesting that dilution does not affect the analysis. Moreover, all data were within the acceptance limits which were set up at ±15% of the average value, thereby allowing application of the developed method to determine the endogenous molecules in different parts of the brain with high precision and accuracy.

4. Concluding remarks

The proposed HPLC–ECD method with a very fast, simple preparation is adequate for simultaneous determination of eight biogenic amines and their metabolites in brain tissues at different concentration levels, using DHBA and 5-HMT as internal standards. In spite of the complexity of the brain tissue matrix, the validation of the analytical method proved that the method is adequate and fulfilling the criteria accepted by the guidelines for linearity, LOQ, stability, selectivity and accuracy in a bio-analytical laboratory.

Optimization of chromatography for the separation of eight endogenous molecules and two internal standards was achieved using two-level fractional factorial experimental design. The optimization of the separation was obtained by dually optimizing the influence of five factors, OSA concentration, pH of the mobile phase, amount of organic modifier, temperature of the column and voltage of detector on the separation factors and capacity factor of each molecule. This approach led to an effective and fast separation of the ten molecules in both matrix and nonmatrix conditions in acceptable time of analysis (30 min) under the following optimal conditions: a mixture of methanol–phosphate–citric buffer (pH 3.2, 50 mM) (9:91, v/v) containing 2 mM OSA as mobile phase, running with a flow rate of 0.05 ml/min, on a microbore ALF-115 column (150 mm × 1.0 mm, 3 μm particle size) filled with porous C_{18} silica as stationary phase, maintained at 32 °C and setting of detector at 650 mV.

Given that the LOQ for all eight molecules was around 10 pg, the proposed analytical method is highly sensitive. The analytical method can save efforts, money and time when monitoring levels of the endogenous molecules in biological samples proposed for research work or clinical examination.

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