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Novel and sensitive reversed-phase high-pressure liquid chromatography method with electrochemical detection for the simultaneous and fast determination of eight biogenic amines and metabolites in human brain tissue

Debby Van Dam[a], Yannick Vermeiren[a], Tony Aerts[a], Peter Paul De Deyn[a,b,c,d,*]

[a] Laboratory of Neurochemistry and Behaviour, Institute Born-Bunge, University of Antwerp, Department of Biomedical Sciences, Universiteitsplein 1, Wilrijk B-2610, Belgium
[b] Biobank, Institute Born-Bunge, University of Antwerp, Universiteitsplein 1, Antwerp 2610, Belgium
[c] Department of Neurology/Memory Clinic, Middelheim General Hospital, ZNA, Lindendreef 1, Antwerp 2020, Belgium
[d] University of Groningen, Department of Neurology and Alzheimer Research Center, University Medical Center Groningen (UMCG), Hanzeplein 1, Groningen 9713 GZ, The Netherlands

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ABSTRACT

A fast and simple RP-HPLC method with electrochemical detection (ECD) and ion pair chromatography was developed, optimized and validated in order to simultaneously determine eight different biogenic amines and metabolites in post-mortem human brain tissue in a single-run analytical approach. The compounds of interest are the indolamine serotonin (5-hydroxytryptamine, 5-HT), the catecholamines dopamine (DA) and (nor)epinephrine ((N)E), as well as their respective metabolites, i.e. 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 5-hydroxy-3-indoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG). A two-level fractional factorial experimental design was applied to study the effect of five experimental factors (i.e. the ion-pair counter concentration, the level of organic modifier, the pH of the mobile phase, the temperature of the column, and the voltage setting of the detector) on the chromatographic behaviour. The cross effect between the five quantitative factors and the capacity and separation factors of the analytes were then analysed using a Standard Least Squares model. The optimized method was fully validated according to the requirements of SFSTP (Société Française des Sciences et Techniques Pharmaceutiques). Our human brain tissue sample preparation procedure is straightforward and relatively short, which allows samples to be loaded onto the HPLC system within approximately 4 h. Additionally, a high sample throughput was achieved after optimization due to a total runtime of maximally 40 min per sample. The conditions and settings of the HPLC system were found to be accurate with high intra and inter-assay repeatability, recovery and accuracy rates. The robust analytical method results in very low detection limits and good separation for all of the eight biogenic amines and metabolites in this complex mixture of biological analytes.

1. Introduction

Given their role in the control and regulation of principal functions and behaviours, the biogenic amines, dopamine (DA), norepinephrine (NE), epinephrine (E) and serotonin (5-HT), together with their precursors and metabolites, have been implicated in the pathophysiology of various neurological and psychiatric conditions [1,2], and may serve interesting biomarkers for disease development or progression and targets for the development of novel therapeutic leads. The simultaneous measurements of these biogenic amines and metabolites in human brain tissue in a single and fast chromatographic run, following a simple sample preparation procedure is therefore of major interest to a broad neuroscience field.

The determination of these biogenic amines and their metabolites in brain tissue requires a highly sensitive and selective method. Over the past decade, several HPLC methods with online sensors, including mass spectrometry (MS), fluorescence (FD)

* Corresponding author at: Laboratory of Neurochemistry & Behaviour, Institute Born-Bunge, University of Antwerp, Department of Biomedical Sciences, Universiteitsplein 1, 2610 Wilrijk, Belgium. Tel.: +32 3 265 26 20; fax: +32 3 265 26 18.
E-mail addresses: debby.vandam@uantwerpen.be (D. Van Dam), yannick.vermeiren@uantwerpen.be (Y. Vermeiren), tony.aerts@uantwerpen.be (T. Aerts), p.p.de.deyn@umcg.nl, peter.dedeyn@uantwerpen.be, dedeyn@skynet.be (P.P. De Deyn).

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and electrochemical detection (ECD), have been developed [3–5]. HPLC combined with ECD (HPLC-ECD) is still considered the method of choice given its potential as a specific and sensitive technique capable of automation, its low cost of analysis, and often simpler sample pretreatment requirements in comparison to for example HPLC with FD [5]. Many applications using ion-exchange reversed-phase HPLC with ECD (RP-HPLC-ECD) have been developed for the determination of these endogenous molecules in biological samples [6–11], but often only a limited number of compounds could be analysed in a single chromatographic run. We previously reported the development, optimization and validation of an ion-exchange reversed-phase HPLC with ECD method to determine eight biogenic amines and metabolites in mouse brain tissue [12]. Given the different matrix conditions in human and mouse brain tissue, as well as the application of a longer chromatographic column to increase efficiency based on augmented resolution, the earlier reported method required additional optimization, as described in this paper. We applied the Antec Leyden (Antec Leyden BV, Zoeterwoude, The Netherlands) dual chromatographic system containing two pumps, two injection loops, two columns and two glassy carbon working electrodes positioned in parallel for the simultaneous determination of the eight molecules: i.e. the indolamine 5-HT, the catecholamines DA, E and NE, as well as their respective metabolites, i.e. 5-HIAA (5-Hydroxyindoleacetic acid; metabolite of the serotonergic neurotransmitter system), DOPAC and HVA (3,4-dihydroxyphenylacetic acid and homovanillic acid, respectively; metabolites of the dopaminergic neurotransmitter system) and MHPG (3-Methoxy-4-hydroxyphenylglycol; metabolite of the (nor)adrenergic neurotransmitter system). Optimization and method validation are important and essential steps when developing new bioanalytical procedures. We report the validation process of our proposed analytical HPLC method in compliance with SFSTP (Société Française des Sciences et Techniques Pharmaceutiques) guidelines [13,14].

2. Material and methods

2.1. Chemicals

Phosphoric acid, citric acid, octan-1-sulfonic acid sodium salt (OSA), and 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), NE (as hydrochloride), E (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 (Millipore, Bedford, MA, USA) was used.

2.2. Human brain sample collection and preparation

Following consented brain autopsy, the left hemisphere was frozen at −80 °C within 6 h after death for neurochemical analyses. With regard to this specific study design, BA22 was chosen for method development and optimization.

Samples needed to be nonacidotic (i.e. pH > 6.1) in order to guarantee high-quality brain tissue since acidosis may induce alterations in neurotransmitter/neuropeptide concentrations and enzyme activity [15]. Several factors such as a prolonged death struggle, pre-mortem stroke and a long post-mortem delay could acidify brain tissue [16]. In this study, pH values of the cerebellar cortex were measured as described by Stan et al. [16] since the cerebellar pH is most representative for the entire brain. If the cerebellar pH were to be below 6.1, pH values of the individual dissected brain regions would be measured.

Sample preparation procedures were previously optimized by our group, taking into consideration the unstable nature of biogenic amines [12]. In brief, frozen BA22 samples (–300 mg) were weighed in 4 ml phosphoric–citric buffer (Section 2.3.2). Next, this mixture was homogenized for 40 s at moderate speed using an Ultra-Turrax TR 50 homogenizer® (Janke & Kunkel, Ika-Werk, Staufen, Germany). The homogenate was then centrifuged (26,000 × *g*, 20 min, 4 °C) and afterwards, the supernatant was filtered using a 0.2-μm Millipore® filter (Millex, Millipore, Ireland) attached to a syringe. Further elimination of proteins was accomplished using 10-kDa Amicon® Ultra Centrifugal Filters (Millipore, Ireland) (14,000 × g, 20 min, 4 °C) which were washed twice beforehand with mobile phase.

2.3. HPLC conditions

2.3.1. Instrumentation

An Alexys™ monoamines analyzer system (Antec Leyden, The Netherlands) consisting of two LC 110 pumps operating at an isocratic flow rate of 40 μl/min was applied. Samples (5 μL) were loaded with an Alexys™ AS 100 Autosampler on two identical microbore ALF-125 columns (250 mm × 1 mm, C_{18}, 3 μm particle size) maintained at a constant temperature. The Decade II electrochemical detector was equipped with two thin layer electrochemical VT03 flow cells each fitted with a glassy carbon 0.7 mm working electrode and an in situ Ag/AgCl (ISAAC) reference electrode. Integration of chromatograms was performed with channel integration M018/EN25B Clarity software (DataApex Ltd., Prague, The Czech Republic).

2.3.2. Preparation of mobile phases

The preparation of phosphoric–citric mobile phases was a two-step procedure: (1) buffer preparation: KCl, H_3PO_4, citric acid, OSA and Na_2EDTA were dissolved in an appropriate volume of water, and the pH was adjusted with 50% NaOH. Water was further supplemented to guarantee the desired concentration of each component if required, i.e. 8 mM KCl, 50 mM H_3PO_4, 50 mM citric acid, 0.1 mM Na_2EDTA, and the desired concentration of OSA during method development, optimization and validation (Table 1). 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: the buffer was mixed with the organic modifier MeOH in a concentration as 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) prior to application in the HPLC system.

2.4. Chromatographic conditions: development and optimization of the analytical method

2.4.1. Experimental design

Building on previous method development and optimization for the detection of biogenic amines in mouse brain tissue using two-level fractional factorial experimental design ($1/2\ 2^K$) [12], five quantitative factors were known to significantly influence chromatographic behaviour: (1) the ion-pair counter concentration, i.e. the concentration of octan-1-sulfonic acid in the mobile phase, [OSA]; (2) the pH of the mobile phase, (pH); (3) the level of organic modifier, i.e. the percentage of methanol added to the mobile phase, [MeOH]; (4) the temperature of the column, (t°);