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Optical fiber biosensor coupled to chromatographic separation for screening of dopamine, norepinephrine and epinephrine in human urine and plasma

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ABSTRACT

An optical fiber biosensor has been developed for the determination of catecholamines (dopamine, norepinephrine and epinephrine) based on the recognition capacity of the enzyme laccase. In this study, a glass tube constituted by a fused silica fiber coated with a film of polystyrene/divinylbenzene resin (PS/DVB) was used for catecholamines separation. Firstly, the analyzer was tested for calibration and its analytical performance for catecholamines detection was compared with a classical analytical method, namely high performance liquid chromatography-electrochemical detector (HPLC-ED). The developed analytical device shows a high potential for catecholamines quantification with a detection limit of 2.1, 2.6 and 3.4 pg mL^{-1} for dopamine, norepinephrine and epinephrine, respectively. The analytical sensitivity, inferred from the slope of the calibration curves established for a range of concentrations between 5 and 125 pg mL^{-1}, was found to be 0.344, 0.252 and 0.140 dB/pg mL^{-1} for dopamine, norepinephrine and epinephrine, respectively. Furthermore, catecholamines speciation with the PS/DVB fiber was completely achieved in 3 min. The analytical performance of the reported sensor was also evaluated and found adequate for catecholamines determination in human urine and plasma samples.

1. Introduction

Biosensors constitute an expanding field of analytical devices for the detection of analytes and other parameters of biological interest, which usually combines a biological component with a physic-chemical transducer.

Optical fiber (OF) biosensors are analytical devices in which the OF serves as a transduction element, showing several analytical advantages due to their high analytical sensitivity, fast response, large geometrical versatility, possibility of continuous monitoring of many chemical parameters, low cost, lightweight associated to small size, and high degree of miniaturization. These advantages make them an excellent alternative to laboratory-scale analytical methods and highly competitive in non-invasive clinical applications.

The use of the enzyme laccase as recognition element towards the development of biosensors for catecholamines determination has received a great deal of attention from several research groups [1–7]. Although some analytical devices have been reported for catecholamines determination which combines the enzymatic recognition with the OF technology, such as for example, an OF biosensor for epinephrine determination based on immobilized laccase catalysis and fluorescence quenching [7], they have not shown yet an adequate analytical selectivity and sensitivity.

Catecholamines, namely dopamine, norepinephrine and epinephrine, play an important role in the central nervous system as neurotransmitters or hormones, and they also affect the regulation of blood pressure, heart rate and lipolysis [4,8]. The determination of catecholamines in different human fluids, such as plasma and urine, is of great interest in medical diagnostics, especially for patients suffering from Parkinson's disease or Phaeochromocytoma and stress. An early and accurate determination of the catecholamines concentration in urine and plasma could prevent unnecessary drug treatment.

This work aimed at the development of an optical fiber biosensor for screening of catecholamines (dopamine, norepinephrine and epinephrine) based on a sensitive cladding of laccase. The OF biosensor, named LacOF biosensor, is composed of a miniaturized column for catecholamines separation, a biocomponent enzymatic cladding of laccase, and an OF component with associated electronics. The results obtained by applying the LacOF biosensor to the analysis of human urine and plasma were compared to those

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obtained by a methodology based on high performance liquid chromatography coupled to an electrochemical detector (HPLC-ED).

2. Experimental

2.1. Development of the LacOF biosensor

2.1.1. Preparation of the optical fiber (OF) and deposition of the enzymatic cladding

A directional 50:50 Y optical coupler was prepared from a single-mode optical fiber (OF) with core and cladding diameters of 9 and 125 μm, respectively. The OF was mechanically uncladded, immersed in dichloromethane in order to remove the protective cladding, and cleaved in a length of 15 mm with a Cleaver V6 (Future Instrument, Spain) precision fiber cleaver. The sensitive coating (enzymatic cladding) was deposited onto the cleaved ended surface of the fiber following the dip-coating technique. The uncladded OF section was dipped into an alginate/enzyme suspension, followed by the immersion into a 0.2 M calcium chloride ($CaCl_2$) solution. The suspension of alginate and laccase of *Trametes versicolor* a white-rot fungus (E.C.1.10.3.2) (22.4 U mg^{-1}), was prepared mixing 400 U mL^{-1} of laccase, with a 3% (w/v) alginic acid solution salt from brown algae and kept stored at 4 °C.

2.1.2. Assemblage of the optical fiber biosensor and the underlying analytical principles

Fig. 1 shows the schematics of the whole LacOF biosensor detection system, including enzymatic cladding at the OF surface which provides the analytical principle underlying this biosensor.

The developed biosensor is constituted by two main components. The first analytical component aiming at the separation of the three catecholamines, includes a pump, a mobile phase, an injection cell (IC), and a glass tube (GT) which contains a fused silica fiber coated with a polystyrene/divinylbenzene resin (50–100 mesh particles size). The second component aiming at the detection of the catecholamines is constituted by an analytical tube (AT) containing the sensitized OF section, an optical coupler (OC), a photodetector (OZ Optics, Spain) and a laser diode (OZ Optics, working in the infrared region (λ = 1550 nm)). The connections between the following system elements: IC-GT; GT-AT and AT-OF, were made of Teflon. The various components of the sensor, excluding the mobile phase, the pump and the laptop were appropriately located inside a home-made aluminum box (height: 14.0 cm, width: 17.7 cm and length: 25.1 cm), providing the analytical system with a very compact design.

The catecholamines standards and samples are injected with a micro-syringe (Hamilton) at the top of a glass cell (injection cell) which are then carried by a constant flow (0.75 mL min^{-1}) of mobile phase to the glass tube and finally reach the analytical tube. The mobile phase consisted of 5% acetronitrile, 0.025 M sodium phosphate, 0.025 M citric acid, 0.001 M heptane and sulphonic acid pH 2.85, being its flow rate controlled by a mass flowmeter (Supelco, Spain).

Fig. 1b shows a schematic design of the OF surface coated with an enzymatic cladding and the oxidation of the catecholamine (epinephrine) by the enzyme (laccase) to the respective aminoquinone (adrenoquinone). The linkage of catecholamines to the enzyme changes the refractive index of the sensitive cladding (laccase incorporated in an alginate matrix) causing changes of the reflected optical power, which are independent of the produced quinone. The optical power changes are measured as the analytical signal by the photodetector and data acquisition and processing performed by a computer (PC) with home-made software.

2.2. Application of a HPLC-ED methodology

The HPLC-ED methodology was carried out in an Aliance 2695 HPLC system (Waters, USA) with a PLRP-S 100 Å 5 μm, 150 mm × 4.6 mm I.D. reversed-phase analytical column (from Polymer Laboratories Inc., USA). The detection of catecholamines was attained by a Waters 2465 electrochemical detector with a glassy working electrode set at 750 mV to a salt-bridge Ag/AgCl reference electrode. The mobile phase at a constant flow rate of 0.75 mL min^{-1} consisted of 5% acetonitrile, 0.025 M sodium phosphate, 0.025 M citric acid, 0.001 M heptane and sulphonic acid pH 2.85, as in the case of the OF system.

2.3. Preparation of standards mixtures of catecholamines

The standard solutions of mixtures of the three catecholamines were prepared in phosphate-citrate buffer pH 5.5 and used for calibration studies, performing five replicates of the various standard concentrations (5, 35, 65, 95 and 125 pg mL^{-1}) of each catecholamine. All reagents used were analytical grade from Sigma–Aldrich and used without further purification.

2.4. Preparation and analysis of human urine and plasma samples

Urine and blood samples were obtained from healthy volunteers in a certificated laboratory of clinical analysis. Blood samples were stored in glass tubes containing EDTA as the anticoagulant, and then

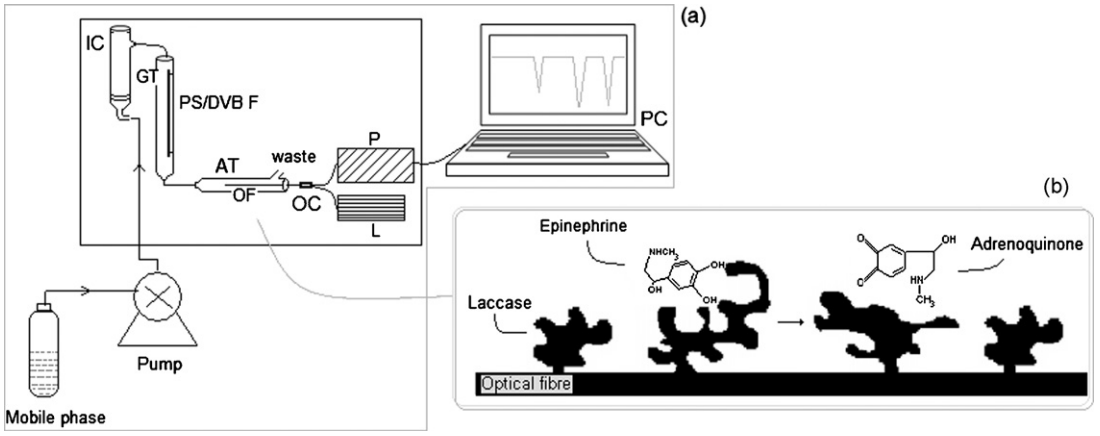

Fig. 1. (a) Schematic configuration of the experimental layout (AT – analytical tube; GT – glass tube; IC – injection cell; L – laser; OC – optical coupler; OF – optical fiber; P – photodetector; PC – laptop for data acquisition and processing; PS/DVB F – styrene/divinylbenzene fiber) and (b) schematics of the enzymatic reaction occurring at the OF surface coated with laccase.

centrifuged at 1400 × *g* for 15 min; the supernatant, i.e. plasma, was then transferred to polypropylene test tubes. Urine and plasma samples were stored at −18 °C until further processing for each of the analytical methodologies implemented in this work (LacOF biosensor and HPLC-ED). The preparation of the samples of urine (2.4.1) and plasma (2.4.2) was carried out by solid phase extraction (SPE) and performed according to Janichy-Deverts et al. [9] and Mercolini et al. [10] procedures, respectively. After the extraction procedure, the obtained solutions were analyzed by LacOF biosensor and HPLC-ED; quantification of the catecholamines in urine and plasma samples was attained by linear regression of the calibration data, performing five repeated measurements for each sample analyzed, exactly in the same way as in the calibration studies. The samples were numbered from 1 to 5 and the same number in urine and plasma analysis means that the sample has the same subject as source.

2.4.1. Preparation of urine samples

Prior to extraction by SPE the thawed samples were centrifuged at 10,000 × *g* for 3 min and diluted with ultra-pure water (SG Water, Germany). Aliquots of 3 mL of the diluted sample 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. Each prepared sample was adjusted to a pH value of approximately 6.5 with 0.5 M of NaOH. The extraction of catecholamines of urine samples was performed by SPE column filled with Bio-Rex 70 cation exchange resin (50–100 mesh) previously washed with dilution reagent and ultra-pure water. Elution of the analytes was achieved by 6 mL of 3.6 mM ammonium pentaborate following a procedure developed by Janichy-Deverts et al. [9].

2.4.2. Preparation of plasma samples

The plasma samples were extracted by SPE on Waters (Milford, Mass., USA) Oasis MAX cartridges (30 mg, 1 mL). The SPE cartridges were preconditioned and activated with 2× 1 mL of carbonate buffer (200 mM Na_2CO_3, pH 9.0) and 2× 1 mL of methanol prior to loading with plasma sample. After that, the cartridges were then washed with 2× 1 mL of carbonate buffer and vacuum dried. Elution of the analytes was achieved with 1 mL of 5% acetic acid in methanol. The eluate was dried using a rotary evaporator and the residue was re-dissolved in 150 μL of mobile phase for analysis.

3. Results and discussion

Fig. 2 shows the optical power changes obtained during the process of preparation of the LacOF biosensor for determination of different amounts of catecholamines. Fig. 2a shows the sensor response for 35 and 95 pg mL^{-1} of dopamine, norepinephrine and epinephrine and Fig. 2b reports the calibration curves obtained for the three catecholamines tested, with estimates of slope and intercept with their respective standard deviation.

The maximum amplitude of the optical power variation recorded before the injection of dopamine, norepinephrine and epinephrine (baseline) was approximately 0.01 dB, which was about 1% of the minimum analytical signal obtained during the lower concentration of catecholamines tested (0.9 dB for 5 pg mL^{-1} of epinephrine).

The total analytical time was found to be around 3 min for the three completely separated catecholamines detected as shown in Fig. 3a.

This analytical system shows a high statistical degree of linearity for the calibration model used, as it can be inferred from the r^2 and respective *p* values also shown in Fig. 2b. The intensity and therefore the sensitivity (measured as the slope of the calibration curve) of the analytical signal obtained for dopamine was higher than the ones obtained for norepinephrine and even for epinephrine.

The results obtained during the calibration process also revealed that the detection capacity of the developed sensor system reaches

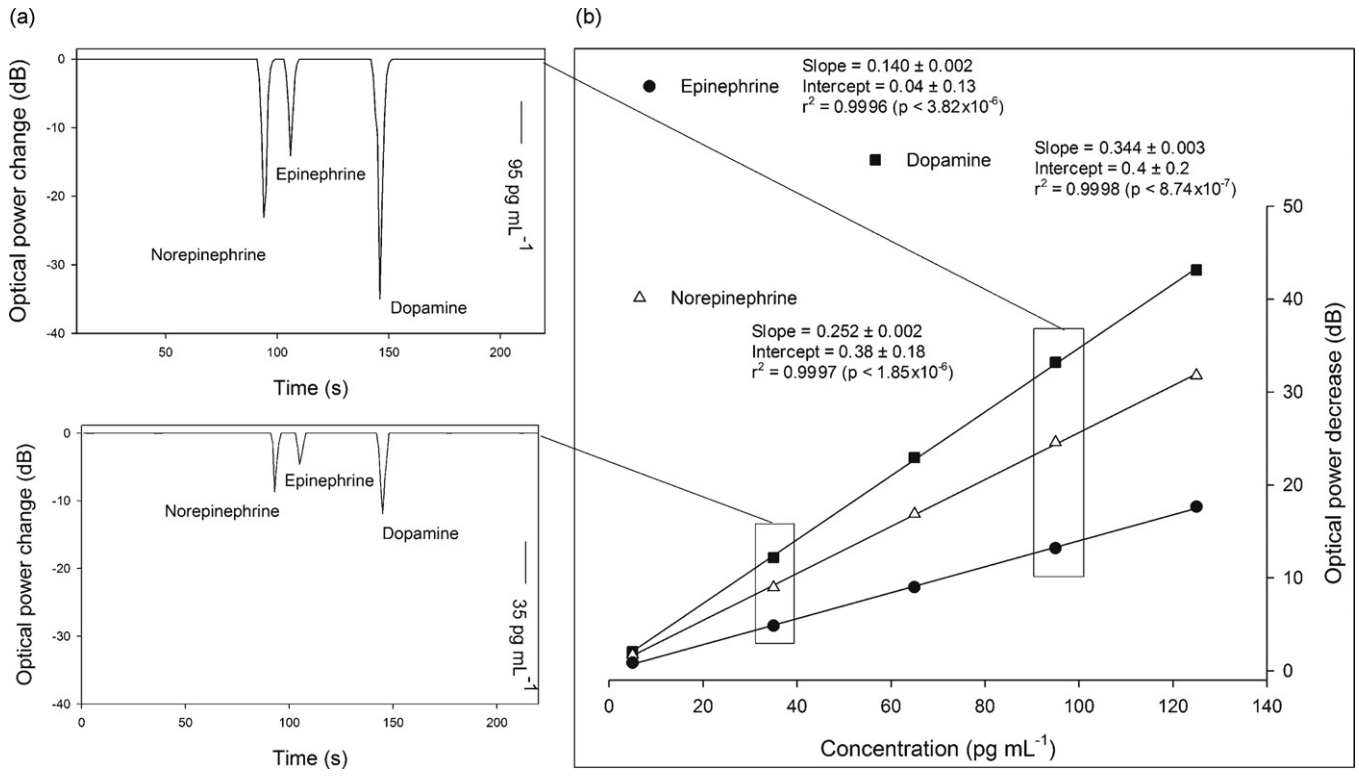

Fig. 2. LacOF biosensor response for different amounts of catecholamines, namely, dopamine, norepinephrine, and epinephrine: (a) example of the biosensor profile obtained for 35 and 95 pg mL^{-1} of catecholamines; (b) calibration of the LacOF biosensor with amounts of catecholamine in a range between 5 and 125 pg mL^{-1}.

Table 1
Results obtained for catecholamines analysis by the LacOF biosensor and the HPLC-ED method.

Standards ($pg\,mL^{-1}$)	Dopamine		Norepinephrine		Epinephrine	
	LacOF biosensor ($pg\,mL^{-1}$)	HPLC-ED ($pg\,mL^{-1}$)	LacOF biosensor ($pg\,mL^{-1}$)	HPLC-ED ($pg\,mL^{-1}$)	LacOF biosensor ($pg\,mL^{-1}$)	HPLC-ED ($pg\,mL^{-1}$)
10	10.1 ± 0.4	10.4 ± 0.6	10.2 ± 0.3	10.6 ± 0.7	10.4 ± 0.2	10.4 ± 0.8
20	20.3 ± 0.3	20.2 ± 1.2	20.1 ± 0.4	20.1 ± 0.5	20.2 ± 0.6	20.2 ± 0.8
30	30.1 ± 0.3	30.0 ± 1.0	30.0 ± 0.3	30.1 ± 0.7	30.1 ± 0.6	30.1 ± 1.8
40	40.1 ± 0.4	39.9 ± 2.4	40.0 ± 0.3	40.0 ± 1.8	40.0 ± 0.5	39.6 ± 3.1
50	50.0 ± 0.6	49.8 ± 3.0	50.1 ± 0.2	50.1 ± 1.8	50.2 ± 0.4	50.1 ± 3.7
60	59.8 ± 0.5	59.3 ± 2.8	60.0 ± 0.3	60.0 ± 1.8	60.2 ± 0.5	60.1 ± 3.1
70	70.1 ± 0.7	69.6 ± 3.3	69.8 ± 0.3	69.7 ± 2.5	69.6 ± 0.6	69.4 ± 3.1
80	79.9 ± 0.2	79.6 ± 4.1	79.9 ± 0.3	79.9 ± 2.5	79.8 ± 0.7	79.7 ± 3.7
90	90.1 ± 0.2	89.4 ± 5.8	90.1 ± 0.3	90.1 ± 2.8	90.2 ± 0.5	90.1 ± 3.5
100	99.9 ± 0.4	99.5 ± 5.8	100 ± 0.6	100.3 ± 6.9	99.9 ± 1.1	99.7 ± 7.5

a plateau in terms of maximum capacity of detection for concentrations of catecholamines higher than about 130 $pg\,mL^{-1}$, with a decrease of the analytical performance of the LacOF biosensor for concentrations above that value.

Since the linkage of the catecholamine to the enzyme is the most relevant factor for the analytical signal generation, the differences in the analytical sensitivities obtained with the developed LacOF sensor are mainly due to the differences in the affinity of laccase to each of the catecholamines under study. Therefore, the highest analytical sensitivity obtained for dopamine could be attributed to the high affinity of the enzyme to this catecholamine, with consequent increase of the optical power change amplitude.

The detection limits obtained for the three catecholamines analyzed, based on three times the residual standard deviation [11] for both LacOF biosensor and HPLC-ED method were, respectively, 2.1 and 5.1 $pg\,mL^{-1}$ for dopamine, 2.6 and 4.5 $pg\,mL^{-1}$ for norepinephrine and 3.4 and 4.5 $pg\,mL^{-1}$ for epinephrine. These values show that the detection limits for both analytical methodologies have the same order of magnitude.

A set of experiments has been carried out for the assessment of the sensor system stability; thus five repeated measurements of catecholamines (35 $pg\,mL^{-1}$) have been performed during different weeks of 2 months of operation with the same system. The developed biosensor showed a relative standard deviation of the performed measurements of less than 5%, suggesting an adequate degree of stability of the analytical system. However, additional studies regarding the monitoring of the LacOF device behavior for periods of operation longer than 2 months showed a slightly analytical signal degradation after the 13th week of the device operation. This observation was not find to be of special concern, since the sensors head (OF section coated with the enzymatic matrix) can be easily replaced, after 12 weeks, avoiding any losses of the analytical signal or decrease of performance caused by extensive utilization for longer periods of the developed LacOF based biosensor.

The analytical performance of the LacOF biosensor was also compared with HPLC-ED method: 10 different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 $pg\,mL^{-1}$) of a standard mixture of catecholamines were injected, five times each, using both methods in order to test the performance of the developed sensor against the HPLC-ED method. The obtained results are displayed in Table 1.

The ANOVA of the results shows that there is no statistically significant difference ($p = 0.431$, 0.832 and 0.704, for dopamine, norepinephrine and epinephrine, respectively) for the effects of differences in the two methods. However, and 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 LacOF biosensor and HPLC-ED, varied between 1.9 and 2.5 $pg\,mL^{-1}$.

The ANOVA can also be confirmed with a regression analysis of the results obtained with the two analytical systems during the determination of different amounts of catecholamines ranging from 10 to 100 $pg\,mL^{-1}$. Assuming the null hypothesis (same sensitivity, i.e. slope equals 1 and no systematic errors, i.e. intercept equals to 0) for the results obtained with both methods the slope, the intercept, r^2 and p of the regression line, of each of the data sets were calculated and included in Table 2.

For all the three catecholamines analyzed the regression line has an intercept not significantly different of zero and a slope not significantly different of 1. The values above mentioned and a correlation coefficient of approximately 1, allows concluding that the results obtained with the two analytical systems cannot be statistically differentiated, as already inferred from the ANOVA.

Both analytical methodologies were also applied to samples of human urine and plasma, for assessing comparatively the concentrations of catecholamines. Figs. 3 and 4 depict the results obtained in the analysis of five samples of human urine and plasma, respec-

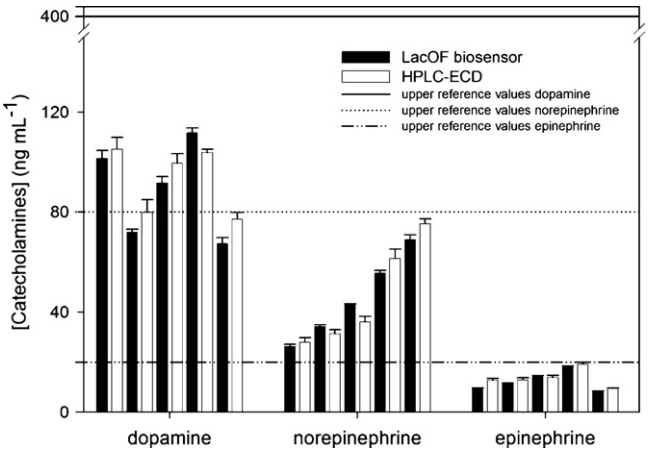

Fig. 3. Results obtained for catecholamines determination of five actual samples of human urine by LacOF biosensor and HPLC-ED methods.

Table 2
Analytical parameters obtained for the comparison of the results obtained with the LacOF biosensor and HPLC-ED method for determination of catecholamines.

	Intercept	Slope	Coefficient of determination (r^2)
Dopamine	0.0 ± 0.1	1.0 ± 0.1	0.99997 ($p < 3.48 \times 10^{-17}$)
Norepinephrine	0.1 ± 0.1	0.998 ± 0.002	0.99998 ($p < 1.15 \times 10^{-17}$)
Epinephrine	0.1 ± 0.1	1.001 ± 0.002	0.99997 ($p < 2.88 \times 10^{-17}$)

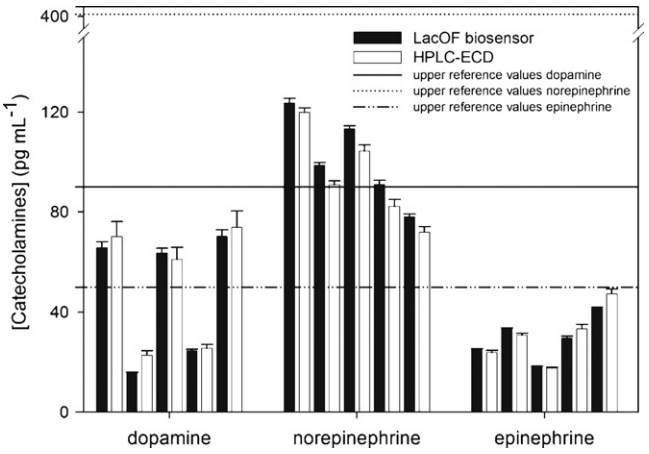

Fig. 4. Results obtained of catecholamines determination for five actual samples of human plasma with five replicates by LacOF biosensor and HPLC-ED methods.

tively by both the LacOF biosensor and the HPLC-ED method for dopamine, norepinephrine and epinephrine.

The levels of catecholamines found in the urine samples analyzed do not exceed the values accepted as normal for these compounds, according to Moyer et al. [12] studies: <400 μg 24 h^{-1} for dopamine; <80 μg 24 h^{-1} for norepinephrine; and <20 μg 24 h^{-1} for epinephrine.

The concentration obtained for dopamine, norepinephrine and epinephrine in plasma are also below the normal values, according to Peaston and Weinkove [13] studies: 0.01–0.475 nmol L^{-1} for dopamine; 0.71–4.02 nmol L^{-1} for norepinephrine; and 0.01–0.328 nmol L^{-1} for epinephrine.

The comparison of the means of the five samples analyzed by the biosensor and the HPLC-ED method, showed that there is no statistically significant difference between the results obtained by the two analytical systems in both urine ($p = 0.460$, 0.868 and 0.848 for dopamine, norepinephrine and epinephrine, respectively) and plasma ($p = 0.970$, 0.995 and 0.889 for dopamine, norepinephrine and epinephrine, respectively).

In order to test the developed biosensor for interferences that could be present in the biological matrices (urine and plasma), the effects of normetanephrine and metanephrine used as example of interferences compounds, on the analytical signal were also evaluated. Each compound tested produces a peak, but in a different analytical time window of the compounds of interest, therefore none of the above mentioned compounds exhibit any interference in the analytical signal. The obtained results allow concluding that the adjustment of the time window could be used for avoiding other potential interferences, as a consequence it is also possible to infer that the developed biosensor could be also applied to determine of a large variety of analytes.

4. Conclusions

An optical fiber biosensor has been successfully developed for determining the concentration of dopamine, norepinephrine and epinephrine. The reported analytical system showed a high analytical signal and a linear response between 5 and 125 pg mL^{-1}, allowing the determination of catecholamines with detection limit less than 3.4 pg mL^{-1}. In addition, the new and compact design of the developed LacOF analytical system has obvious analytical advantages, constituting an excellent alternative to the more classical methods for catecholamines detection.

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