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High performance liquid chromatography coupled to an optical fiber detector coated with laccase for screening catecholamines in plasma and urine

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ABSTRACT

An analytical method based on separation by high performance liquid chromatography (HPLC) and detection by optical fiber (OF) coated with an enzyme (laccase), has been developed for separation and quantification of catecholamines, namely epinephrine, dopamine and norepinephrine. The application of OF as a detector in this analytical system relies on the variation of the reflected optical power detected when the catecholamines eluted from the HPLC column act as the substrate of the laccase immobilized on a tip of a single-mode OF. The developed method shows a high linearity in a range between 5 and 125 pg/mL and detection limits of 3.5, 2.9 and 3.3 pg/mL for epinephrine, dopamine and norepinephrine, respectively. The analytical performance of the proposed method was compared with a classical analytical method, namely high performance liquid chromatography-electrochemical detector (HPLC-ED) regarding catecholamines detection, showing great analytical advantages such as low cost of equipment. Additionally, the proposed method was applied to catecholamines determination in actual samples of plasma and human urine.

1. Introduction

Catecholamines of clinical interest such as, epinephrine (adrenaline), dopamine and norepinephrine (noradrenaline) are important neurotransmitters in the sympathetic nervous system [1]. They are monoamines linked to a catechol group (a benzene ring with two hydroxyl groups), sensitive to light, easily oxidized and naturally fluorescent due to their particular chemical structure [1].

Because of their transmitter function in the brain, the assessment of catecholamines concentrations in body fluids may serve as biochemical indicators for several neurological disorders and thus support their pharmacological treatment [2–4]. In addition, the determination of catecholamines and their metabolites levels in plasma and urine is especially relevant for the clinical diagnosis, particularly in tumors cases [5]. The catecholamines levels in these patients can exceed, by several orders of magnitude, the normal values, which should vary between 0.02–0.46 nmol/L for epinephrine, 0.01–0.48 nmol/L for dopamine and 0.45–2.49 nmol/L for norepinephrine in plasma [1]. Therefore, an early diagnosis is absolutely necessary to avoid the complications associated with the excessive release of catecholamines into the circulation system.

High performance liquid chromatography (HPLC) coupled to electrochemical detection (ED) has been widely used as a method for catecholamine speciation in biological and synthetic matrices [2,6–17]. Liquid chromatography coupled to mass spectrometry or tandem mass spectrometry detectors [18–25] also constitutes an excellent analytical methodology for low concentrations of catecholamines and metanephrines. In the last few years some innovative methods have been reported [26–29], with high analytical performance for catecholamines determination based on fluorescence and chemiluminescence detection. Although sensitive, these methods are expensive and usually they do not allow remote data acquisition.

Optical fiber (OF) analyzers could provide an excellent alternative to laboratory-scale devices due to its fast response time, high sensitivity, immunity to electrical and magnetic interferences, low cost, small size and compact design [30]. The conjugation of this technology with a sensing biocomponent, such as enzymatic matrices could provide the basis for accurate and sensitive measurement of different analytes for clinical applications.

This work reports for the first time an analytical methodology which combines the HPLC separation technique with an OF detection system using laccase as sensing biocomponent (HPLC–LacOF). To meet this goal a LacOF detector was developed and coupled to a HPLC system, for quantification of catecholamines (epinephrine, dopamine and norepinephrine) in biological fluids (i.e. plasma and urine).

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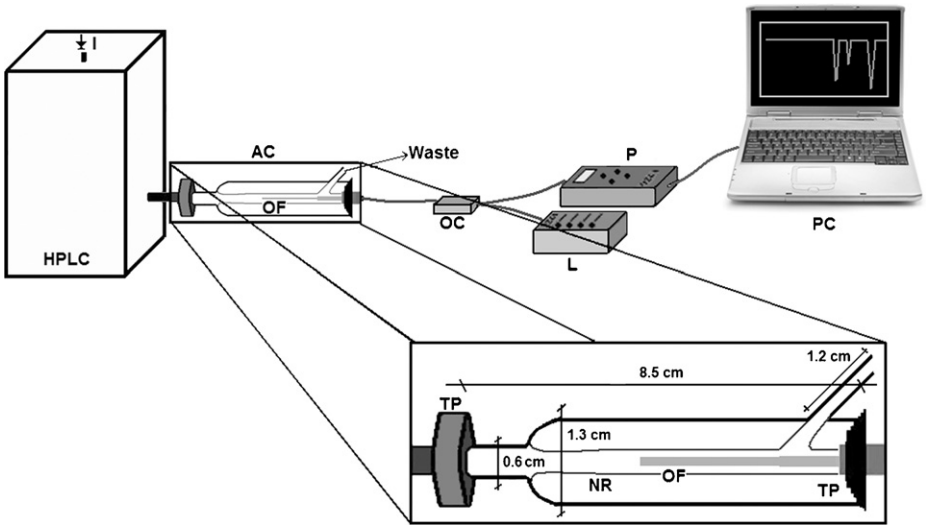

Fig. 1. Experimental apparatus for the developed analytical method based on the HPLC–LacOF; AC: analytical cell; I: injector; L: laser; NR: narrowed region; OC: optical coupler; OF: optical fiber; PC: computer; P: photodetector; TP: Teflon plug.

2. Experimental

2.1. Analytical details and experimental apparatus for catecholamines detection by HPLC–LacOF

Fig. 1 shows the experimental apparatus used for the proposed analytical method, highlighting a detailed view of the analytical cell (AC), which design includes an internal narrowed region of 0.4 cm of diameter and 6.5 cm long. The developed analytical apparatus is constituted by two main components, the HPLC separation system and the OF laccase coated (LacOF) detector.

The LacOF detection component is constituted by an optical source (laser diode (Oz Optics, Ottawa, Canada) with the working wavelength set at 1550 nm) to generate the interrogating signal and a photodetector (Oz Optics, Ottawa, Canada) to measure the intensity of the modulated signal. The optical component of the detector is constituted by a monomode optical fiber pigtail, core and cladding diameters of 9 and 125 μm, respectively, integrated into a directional 50:50 Y optical coupler (OC). A 15 mm of the optical path cord was uncladded and cleaved with a Cleaver V6 (Future Instrument, Barcelona, Spain) precision fiber cleaver. An alginate/laccase matrix was deposited in this fiber section resulting in the sensitive component of the developed detector. The enzymatic cladding was deposited by dip-coating technique, dipping the OF section into the alginate/enzyme suspension, and then into a 0.2 M calcium chloride ($CaCl_2$) solution. The suspension of alginate/enzyme was prepared by mixing 400 U/mL of *Trametes versicolor* laccase (E.C.1.10.3.2; Fluka, Steinheim, Germany), a white-rot fungus enzyme (22.4 U/mg), with a 3% (w/v) alginic acid solution salt from brown algae and kept stored at 4 °C. Finally the sensitized OF section was introduced into an analytical cell connected to the HPLC column PLRP-S 100 Å 5 μm, 150 mm × 4.6 mm ID reversed-phase (Varian, Middelburg, The Netherlands). The mobile phase consisted of the following: 5% acetronitrile, 0.025 M sodium phosphate, 0.025 M citric acid, 0.001 M heptane and sulphonic acid pH 2.85, used at a constant flow rate of 0.75 mL/min.

2.2. The HPLC-ED methodology

The HPLC-ED methodology was implemented in an Aliance 2695 HPLC connected to a Waters 2465 electrochemical detector (Waters, Milford, MA, USA) with a glassy carbon working electrode, set at 750 mV to a salt-bridge Ag/AgCl reference electrode. A 35 μL volume was injected into a Rheodyne loop injector from an Aliance 2695 HPLC system (Waters, Milford, MA, USA). The characteristics of the column and mobile phase used in this method were the same as those implemented in the HPLC–LacOF system.

2.3. Preparation of standards mixtures of catecholamines

Catecholamines solutions of 5, 35, 65, 95 and 125 pg/mL were prepared in phosphate–citrate buffer pH 5.5 for calibration studies, performing five replicates of each concentration tested. All reagents used were analytical grade from Sigma–Aldrich (Steinheim, Germany) and used without further purification.

2.4. Preparation and analysis of human plasma and urine samples

Blood and urine samples were obtained from healthy male and female volunteers in a certificated laboratory of clinical analysis and were stored and kept frozen at −18 °C until analysis. Before extraction and separation by solid phase extraction (SPE) and HPLC, the blood samples were thawed at room temperature and then centrifuged at 1400 × *g* for 15 min. The plasma (supernatant) pretreatment procedure, based on Mercolini et al. [27], was carried out by SPE using Oasis MAX cartridges (30 mg, 1 mL) (Waters, Milford, MA, USA). Cartridges were activated and conditioned with 2× 1 mL of methanol and 2× 1 mL of pH 9.0, 200 mM carbonate buffer, respectively. The cartridges were then washed with 2× 1 mL of pH 9.0, 200 mM carbonate buffer and dried applying vacuum. Analyte elution was carried out with 1 mL of 5% acetic acid in methanol, applying vacuum. The eluate was brought to dryness with a rotary evaporator and the residue was re-dissolved in 150 μL of mobile phase and injected in the Aliance 2695 HPLC system.

The preparation of the urine samples was performed according to Janichy-Deverts et al. [11] procedure. Aliquots of 20 mL from urine samples were stored and kept frozen at −18 °C until analysis. Prior to extraction and separation by SPE and HPLC the urine samples were thawed at room temperature and centrifuged at 10,000 × *g* for 3 min; aliquots of 50 μL of urine samples were added to 50 mL of milli-Q water. In preparation for SPE, 3 mL aliquots of the diluted urine were mixed with 5 mL of dilution reagent (30 mM ammonium acetate, 2.7 mM EDTA; pH 7.5) and 100 μL of 0.5 M NaOH. The pH of each mixture was adjusted to 6.5 ± 0.5 by adding 0.5 M NaOH. The solutions were adsorbed onto a SPE column filled with Bio-Rex 70 cation exchange resin (50–100 mesh) and allowed

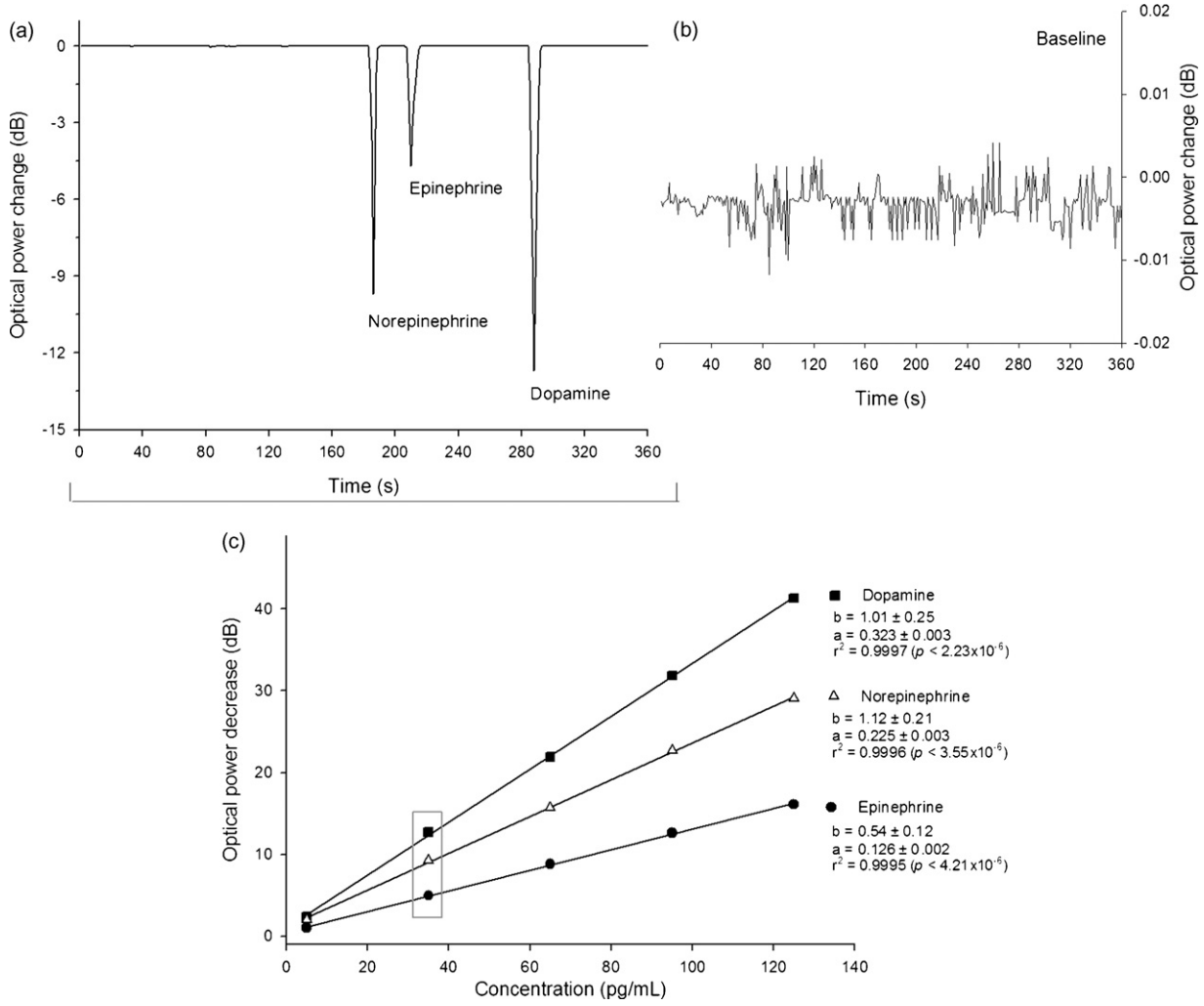

Fig. 2. Analytical response obtained with the developed LacOF detector; (a) analytical signals obtained for a standard mixture solution of 35 pg/mL of epinephrine, dopamine and norepinephrine; (b) LacOF detector baseline; (c) calibration of the LacOF detector with amounts of catecholamines in a range between 5 and 125 pg/mL.

to drain. The columns were washed with dilution reagent and milli-Q water and eluted with 6 mL of 3.6 mM ammonium pentaborate. The eluate was collected and injected into the Aliance 2695 HPLC system.

Quantification of plasma and urine samples was attained by linear regression of the calibration data. Five repeated measurements were performed for each plasma and urine sample analyzed. The samples were numbered from 1 to 5 and the same number in plasma and urine analysis means that the sample has the same subject source.

3. Results and discussion

3.1. Detection principle and analytical performance of the HPLC–LacOF method for catecholamines determination

The performance of the enzyme based-optical fiber detector was evaluated for different amounts of three catecholamines and compared to the HPLC-ED method. Fig. 2a shows the decrease in optical power obtained with HPLC–LacOF for 35 pg/mL of epinephrine, dopamine and norepinephrine.

The sensing principle underlying the developed analytical device is based on changes in the refractive index of the OF sensitive cladding (laccase + alginate matrix), caused by the linkage of the catecholamines to the laccase enzyme, which oxidizes these subtracts generating the correspondent quinone. The changes in the refractive index of the OF sensitive enzymatic cladding leads to changes in the reflected optical power, measured as the analytical signal. The linkage of the catecholamine to the enzyme is the most relevant factor for the analytical signal generation, since it is independent of the produced quinone and returns to its initial value after the catecholamine oxidation. Therefore, the highest analytical signal and sensitivity obtained for dopamine when compared to the other catecholamines under study could be attributed to the high affinity of the enzyme to this catecholamine, with consequent increase of the optical power change amplitude.

The chromatogram displayed in Fig. 2a highlight that all the three catecholamines tested were well separated in retention times of 186, 210 and 288 s for norepinephrine, epinephrine and dopamine, respectively, of a total analytical time of around 7 min. Moreover, the high intensity of the analytical signal in terms of height demonstrates the suitability of the developed detection device for quantification of catecholamines. Observing Fig. 2a it can be also noted the potential of this analytical system for other analytes (i.e. normetanephrine and metanephrine) measurement, by its inclusion in the analytical window showed in this figure.

The graph in Fig. 2b depicts the optical signal baseline recorded before the injection of the catecholamines standard mixtures. The optical signal varies from -1.2×10^{-2} to 4.1×10^{-3} dB, which represents an amplitude of 1.6×10^{-2} dB in terms of optical power variation. When comparing this value with the average value in dB of the optical power decrease obtained for the lower concentration

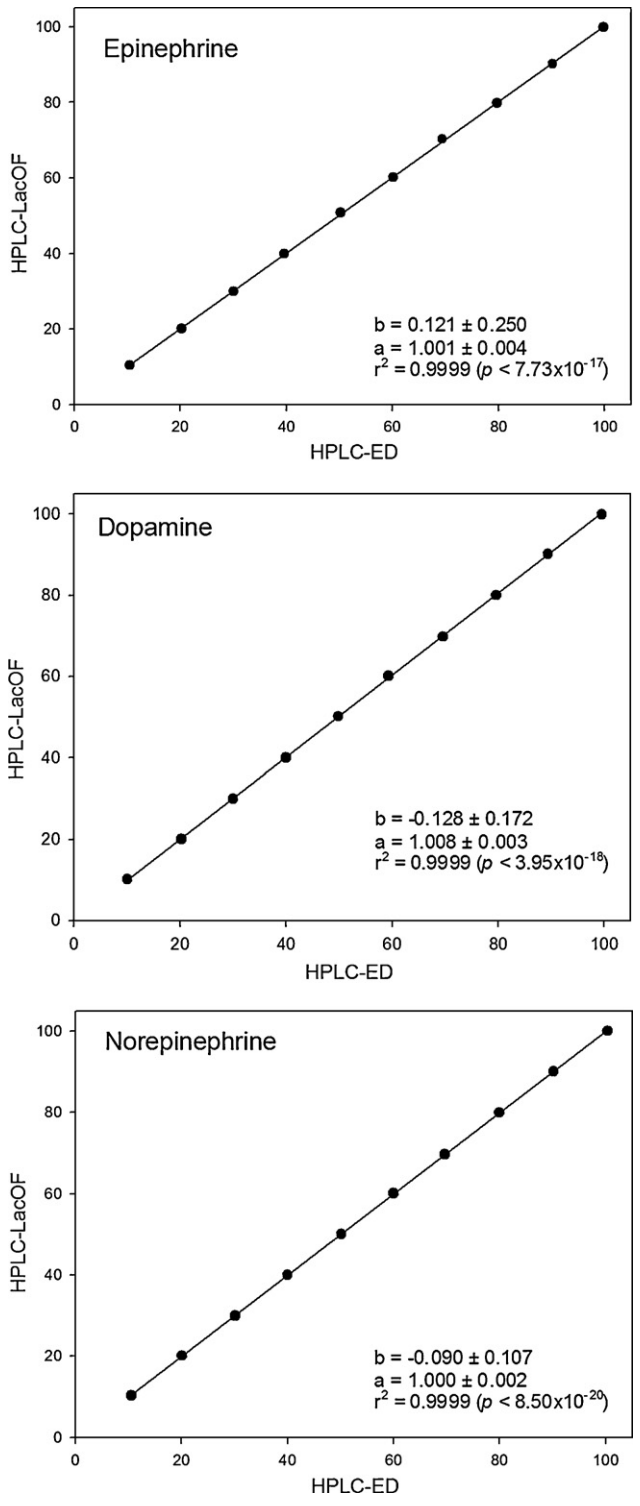

Fig. 3. Comparison of the results obtained with the HPLC–LacOF and HPLC-ED methods for catecholamines.

of catecholamines tested (1.45 dB for 5 pg/mL of epinephrine), it can be conclude that the baseline noise of the LacOF detector was around 1.5% of the analytical signal.

Fig. 2c shows the calibration curves obtained with HPLC–LacOF for the three tested catecholamines. The analytical sensitivity, measured as the slope of the calibration curve, increases according to the following order: epinephrine < norepinephrine < dopamine.

The detection limits based on three times the residual standard deviation [31], obtained for the three catecholamines analyzed by the HPLC–LacOF and HPLC-ED method were found to be 3.5 and 4.5 pg/mL for epinephrine, 2.9 and 5.1 pg/mL for dopamine and 3.3 and 4.5 pg/mL for norepinephrine, respectively. The detection limits values obtained for both evaluated methods are very similar, thus the two methods cannot be differentiated in terms of this figure of merit.

The test for stability of the developed detector, performed by injecting a standard mixture of 35 pg/mL of the three catecholamines under study during two months of continuous operation, showed no variations in the optical signal and in the analytical performance. Additional experiments regarding the sensor behavior for longer periods of operation revealed a slight decrease of the analytical signal of 2 dB at the end of six months of continuous utilization. However, the sensitive region of the newly detector, that is, the OF coated with the enzymatic matrix could be easily replaced, maintaining in this way the high analytical performance and signal stability of the LacOF detector. Regarding the target compounds in acidified urine samples, stored at −18 °C, they showed no degradation up to two months of storage time, and relative standard deviation calculated from these analyses was always less than 5%, demonstrating the robustness of the analytical procedure.

3.2. Comparison with HPLC-ED method

In order to test and compare the performance of the proposed method (HPLC–LacOF) with HPLC-ED, ten different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 pg/mL) of a standard mixture of catecholamines were determined with both methods performing five repeated evaluations for each concentration tested. The comparison of the results obtained with the HPLC–LacOF and HPLC-ED methods for catecholamines are displayed in Fig. 3.

Assuming the null hypothesis (same sensitivity, i.e., slope equals to 1 and no systematic errors, i.e., intercept equals to 0) for the results obtained with both methods the slope (a), the intercept (b), r^2 and p of the regression line, of each of the data sets were calculated and included in Fig. 3.

For the three catecholamines analyzed the regression line has an intercept not significantly different of zero and a slope and a correlation coefficient not significantly different of 1, allowing to conclude that the results obtained with the two analytical methods cannot be statistically differentiated. This fact was also corroborated by the results of an ANOVA (using SigmaStat 3.0 [32]) applied to all data obtained on catecholamines analysis by HPLC–LacOF and HPLC-ED method. The ANOVA of the results shows that there is no statistically significant difference ($p = 0.643$, 0.699 and 0.735 for epinephrine, dopamine and norepinephrine, respectively) for the effects of differences in the two methods. However, as expected there is a significant statistic difference ($p < 0.001$) between the different levels of the concentrations. The analytical error, measured as the residual standard deviation of both methods, varied between 1.7 and 2.8 pg/mL.

3.3. Analysis of catecholamines in plasma and urine samples: application and comparison of the HPLC–LacOF and HPLC-ED methodologies

Fig. 4 depicts the analytical response obtained with the HPLC-LacOF method for catecholamines in human plasma and urine samples. Tables 1 and 2 show the results obtained by the proposed HPLC–LacOF method and the HPLC-ED method when applied to five actual samples of human plasma and urine, respectively.

According to Peaston and Weinkove [1] study, the concentration obtained for epinephrine, dopamine and norepinephrine in plasma are below the normal values (0.02–0.46 nmol/L for epinephrine, 0.01–0.48 nmol/L for dopamine and 0.45–2.49 nmol/L for norepinephrine) of these catecholamines in this biological

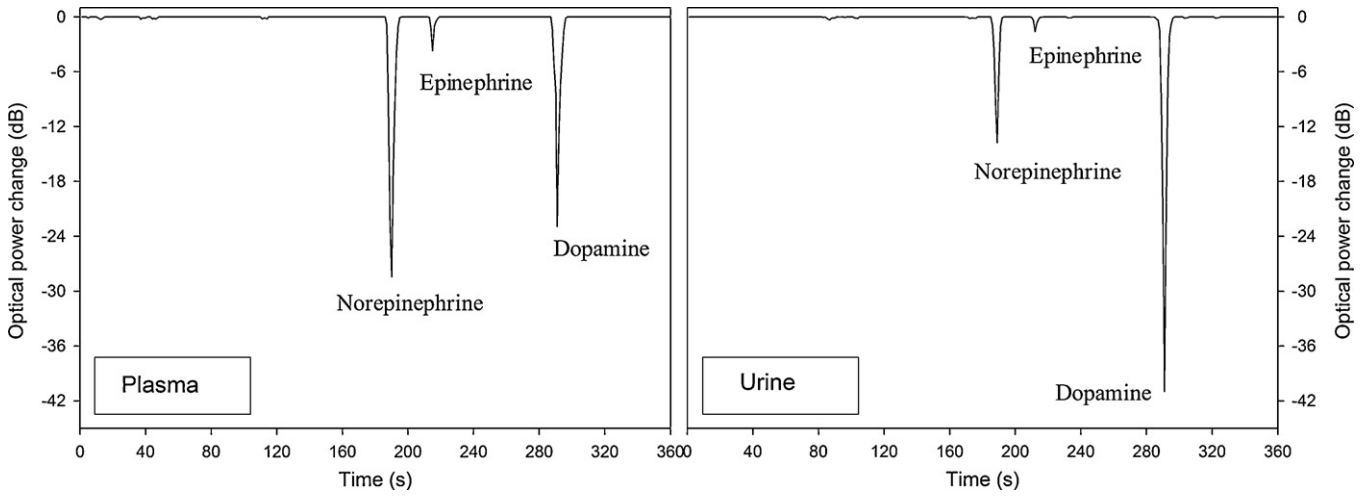

Fig. 4. Chromatograms obtained during the analysis of catecholamines by HPLC–LacOF in plasma and urine samples.

Table 1
Results obtained for five actual samples of plasma by both HPLC–LacOF and HPLC-ED method.

Samples	Epinephrine		Dopamine		Norepinephrine	
	HPLC–LacOF (pg/mL)	HPLC-ED (pg/mL)	HPLC–LacOF (pg/mL)	HPLC-ED (pg/mL)	HPLC–LacOF (pg/mL)	HPLC-ED (pg/mL)
1	25.3 ± 0.2	23.9 ± 0.8	66 ± 2	70 ± 6	124 ± 2	120 ± 2
2	33.3 ± 0.4	30.7 ± 0.7	15.5 ± 0.5	23 ± 2	99 ± 1	91 ± 2
3	18.0 ± 0.4	17.6 ± 0.4	64 ± 2	61 ± 5	113 ± 1	104 ± 2
4	29.6 ± 0.9	33 ± 2	24.6 ± 0.6	26 ± 2	91 ± 2	82 ± 3
5	41.5 ± 0.4	47 ± 2	70 ± 3	74 ± 7	78 ± 1	72 ± 2

Table 2
Results obtained for five actual samples of human urine by both HPLC–LacOF and HPLC-ED method.

Samples	Epinephrine		Dopamine		Norepinephrine	
	HPLC–LacOF (ng/mL)	HPLC-ED (ng/mL)	HPLC–LacOF (ng/mL)	HPLC-ED (ng/mL)	HPLC–LacOF (ng/mL)	HPLC-ED (ng/mL)
1	8.7 ± 0.2	7.6 ± 0.8	120 ± 3	111 ± 2	56.6 ± 0.7	58 ± 2
2	10.0 ± 0.4	9.7 ± 0.7	70 ± 2	71 ± 7	31 ± 1	31 ± 2
3	10.6 ± 0.4	10.5 ± 0.6	121 ± 3	127 ± 11	46 ± 1	44 ± 2
4	6.3 ± 0.9	6.1 ± 0.4	81 ± 2	79 ± 7	23.9 ± 0.7	24 ± 2
5	18.3 ± 0.4	16 ± 2	117 ± 3	109 ± 9	25 ± 1	25 ± 2

matrix. Additionally, no significant difference ($p = 0.875$, 0.868 and 0.559, epinephrine, dopamine and norepinephrine, respectively) was observed between the results obtained by the utilized analytical methodologies for the analysis of catecholamines in plasma.

The obtained results for the three catecholamines analyzed do not exceed significantly the normal values for these compounds in urine of <20 μg/24 h for epinephrine, <400 μg/24 h for dopamine and <80 μg/24 h for norepinephrine, according to Moyer et al. [33] and Peaston and Weinkove [1] studies. Furthermore, no significant difference ($p = 0.746$, 0.885 and 0.992, epinephrine, dopamine and norepinephrine, respectively) was observed between the results obtained with the two analytical methods for each catecholamine.

4. Conclusions

The developed analytical system based on LacOF showed an adequate linearity between 5 and 125 pg/mL, allowing the detection of catecholamines at trace levels. The analytical performance achieved for the HPLC–LacOF method was found comparable to the HPLC-ED reference method, with an analytical error less than 2.8 pg/mL. The HPLC–LacOF method showed high suitability for analysis of catecholamines in actual samples of plasma and human urine, with notable analytical advantages regarding catecholamines analysis, due to the lower cost of equipment and detection components as well as higher potential for miniaturization of the versatile design and low-scale detector, in comparison to the classical methodology based on HPLC-ED.

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