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Perspectives to evaluate the impact of physical activity on mental health using a HPLC method for the monitoring of biogenic amine levels

Perspective pentru evaluarea impactului activității fizice asupra sănătății mintale folosind o metodă HPLC pentru monitorizarea nivelului de amine biogene

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Abstract

Background. Among multiple regulatory systems involved, changes in the homeostasis of neurotransmitters are strongly involved in the beneficial effects of exercises on mental health status. On the other hand, recent studies highlight the alarming prevalence of mental health disturbances among athletes. As biogenic amines are extremely sensitive to many factors, their analysis requires specific and very sensitive bioanalytical methods, justifying the development of new methods for their accurate analysis.

Aims. The aim of this study was the development of a reversed phase chromatographic method (RP-HPLC) with fluorescence detection (FLD) for the simultaneous analysis of epinephrine (E), norepinephrine (NE), dopamine (DA) and serotonin (ST).

Methods. A two-step derivatization reaction was applied using a mixture of benzylamine and 1,2-diphenylethylenediamine. Chromatographic separation of the fluorescent derivatives was developed and optimized using: an internal standard, a new C_{18} stationary phase, acetonitrile and a 10 mM acetate buffer containing 1 mM heptane-1-sulfonic acid sodium salt (pH=5.30) in gradient mode with 0.80 mL/min flow rate. Fluorescent derivatives were detected at λex=345 nm/λem=480 nm.

Results. Chromatographic analysis of biogenic amines was performed in 16 min run time at ng/mL level. The relative standard deviation was found to be lower than 3%, when testing repeatability and reproducibility. Additionally, the regression analysis proved the method linearity in a range of 2.5 to 15.2 ng/mL for NE, 30 to 210 ng/mL for ST, 6.8 to 15.8 ng/mL for E and 10.8 to 63.0 ng/mL for DA, with regression coefficients greater than 0.999 for all derivatives.

Conclusions. A RP-HPLC-FLD method, which allows simultaneous quantification of biogenic amines, was developed and pre-validated. After complete validation, this method will be applied for the analysis of biogenic amines in urine.

Keywords: athletes, catecholamines, serotonin, fluorescence derivatization, HPLC, psychiatric disorder.

Rezumat

Premize. Alături de alte sisteme de reglare, modificările în homeostazia neurotransmițătorilor sunt puternic implicate în efectele benefice ale exercițiilor fizice asupra stării psihice. Pe de altă parte, studii recente evidențiază prevalența alarmantă a tulburărilor de sănătate mintală în rândul sportivilor. Întrucât aminele biogene sunt extrem de sensibile la diferiți factori, analiza lor necesită metode bioanalitice specifice și foarte sensibile, fapt care justifică dezvoltarea de noi metode de analiză.

Obiective. Obiectivul acestei lucrări a fost elaborarea unei metode cromatografice de lichide de înaltă performanță pe fază inversă cu detecție de fluorescență, pentru analiza simultană a epinefrinei (E), norepinefrinei (NE), dopaminei (DA) și serotoninei (ST).

Metode. A fost aplicată o reacție de derivatizare în două etape cu benzilamină, respectiv 1,2-difeniletilendiamină. Dezvoltarea și optimizarea metodei de separare cromatografică a derivaților fluorescenți a presupus utilizarea unui standard intern, a

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unei faze staționare C_{18} de ultimă generație, acetonitril și un tampon acetat 10 mM conținând sarea acidului heptan-1-sulfonic (pH=5,30) ca fază mobilă în gradient cu debit de 0,80mL/min. Fluorescența derivaților a fost monitorizată la λex=345nm/ λem=480nm.

Rezultate. Analiza cromatografică a fost efectuată în timp de 16 minute la nivele de ng/mL. Deviația standard relativă a fost sub 3% pentru repetabilitate și reproductibilitate. Liniaritatea metodei a fost demonstrată pe baza coeficientului de corelație de 0,999 obținut pentru toate aminele biogene în următoarele domenii 2,5-15,2 ng/mL pentru NE, 30-210 ng/mL pentru ST, 6,8-15,8 ng/mL pentru E și 10,8-63,0 ng/mL pentru DA.

Concluzii. A fost dezvoltată și prevalidată o metodă cromatografică de înaltă performanță de fază inversă cu detecție de fluorescență pentru analiza simultană a aminelor biogene. După validare, aceasta va fi aplicată la analiza aminelor biogene din urină.

Cuvinte cheie: sportivi, catecolamine, serotonină, derivatizare, fluorescență, HPLC, boli psihice.

Introduction

There is clear evidence that the most important mainstay of mental health disease prevention for all people is physical activity. Moderate and regular physical activity has been proved to maintain mental health status (Peluso & Guerra De Andrade, 2005; Lin & Kuo, 2013; Dunn & Jewell, 2010), and even to have therapeutic benefits for psychiatric illnesses (Archer et al., 2014; Ruo et al., 2004; Rimer et al., 2012; Bruja et al., 2013; Dunn & Jewell, 2010) and neurodegenerative diseases (Sahlin & Lexell, 2015).

As recently reviewed (Lin & Kuo, 2013), the levels of biogenic amines, such as catecholamines norepinephrine (NE), dopamine (DA) and the indoleamine serotonin (ST) change after performance of exercise. Moderate exercise increases DA and ST levels, while NE levels decrease, but on the other hand, overtraining stimulates the hyperactivity of biogenic amines and causes fatigue (Carfagno & Hendrix, 2014; Lin & Kuo, 2013).

More accurately, the interconnection of the NE system and exercise is supported by an enhancement of neuronal adaptation against stress stimuli. An explanation of the protective effect against stress is attributed to the secretion of galanin after exercise and the consequent inhibition of NE release, which reduces anxiety. Regarding exercise and the DA system, it has been shown that exercise increases DA levels. Furthermore, it determines calcium/ calmodulin-dependent DA synthesis after tyrosine hydroxylase activation, increases DA and DA receptor binding affinity and also has a protective effect against the toxicological degradation of DA neurons. The ST system is also modulated by exercise. ST levels increase only after intense exercise and determine central fatigue, while after moderate physical activity they remain unaltered. Among the properties of the ST system, its antidepressant and anxiolytic roles support the benefits of exercise for mental health status. Above all, biogenic amines modulate the activity of each other, sustaining their common interplay in physical exercise (Lin & Kuo, 2013).

Although moderate and regular exercises have proved to counteract symptoms of depression (Archer et al., 2014; Ruo et al., 2004; Rimer et al., 2012; Bruja et al., 2013), genetic susceptibility seems to be a cause for the development of depression (Haslacher et al., 2015), anxiety and panic disorder (Sardinha et al., 2011), neurological disabilities (Sahlin & Lexell, 2015), and even neurocognitive dysfunction in bipolar disease. Recent studies highlight the alarming prevalence (46.4%) of mental health disturbances among athletes (Gulliver et al., 2015). Thus, the prevalence of depression ranged up to 27.2% (Gulliver et al., 2015; Gouttebarge et al., 2015; Weigand et al., 2013) in active athletes and up to 39% in former athletes (Gouttebarge et al., 2015; Weigand et al., 2013). Other depression related conditions among athletes were also observed: eating disorders (22.8%), general psychological distress (16.5%), social anxiety (14.7%), generalized anxiety disorder (7.1%) and panic disorder (4.5%) (Gulliver et al., 2015).

Furthermore, the idealization of athletes and their health status has led to the generalized assumption of a low prevalence of depression experienced by athletes and above all, athletes themselves are taught to be tough and to focus on physical performance. From the research findings, it is well understood that an athlete's mental state plays a crucial role in their ability to perform (Bar & Markser, 2013). Therefore, well documented causes of depression among athletes, such as: a high pressure environment focused on winning and achieving progress, overtraining syndrome (Carfagno & Hendrix, 2014), concussions and injuries (Vargas et al., 2015; Roiger et al., 2015), support that mental health among athletes has gained increasing attention in recent years.

Unfortunately, evaluation of the mental health status of athletes relies on online self-reported questionnaires such as: a self-report Internet-based survey (Gulliver et al., 2015), the Distress Screener, the Utrecht Burn-Out Scale, the 12-item General Health Questionnaire, Rosenberg's Self-Esteem Scale (Gouttebarge et al., 2015), a cross-sectional online survey including Wakefield Depression Inventory (Weigand et al., 2013) and The Beck Depression Inventory (Vargas et al., 2015), while no clinical parameter was included in these assessments.

The main disadvantage of these tools is their partiality. The patients' interpretation of their emotional terms and cultural conception of depression influences screening reliability. Therefore, under- or overreporting of symptom severity often occurs, leading to inaccurate evaluation. Moreover, these questionnaires cannot be completed by individuals with physical debility or compromised cognitive function, such as injured athletes (Kerr & Kerr, 2001).

The clinical relevance of these studies could be significantly increased by applying a biological parameter analysis, such as the simultaneous analysis of biogenic amines from urine, due to the fact that biofluids such as urine are an easy, rapid and non-invasive matrix to collect.

Given that biogenic amines are characterized by extremely low concentrations, low chemical stability, high susceptibility for spontaneous oxidation and decomposition

at high pH level, their analysis requires specific and very sensitive bioanalytical methods. Concurrent studies showed tremendous interferences from the matrix (such as plasma, serum or urine), which affected both the sensitivity and specificity of the developed methods (Bicker et al., 2013).

Radioenzymatic and immunological assays were replaced by more sensitive and selective chromatographic methods, while high-performance liquid chromatography represents the standard method for separation and quantification in biological samples, coupled with electrochemical (Patel et al., 2005; Duncan et al., 1984; Holmes et al., 1994; Raggi et al., 1999; Unceta et al., 2001; Willemsen et al., 2003; Sabbioni et al., 2004; Kumar et al., 2011), fluorescence (Mitsui et al., 1985; Jeon et al., 1992; Ishida et al., 1993; Kehr, 1994; Yamaguchi et al., 1998; Fujino et al., 2003; Zhao & Suo, 2008), chemiluminescence (Nalewajko et al., 2007) or mass spectrometry detection (Törnkvist et al., 2004; Bourcier et al., 2006; De Jong et al., 2007; Gu et al., 2008; Cai et al., 2010; Clark & Frank, 2011; He et al., 2011; He & Kozak, 2012; Fang et al., 2012).

One successful approach for both eliminating interferences and increasing selectivity and sensitivity relies on a derivatization step prior to analysis. Two derivatization agents, benzylamine (BA) and 1,2-diphenylethylenediamine (DPE), were used in several HPLC-FLD methods for the derivatization of biogenic amines.

DPE was proposed as a derivatization agent for the simultaneous analysis of NE, E and DA, and the method was employed for the evaluation of catecholamine levels in plasma from healthy humans (Mitsui et al., 1985) and urine from patients with Alzheimer's disease (Liu et al., 2011). Several parameters, such as additional buffers, new reagents and even automatization of the derivatization process, were documented (Liu et al., 2011; Kehr, 1994).

BA was used as a derivatization agent for the simultaneous determination of 5-hydroxyindole-3-acetic acid (5HIIA), ST, NE and E in human urine, under mild conditions (Yamaguchi et al., 1998). A postcolumn derivatization method with BA was developed for the monitoring of basal ST release in rat brain microdialysates. This method employed for the first time an ion-pairing reagent in the mobile phase and ensured applications for low volume samples (Yoshitake et al., 2001).

The combination of these two derivatization reagents, BA and DPE, and the chemistry behind it (Yoshitake et al., 2006) allow the simultaneous derivatization of NE, E, DA, ST and some of their metabolites. A HPLC-FLD method was developed to quantify biogenic amines from rat prefrontal cortex microdialysate samples after treatment, in a run time of about 40 min (Fujino et al., 2003; Yoshitake et al., 2004).

Hypothesis

Since biogenic amines are strongly related to the effects of exercise on health status, an accurate evaluation of these effects should be based on the monitoring of biogenic amine levels from biofluids. The aim of this study was the development of a new reversed phase chromatographic method (RP-HPLC) with fluorescence detection for the simultaneous analysis of E, NE, DA and ST in a shorter run time, using recent advances in the chemistry of stationary phases.

Material and methods

Chemicals: deionized and bidistilled water purified with a Millipore Milli-Q50 system, 0.1 N hydrochloric acid (HCl), methanol (MetOH) (Chimopar), acetonitrile (ACN) HPLC gradient grade, acetic acid 100%, benzylamine hydrochloride(BA),3-cyclohexylamino-1-propanesulfonic acid (CAPS), meso- 1,2-diphenylethylenediamine (DPE), Glycine (Gly) (Sigma-Aldrich), potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), sodium acetate trihydrate, sodium heptane-1-sulfonic acid salt (Merck), epinephrine (E), norepinephrine (NE), dopamine DA (Fluka), serotonin ST, (−)-3,4-dihydroxynorephedrine DHN (internal standard) (Sigma-Aldrich).

Reagents: 0.3M BA and 0.3M CAPS pH=10.00 in water:methanol (90:10v/v); 20 mM $K_3[Fe(CN)_6]$ in water:methanol (50:50v/v), 0.05 M DPE in 0.1 M HCl, 0.3 M Glycine in water; Derivatization reagents (DR): DRI was a mixture of 0.3 M BA: 0.03 M CAPS: 0.02 M $K_3[Fe(CN)_6]$: methanol 2:6:3:24 (v/v) and DRII was a mixture of 0.05 M DPE: 0.3 M Gly 2:1 (v/v).

Standard solutions: 1 mg/mL E, NE, DA, ST, DHN in 0.1 M HCl and further diluted with water to desired concentrations before use.

Equipment: HPLC system (Waters 2695 Alliance), fluorescence detector (Waters 2475 Multi λ Fluorescence Detector), chromatographic column: XBridge C_{18} (4.6x150 mm, 3.5 µm), XBridge guard column (4.6x20 mm, 3.5 µm).

Derivatization was performed as described (Fujino et al., 2003). In brief, 40 µl of DRI were added to 100 µl of an aqueous mixture of epinephrine (20 ml), norepinephrine (20 ml), dopamine (20 ml), serotonin (20 ml) and internal standard (20 ml) placed in a 1.5 mL test tube. After reacting at room temperature for exactly 2 min, 40 µl of DRII were added; the test tube was homogenized, tightly sealed and heated at 50ºC for exactly 20 min. The derivatization reaction was quenched by transferring the mixture to a pre-cooled amber vial with 300 µl insert and kept on ice for 20 min. For the blank sample, 220 µl water were subjected to the same procedure; 20 µl of each sample were injected into the chromatograph.

Stability of the fluorescent derivatives was evaluated by analysis of the same sample at t=0 (immediately after derivatization), t=4 h and t=24 h while stored at 5°C in the autosampler.

Chromatographic conditions. Biogenic amine fluorescent derivative separation, on the new chromatographic column XBridge C_{18}, 4.6x150 mm, 3.5 µm, protected by a guard column XBridge 4.6x20 mm, 3.5 µm (Waters), was evaluated in terms of peak resolution, retention time (t_R) and peak area (fluorescence intensity).

The mobile phase for isocratic elution was acetonitrile (mobile phase A): acetate buffer (10 mM acetate buffer, 1 mM heptane-1-sulfonic acid sodium salt, pH=5.30) (mobile phase B) in a ratio of 35:65 (v/v). The flow rate was set to 0.8 mL/min and the column was kept at 30°C. The elution gradient was from 32% (0 min) to 52% A (20 min), using the same mobile phases, flow rate and column

temperature.

The samples were maintained in an autosampler set at 5ºC during analysis, and detection was at λex=345 nm/ λem=480 nm.

Method validation. The linearity, accuracy and precision of the developed method were evaluated according to the accuracy profile method (Hubert et al., 2007; Hubert et al., 2004; Hubert et al., 2008).

Results

Fluorescent derivative stability in time was the first parameter evaluated. We measured the fluorescence intensity (peak area) of the same mixture at the two points in time (t=0 h and t=4 h). After derivatization, the vial with the test mixture was kept in the freezer (-22°C) for 20 min. Data showed that the peak areas of NE, E and DHN slightly increased, ST was constant, while DA decreased (Figure 1).

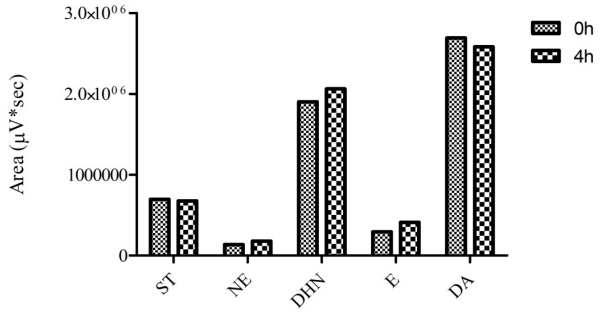

Fig. 1 – Evaluation of biogenic amine fluorescent derivative stability.

Based on the variation observed, we conducted a comparative evaluation of reaction quenching effectiveness: transferring the mixture after derivatization to a vial and placing it on an ice bath or transferring the mixture directly to a pre-cooled (-22°C) vial and keeping it on the ice bath for 20 min. The last reaction quenching method led to 24 h stable derivatives stored at 5ºC.

In isocratic mode, the fluorescent derivatives of all biogenic amines, ST, NE, E, DA, and the internal standard DHN eluted at the following retention times: NE t_R=6.29 min, ST t_R=7.26 min, DHN t_R=8.94 min, E t_R=14.63 min and DA t_R=22.44 min (Figure 2).

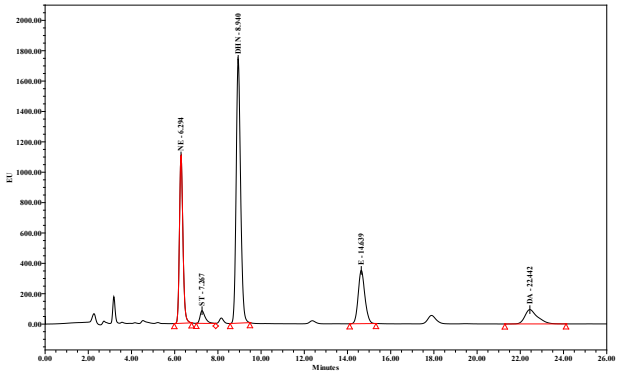

Fig. 2 – Separation of biogenic amine fluorescent derivatives in isocratic elution NE 49.50 ng/mL, ST 56.50 ng/mL, DHN 49.86 ng/mL, E 47.83 ng/mL and DA 50.08 ng/mL.

Isocratic elution resolved separation of all analytes in a 26 min run time with a good resolution, but with low sensitivity and peak symmetry, especially for DA. In order to improve separation, a gradient elution was chosen.

The separation achieved in gradient elution and the corresponding retention times are shown in Figure 3, where: NE t_R=6.70 min, ST t_R=7.35 min, DHN t_R=8.48 min, E t_R=11.77 min and DA t_R=15.26 min. Also, we detected additional peaks on the chromatogram, corresponding to the excess of derivatization reagents.

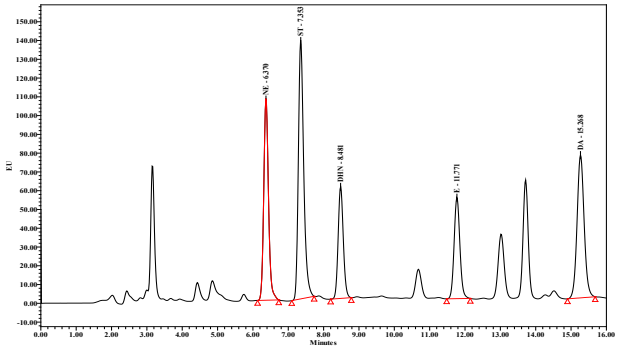

Fig. 3 – Separation of biogenic amine fluorescent derivatives in gradient elution NE 6.75 ng/mL, ST 90.00 ng/mL, DHN 3.12 ng/mL, E 6.82 ng/mL and DA 28.80 ng/mL.

Further, we evaluated the linearity, accuracy and precision of the developed method.

The linear relation between peak areas and concentrations across the range of 2.5 to 15.2 ng/mL for NE, 30 to 210 ng/mL for ST, 6.8 to 15.8 ng/mL for E and 10.8-63.0 ng/mL for DA was estimated (Table I). The correlation coefficient, y-intercept and slope of the regression line for each biogenic amine were: y=9.10e-001x-2.52E-001, r=0.999 for NE, y=9.04E-002x-4.08E-001, r=0.999 for ST, y=5.58E-001x-2.17E001, r=0.999 for E and y=2.08E-001x+1.10E-001, r=0.999 for DA.

Accuracy was evaluated at three levels of concentration for each analyte and the results are presented as recovery in Table I. Repeatability was evaluated for each analyte at one level of concentration based on three runs and the coefficients of variation were: 1.66% for NE, 2.36% for ST, 1.46% for E and 2.25% for DA.

Also, the detection limit and quantification limit for each analyte were calculated based on calibration curve slopes and standard deviation of area at the lowest concentration of calibration curves (low limit of detection (LLOD) and low limit of quantification (LLOQ) (Table I).

Table I

Evaluation of accuracy, limit of detection and limit of quantification.

Compound	Concentration (ng/mL)	Calculated value	Recovery (%)	LLOD (ng/mL)	LLOQ (ng/mL)
NE	2.587	2.666	103.054	0.225	0.560
	4.725	4.620	97.778		
	15.197	15.179	99.882		
ST	31.500	31.107	98.752	2.250	4.500
	90.000	90.577	100.641		
	213.750	213.564	99.913		
E	6.825	6.940	101.685	0.210	0.525
	12.600	12.523	99.389		
	15.750	15.967	101.378		
DA	10.800	10.481	97.046	0.900	2.250
	19.800	19.911	100.561		
	63.000	61.894	98.244		

Discussions

The most common mental health disorder is depression, which has a significant negative effect on the quality of life of more than 350 million people of all ages worldwide, according to a 2012 report of the World Health Organization. By 2030, depression will be the leading cause of disease burden globally (1).

Since the 1960's, depression has been linked to imbalances in the brain with regard to biogenic amines represented by catecholamines - epinephrine, norepinephrine, dopamine and the indoleamine - serotonin, which act as neurotransmitters and hormones both at peripheral and central level. This "monoamine deficiency" theory (Coppen, 1967) is still supported considering that the high-affinity transporters for these amines are primary targets for antidepressant drugs and the increase in extracellular levels of these biogenic amines induces therapeutic benefits (Hensler et al., 2013).

As recently reviewed (Haenisch & Bonisch, 2011), disturbances in the fine balance between biogenic amines can be monitored in biofluids, which can support their role as potential clinical screening tools and biomarkers for diagnosis, prediction or prognosis of depression (Marc et al., 2011; Hensler et al., 2013).

The aim of this study was to develop a new separation and quantification method with fluorescence detection for the simultaneous analysis of epinephrine, norepinephrine, dopamine and serotonin. The main advantages of this method are its shorter run time and the possibility of quantifying low ng/ml concentrations, using recent advances in the chemistry of stationary phases.

Biogenic amines are low-molecular weight molecules with a very polar nature and positive charge under acidic conditions. Although a great diversity of chromatographic columns are available, reversed-phase C18 columns are still the standard choice because of the fast elution of polar compounds and the relatively short run time (Bicker et al., 2013).

A high-resolution separation, in only 16 min run time, and very symmetrical peaks were obtained. Also, the method allows the quantification of biogenic amines with good linearity, accuracy and precision. Furthermore, the range of quantification for all biogenic amines makes this method suitable for simultaneous monitoring of biogenic amines in urine and the shorter run time is an advantage for routine clinical use. In current laboratory analysis, urinary levels of biogenic amines are employed for the diagnosis of pheochromocytoma (2),(3) and physiological concentrations are reported to be found at ng/mL level.

Considering this, the developed method can be successfully applied for the evaluation of the impact of physical exercise on biogenic amine levels in urine and its effects on the mental health status of athletes.

Conclusions

1. In conclusion, we developed a new RP-HPLC-FLD method for simultaneous determination of biogenic amines. The method is simple, rapid, accurate, sensitive and suitable for routine analysis, ensuring a very good separation and quantification of E, NE, DA and ST at ng/mL levels.

2. It will undergo complete validation using urine as a matrix and will be further employed as an excellent clinical tool for biomarker discovery. It will serve as a promising tool that will help clinicians to monitor the effects of exercise on mental health status in athletes and their predisposition to depression or its onset, and to guide the selection of the most effective therapies.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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