DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE MEASUREMENTS OF BIOGENIC AMINES

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Abstract

Many important biogenic amines (dopamine, noradrenaline and serotonin) are produced from amino acids by enzyme-catalysed processes and play a prominent role in neuronal functions and therefore, they serve as pharmacological target for the treatment of neurological disorders, such as Alzheimer's disease or Parkinson's disease.

The aim of the current study was to optimize a high-performance liquid chromatography method that allows selective separation of eight biogenic amines and some of their metabolites (levodopa, 3,4-dihydroxyphenylacetic acid, noradrenaline, 5-hydroxyindoleacetic acid, homovanillic acid, dopamine, serotonin and 3-methoxythyramine) using 3 internal standards with electrochemical detection. During the development of our method, we optimized the amount of ion pairing component, pH and the amount of organic phase. Several selective methods were tested, but the most effective one was used for validation process for mouse and rat brain regions, including the striatum, cortex and hippocampus.

During validation, the limit of detection, the limit of quantification, recovery, intraday and interday precisions were determined for the eight analytes. The ranges of recovery were between 87 and 120%, the intraday and interday precision were < 10% in all cases. The limit of detection and quantification ranged around 2 and 10 ng/ml, respectively.

The developed and optimized method ensures the measurement of the aforementioned biogenic amines from mouse and rat brain regions.

Introduction

Monitoring of the concentration of biogenic amines may have a great importance from several aspects [1]. The measurement of these metabolites from biological samples requires highly selective and sensitive methods because of their considerably low concentrations [1, 2]. High-performance liquid chromatography (HPLC) methods have been widely applied [3] for this purpose. HPLC combined with electrochemical detector (ECD) is one of the best alternatives for the quantitative detection of monoamines and related compounds in biological samples because of their electroactive function groups and the exceptional sensitivity of the ECD.

The aim of the current study was to optimize our latest HPLC-ECD method [3] to be able to determine 8 biogenic amines and some of their metabolites (levodopa (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), noradrenaline (NA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), dopamine (DA), serotonin (5-HT), 3-methoxythyramine (3-MT)) from different biological samples. It is essential to apply internal standard(s) (IS) for HPLC measurements [4, 5], therefore, based on the recommendations [4], we decided to use 3 (3,4-dihydroxybenzylamine (DHBA), isoproterenol (IPR) and N-methyl serotonin (NM-5HT) instead of the previous one. After the successful extension of the method, we applied it to different biological samples, i.e., mice and rat brain regions and the validation process was carried out as well.

Materials and methods

L-DOPA, 5-HT and their metabolites, DA, 3-MT, DOPAC, HVA, NA and 5-HIAA were measured from the striatum, cortex and hippocampus of C57Bl/6 mice and Wistar rat animals. We used an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) combined with a Model 105 ECD (Precision Instruments, Marseille, France) under isocratic conditions. The brain regions were weighed and then homogenized in an ice-cold solution (striatum: 60 µL/mg; cortex: 25 µL/mg and hippocampus: 18.75 µL/mg) containing perchloric acid (3.4 w%, Sigma Aldrich, Saint Louis, MO, USA), sodium-metabisulfite (400 µM, Fluka, Sigma-Aldrich, Saint Louis, MO, USA), ethylenediaminetetraacetic acid disodium salt (Na_2EDTA, 500 µM, Lach-Ner, Neratovice, Czech Republic), distilled water and ISs: 50 ng/ml DHBA, 200 ng/ml IPR and 100 ng/ml NM-5HT (Sigma Aldrich, Saint Louis, MO, USA). The homogenate was centrifuged at 12,000*g* for 30 min at 4°C. The supernatants of individual brain regions were pooled and spiked with standard solution in three different concentration levels. The working potential of the detector was set at +750 mV, using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase contained sodium-dihydrogenphosphate (NaH_2PO_4; 75 mM, Reanal, Budapest, Hungary), Na-octylsulphate (NaOS, 2.2 mM, Sigma Aldrich, Saint Louis, MO, USA) and Na_2EDTA (50 µM, Lach-Ner, Neratovice, Czech Republic) was supplemented with acetonitrile (ACN; 6.25% v/v, VWR International, Radnar, PA, USA) and the pH was adjusted to 3.0 with phosphoric acid (H_3PO_4; 85% w/w, Sigma Aldrich, Saint Louis, MO, USA). The mobile phase was delivered at a rate of 1.5 ml/min at 40°C onto the column (Zorbax Eclipse Plus C18, 100 x 4.6 mm, 3.5 µm particle size; Agilent Technologies, Santa Clara, CA, USA) after passage through a pre-column (SecurityGuard, 4×3.0 mm i.d., Phenomenex Inc., Torrance, CA, USA)). 10 µL aliquots were injected by the autosampler with the cooling module set at 4°C.

Results

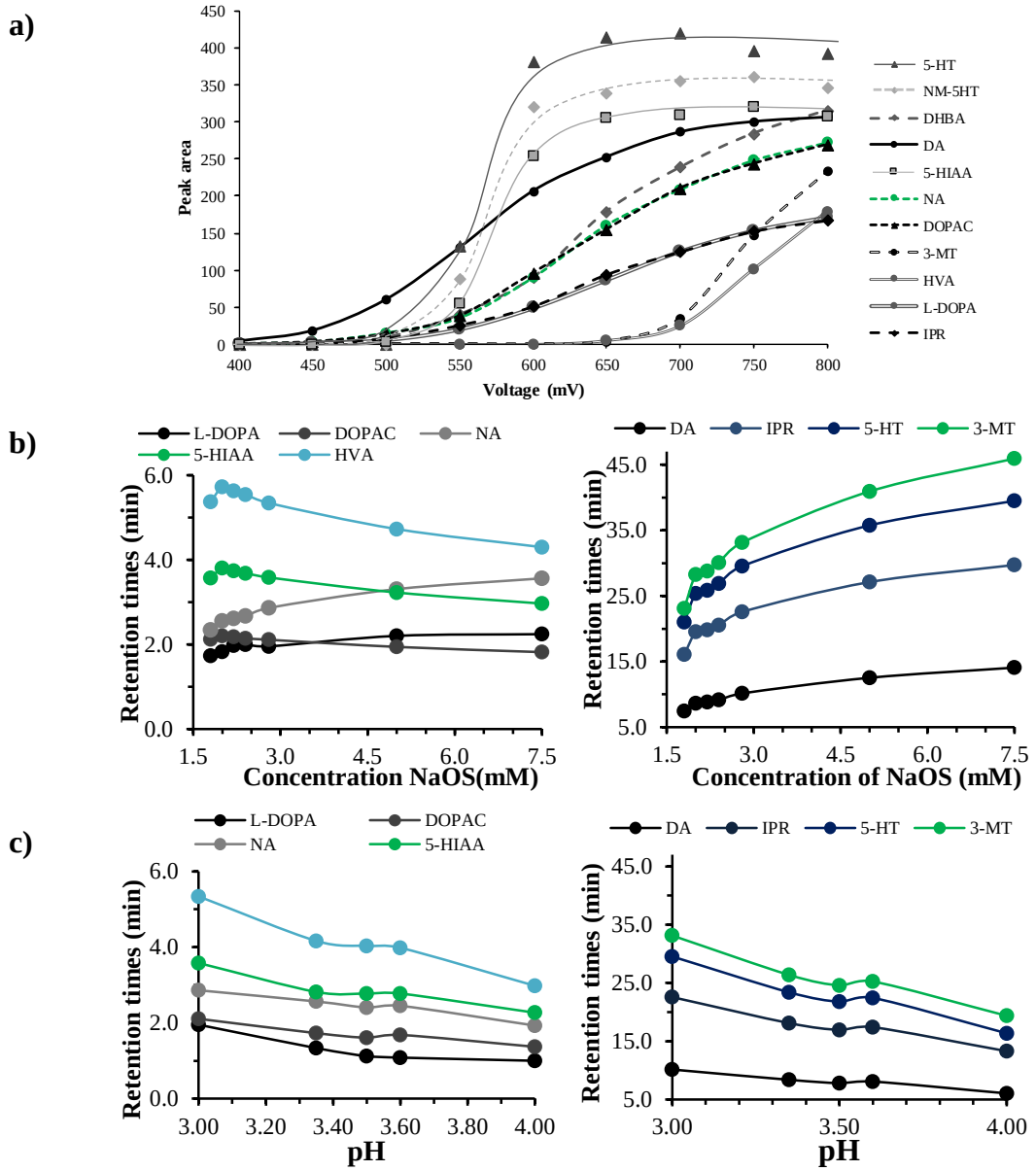
Before validation, the optimal working potential was investigated. The detector was set from 400 mV to 800 mV with 50 mV increments (**Fig 1a**). Although 800 mV would be the best working potential for HVA and 3-MT, the signal-to-noise ratio also increases with the applied working potential, so we decided to set the detector at +750 mV.

First we determined the effect of the change in NaOS concentration (**Fig.1b**) with a result that the increasing amount has ambivalent effect on analytes: increased retention times were observed in case of L-DOPA, NA, DA, 5-HT, 3-MT, IPR (IS), whereas in case of DOPAC, 5-HIAA and HVA the retention time decreased. The concentration of 7.5 mM was selective for all the compounds as well, however, the run time was more than 45 min.

Then we checked the influence of the pH value of the mobile phase (**Fig.1c**). As it can be seen, the increasing value of pH from 3.0 to 4.0 reduced the retention time of all the analytes.

These results showed that the best choice is to keep the pH at 3.0 and NaOS in the concentration range between 2.10 mM and 2.20 mM with 5 or 6 v/v% ACN. With the 2 new internal standards (DHBA and NM-5HT), the optimal mobile phase consists of 2.20 mM NaOS, 75 mM NaH_2PO_4, 50 µM Na_2EDTA and 6.25 v/v% ACN. Before adding ACN, the pH value of water phase was set to 3.0 with 85 w/w% H_3PO_4. Test runs showed that mice and rat brains can be measured well with this method.

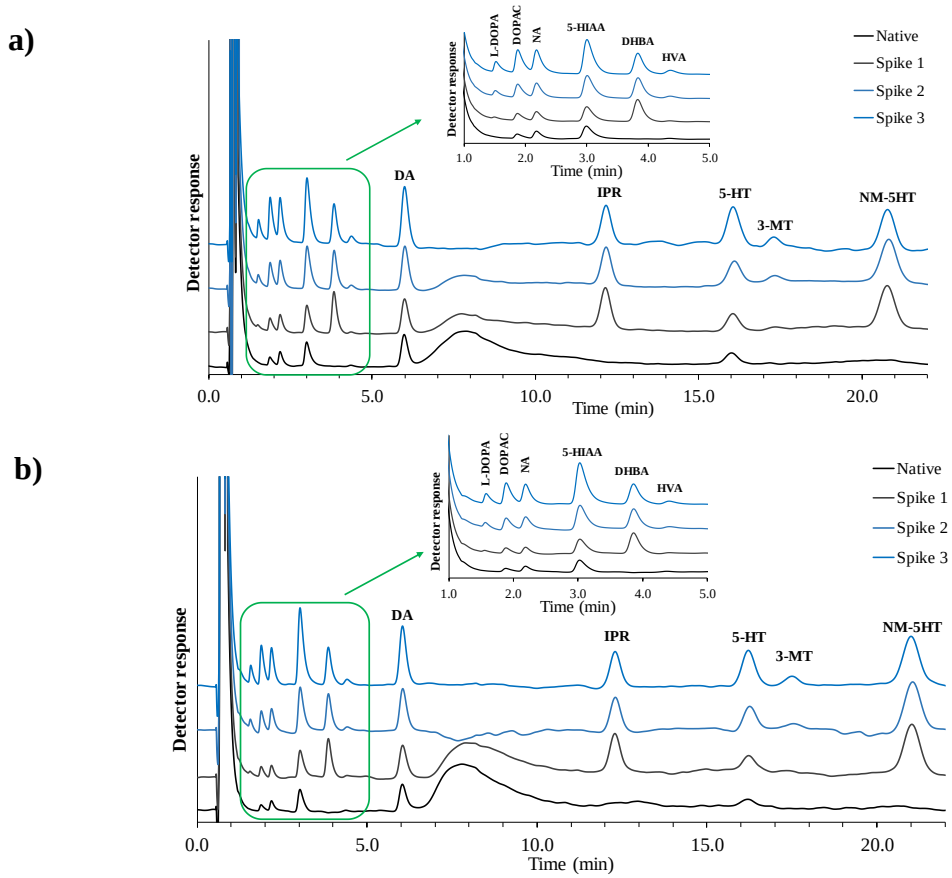
Fig. 1 Voltage vs. peak area responses of the analytes and internal standards (**a**) and the effect of the concentration of NaOS (**b**) or pH (**c**) in mobile phase to retention times of analytes and internal standard. The pH was set to 3.0 and ACN was 5 v/v%.



ACN acetonitrile; *DA* dopamine; *DHBA* 3,4-dihydroxybenzylamine; *DOPAC* 3,4-dihydroxyphenylacetic acid; *HVA* homovanillic acid; *IPR* isoproterenol; *L-DOPA* levodopa; *NA* noradrenaline; *NaOS* Na-octylsulphate; *NM-5HT* N-methyl serotonin; *3-MT* 3-methoxythyramine; *5-HIAA* 5-hydroxyindoleacetic acid and *5-HT* serotonin.

We only demonstrate the results of the striatum from mice and rats. The results of validations are presented in **Table 1**. In **Fig. 2** the native and the spiked chromatograms are demonstrated. Native sample is from pooled mice or rat striatum, cortex, hippocampus, cerebellum and brainstem regions.

Fig. 2 Native and spiked chromatograms of pooled mice (**a**) and rat (**b**) brain regions.



DA dopamine; *DHBA* 3,4-dihydroxybenzylamine; *DOPAC* 3,4- dihydroxyphenylacetic acid; *HVA* homovanillic acid; *IPR* isoproterenol; *L-DOPA* levodopa; *NA* noradrenaline; *NM-5HT* N-methyl serotonin; *3-MT* 3-methoxythyramine; *5-HIAA* 5-hydroxyindoleacetic acid and *5-HT* serotonin.

Table 1. Summary of validation parameters of HPLC-ECD method for biogenic amines in mouse and rat striatum.

Validation parameters		L-DOPA	DOPAC	NA	5-HIAA	HVA	DA	5-HT	3-MT
Linear range (ng/ml)	mouse	5-150	5-80	5-80	10-100	10-100	5-200	5-100	10-200
	rat								
Correlation coefficient	mouse	1.000	0.999	0.999	0.998	0.994	0.999	0.999	0.998
	rat	0.999	0.999	0.999	0.995	0.993	1.000	0.999	0.997
LOD (ng/ml)	mouse	1.3	0.5	0.7	5.5	7.4	2.0	3.8	9.8
	rat	3.1	3.6	7.1	7.9	8.6	2.6	3.8	12.8
LOQ (ng/ml)	mouse	3.9	1.5	2.1	16.5	22.3	6.0	11.6	29.7
	rat	9.3	10.9	21.7	23.9	26.1	7.9	11.4	38.7
Recovery (%)	mouse	100.7	108.0	110.6	108.9	104.3	109.0	104.4	102.0
	rat	108.0	104.9	106.1	109.0	95.3	106.1	103.5	97.3
Intraday precision	mouse	3.19	1.81	3.68	3.82	8.23	1.59	5.36	6.70
	rat	1.96	2.57	3.17	2.76	7.28	1.85	3.60	9.74

(CV%)									
Interday precision (bias%)	mouse	3.24	1.83	3.89	3.85	8.59	1.63	5.42	6.85
	rat	1.98	2.57	3.17	2.75	7.42	1.84	3.62	9.52

DA dopamine; *DOPAC* 3,4- dihydroxyphenylacetic acid; *HPLC-ECD* high-preformance liquid chromatography with electrochemical detector; *HVA* homovanillic acid; *L-DOPA* levodopa; *LOD* limit of detection; *LOQ* limit of quantification; *NA* noradrenaline; *3-MT* 3-methoxythyramine; *5-HIAA* 5-hydroxyindoleacetic acid and *5-HT* serotonin.

Discussion

We successfully extended our latest method with 5 new compounds, of which 2 are internal standards. The applied method is suitable for the measurements of brain regions of mice and rats. The validation parameters are in line with international guidelines. Based on our results, this method will be a valuable tool for multiple experiments, such as, rodent toxin models of neurological disorders.

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