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Flow and Sequential Injection—Luminescence Detection

FLOW-BATCH ANALYZER FOR THE CHEMILUMINESCENCE DETERMINATION OF CATECHOLAMINES IN PHARMACEUTICAL PREPARATIONS

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A novel, simple, cheap, flexible, versatile, and highly sensitivity flow-batch analyzer (FBA) with chemiluminescence detection was developed for determination of dopamine, norepinephrine, and epinephrine in pharmaceutical preparations. The method was based on the inhibitory effect of the mentioned catecholamines on a luminol-potassium hexacyanoferrate (III) chemiluminescence system in alkaline medium. The optimization of the chemical variables affecting this chemiluminescence inhibition effect has been carried out using a Box-Behnken experimental design. The sample throughput was 28 h^{-1}. The system allowed the automatic preparation of standard solutions and analytical process can be accomplished just by changing the operational parameters in FBA control software.

Keywords: Box-Behnken design; Catecholamines; Chemiluminescence; Flow-batch analyzer

INTRODUCTION

The minimization of human interaction in analytical procedures is an exhaustively persecuted target by the modern instrumental analytical chemistry studies, mainly when a large number of samples are involved. In general, the automated procedures are independent of errors caused by the operator and provide high

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repeatability (Burguera and Burguera 2001). Several flow analyzers (FA) have been developed in order to automate and to simplify analytical procedures (Trojanowicz 2008; Ruzicka and Hansen 1988; Valcárcel and Luque de Castro 1987). Automated micro batch (AMBA) and flow-batch analyzers (FBA) proposed by Sweileh and Dasgupta (1988) and Honorato et al. (1999), respectively, can be an excellent alternative to automate different methods of analysis.

The AMBA and FBA allow different analytical processes to be carried out without significant alterations in the physical configurations of analyzers. All these can be accomplished just by changing the operational parameters in the control software. Nowadays, these flexible and versatile systems (multi-task characteristic) are used to put in practice several analytical procedures such as: titrations (Honorato et al. 1999; Honorato, Araújo, et al. 2000; Pasquini et al. 2007), analyte addition (Almeida et al. 2003a; Almeida et al. 2003b), internal standard (Da Silva et al. 2006), screening analysis (Lima et al. 2004), exploitation of concentration gradients (Medeiros et al. 2004), on line matching of pH (Honorato, Carneiro, and Zagatto 2001) and salinity (Carneiro et al. 2002), sample digestion (Honorato, Carneiro, and Zagatto 2000), liquid–liquid extraction (Sweileh and Dasgupta 1988), distillation of volatile analyte (Sweileh and Dasgupta 1988), preparation of calibration solution (Almeida et al. 2007; Grünhut et al. 2008), enzymatic analysis (Grünhut et al. 2008), and kinetic approach (Honorato et al. 1999). However, this new methodology has still not been used to automate chemiluminescence processes. The AMBA and FBA combine the favorable characteristics of both flow and batch analyzers (FA and BA, respectively). The transportation of reagents, samples, or other solutions are carried out in a flow mode as FA, and all the processes of the sample is performed in a mixing chamber (MC) as BA. In AMBA, an injecting loop is used on the sampling stage (as in FA), while in FBA the sample amounts are added into the MC by controlling the ON switching time of one solenoid valve.

In general, FBA present the following characteristics: the employment of solenoid valves and MC; highly precise fluid aliquots can be delivered by microcomputer controll of the ON valve's switching times; high sensitivity due to the physical and chemical equilibria inherent to the analytical processes that may be attained, and the dispersion and/or dilution of the samples that may be negligible. One advantage is the measures of the analytical signal can be performed directly inside MC. This is important for the automation of methods with chemiluminescence detection. In addition, the multicommutation (Reis et al. 1994; Jerônimo et al. 2004) may be used to manipulate the fluids in a simultaneous and/or in an intermittent way. The FBA presents good precision and accuracy, high sample throughput and low contamination, consumption, manipulation of reagents and samples, cost per analysis, waste liberation for the environment, and so forth.

The catecholamines represent a group of compounds such as dopamine (DOP), norepinephrine (NOR), and epinephrine (EPI) that are produced in the adrenal gland and the nerve endings. The circulating catecholamines are involved with the activity of the sympathetic nervous system and the response to stress. In pharmaceutical preparations, these compounds emulate the action of the endogenous catecholamines stimulating the sympathetic nervous system (Portoles and Vargas 1993; Van Boxtel, Santoso, and Edwards 2001). Several methods have been reported in the literature for the assay of these catechol derivatives in biological samples and

pharmaceutical preparations (Kojlo and Calatayud 1995; Aman et al. 1998; Tsuchiya et al. 1997; Parsons, Kerr, and Weiss 1998; Nozaki, Iwaeda, and Kato 1996; Takezawa et al. 2000; Kozminski et al. 1998). Some involve the application of automation for simplification of analytical methodologies (Berzas Nevado, Lemus Gallego, and Buitrago Laguna 1996; Garrido, Lima, and Delerue-Matos 1997; Zhang et al. 1999; Michalowski and Halaburda 2001; Li, Zhang, and Jin 2002; Bezerra et al. 2003; Wang et al. 2004; Nalewajko, Wiszowata, and Kojlo 2007), but their flexibility and versatility are still limited.

The advantages in using a multivariate approach to optimizing an analytical method include reductions in the number of experiments, improved statistical interpretations (particularly with the current availability of statistical software packages), and reduced time requirements. Furthermore, interaction effects between parameters can be investigated with multivariate experiments, which would be impossible to do with a univariate approach (Morgan 1991; Deming and Morgan 1993). The Box-Behnken designs (Box and Behnken 1960) are a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs. In these designs, each factor requires only three levels instead of the five required for central composite designs (unless alpha is equal to one), which may be experimentally more convenient and less expensive to run than central composite designs with the same number of factors. Furthermore, Box-Behnken designs do not contain combinations where the factors are all at their higher or lower levels, simultaneously. The application of Box–Behnken designs has been recorded in optimization of food technology processes (Trinca and Gilmour 1999), microbiological studies (Nagaragan and Natarajan 1999), and pharmaceutical formulation development work (Bodea and Leucuta 1998), among others.

In the present paper a simple, cheap, flexible, versatile, and highly sensitivity flow-batch analyzer (FBA) with chemiluminescence detection is proposed. The feasibility of the proposed FBA is demonstrated for determination of dopamine (DOP), norepinephrine (NOR), and epinephrine (EPI) in pharmaceutical preparations. The method is based on the inhibition effect of the mentioned catecholamines on a luminol-potassium hexacyanoferrate (III) chemiluminescence system in alkaline medium. The optimization of the chemical variables affecting this chemiluminescence inhibition effect was carried out using a statistical model, based on the application of a Box-Behnken experimental design.

EXPERIMENTAL

Apparatus and Software

The chemiluminescence measurements were carried out by using an Aminco Bowman® Series 2 luminescence spectrometer. The photomultiplier was operated at 700 V and the emission of photons was registered at 425 nm.

A model 710 A Orion® pHmeter with an Orion-Ross® model 81-02 electrode was used to carry out the pH measurements.

High-performance liquid chromatographic procedures were carried out on a Gilson liquid chromatograph equipped with a Gilson 322 pump operating at 1.5 mL min^{-1}, a Rheodyne 7725i injector with a 20 µL sample loop, and a

variable-wavelength UV-Vis 156 Gilson detector measuring at 280 nm. A 5 μm Restek C_{18} column (250 mm × 4.6 mm i.d.) was used at room temperature.

Calculations were performed using Matlab 6.0 software. The surface responses were graphed using Statistica 6.0 software.

Reagents and Solutions

All reagents were of analytical grade. Ultra pure water (18 MΩ) was used.

A 0.04 mol L^{-1} luminol stock solution was prepared by dissolving 0.709 g of luminol (Fluka), in 100 mL of a 0.1 mol L^{-1} potassium hydroxide solution. Luminol working solution was daily prepared by appropriate dilution of stock solution in 1.0 mol L^{-1} of potassium hydroxide. A 2.0×10^{-4} mol L^{-1} potassium hexacyanoferrate (III) stock solution was prepared by dissolving 33 mg of potassium hexacyanoferrate (III) (Merck) in 500 mL of water. Working solution was daily prepared by appropriate dilution with water. A 1.0 mol L^{-1} potassium hydroxide solution was prepared by dissolving 56.11 g of potassium hydroxide (Merck) in 1000 mL of water.

Stock solutions of dopamine hydrochloride (Sigma), norepinephrine bitartrate monohydrate (Sigma) and epinephrine bitartrate (Sigma) of 2.90 mg mL^{-1}, 1.28 mg mL^{-1} and 0.95 mg mL^{-1} respectively, were prepared in water. All stock solutions were protected from light and stored at 4°C. The working standard solutions were prepared by adequate dilutions of the stock solutions with water.

Flow-batch Analyzer

A schematic diagram of the proposed FBA is shown in Fig. 1a. Five NResearch three-way solenoid valves were used: four of them (V_W, V_S, V_{LU} and V_{HE}) for water, sample, luminol, and potassium hexacyanoferrate (III) flowing toward the mixing chamber (MC). The fifth valve ($V_{W/MC}$) was used to select the stream flowing of water or mixture which comes from the MC to the waste. A Pentium 166 MHz microcomputer furnished with a laboratory-made parallel interface card was used to control the peristaltic pump and valves. Also, it was used to perform the acquisition and treatment of data. The software was developed in Delphi 7.0 language. A made-lab electronic actuator (EA) increased the power of the microcomputer signal in order to control the valves.

A Minipuls 3 Gilson peristaltic pump, equipped with five pumping channels was used. Tygon® tubes, three of 1.29 mm i.d. and two of 2.06 mm i.d. were used. A home-made mixing chamber (MC) with an inner volume of approximately 4 mL was made in Teflon®. A transversal view of the MC is showed in Fig. 1b. This view allows seeing the quartz window and the outlet channel. The lines linking the valves V_W, V_S, V_{LU}, and V_{HE} to MC and $V_{W/MC}$ to waste were implemented as short as possible using 0.8 mm i.d. Teflon® tubing.

Flow-batch Procedure

One step inherent to flow-batch technique implementation is to obtain the signals that are used to correct the responses for volume changes or the flow-rates of the channels and then performing the calibration and subsequent sample analysis. This step can be easily implemented through the procedure described by Almeida

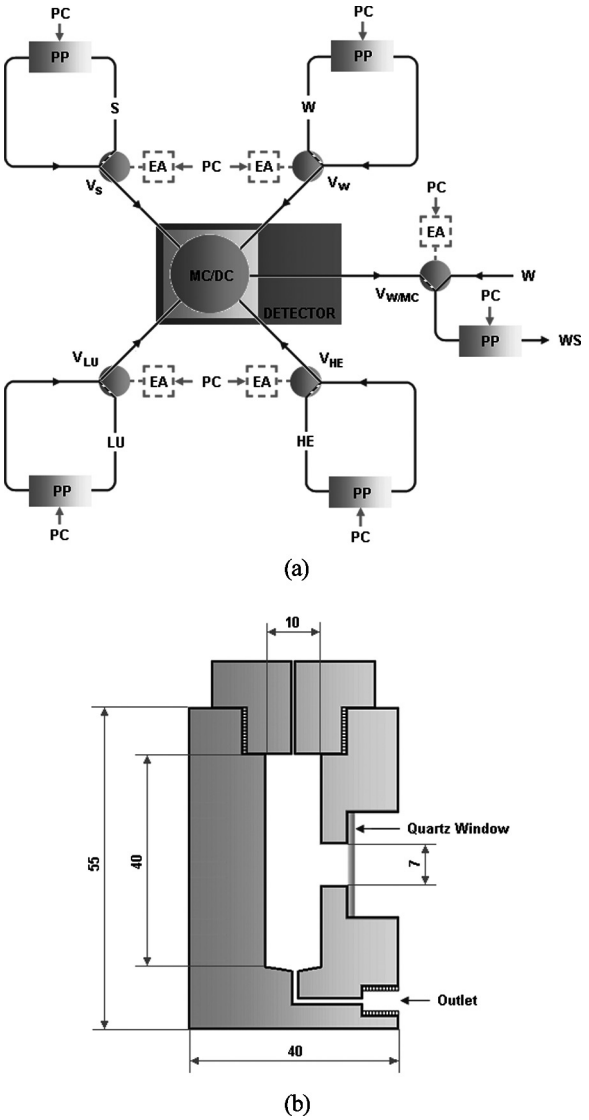

Figure 1. (a) Diagram of the proposed FBA. EA: electronic actuator; HE: potassium hexacyanoferrate (III); LU: luminol; MC/DC: mixing chamber/detection cell; PC: microcomputer; PP: peristaltic pump; S: sample; V: solenoid valves; W: water; WS: waste. The arrows and the dotted lines indicate the direction of the fluids and the control lines, respectively. (b) A transversal view of the mixing chamber (MC) used in the FBA proposed. The dimensions are expressed in mm.

et al. (2003b). While the ratio between the flow rates of two channels varied in a range of 1.00 to 1.10, it was necessary to apply a correction factor to the timing control of the delivered volumes in some channels.

Before starting the procedure, all solutions in their respective channels, were pumped and recycled toward their flasks (Fig. 1a). Then, each valve was switched ON during an interval time of 2 s and the solutions were pumped towards MC in order

to fill the channels between the V_W, V_S, V_{LU}, and V_{HE} valves and MC. Immediately, $V_{W/MC}$ was switched ON and the excess of the solutions into MC were aspirated to waste during 5 s. This operation, denominated "fill channels", consumed a total time interval of 7 s, and it was completed when the solution in each channel was changed.

The MC cleaning was carried out by switching ON V_W valve during 30 s. The total emptying of the MC was assured by switching ON $V_{W/MC}$ valve during 35 s. The system was always cleaned between measurements and the stirrer was ON during all the steps. After performing the filling channels and cleaning procedures, the system was ready to carry on the preparation of the standard solutions and analysis of the samples.

Initially, all the valves were switched OFF, so that the water, sample (or stock solutions), luminol, and potassium hexacyanoferrate (III) were continuously pumped into their channels and returned to their respective recipients.

The blank signal was measured by sequentially switching ON the V_W, V_{LU}, and V_{HE} valves for suitable times (t_W, t_{LU}, and t_{HE}). Then, water, luminol and potassium hexacyanoferrate (III) were pumped towards MC and the signal was measured as a peak height.

The decrease in the chemiluminescence intensity was produced when a solution containing dopamine, norepinephrine, or epinephrine was present into the MC. This decrease was evaluated in relation to the original chemiluminescence emission corresponding to a blank and it was proportional to the dopamine, norepinephrine, and epinephrine concentration. Then, the valves V_W and V_S were sequentially switched ON during previously defined time intervals for each valve (t_W and t_S) and aliquots of each fluid were pumped toward the MC. The mixture was homogenized by stirring for 4 s, and then, V_{LU} valve was ON switched during a t_{LU} time. Finally, the chemical reaction started, when the V_{HE} valve was ON switched for the t_{HE} time and the peaks corresponding to each mixture yielded was recorded. The mixture remained in the MC for 2 s, and, after that, it was aspirated toward the waste, by switching ON $V_{W/MC}$ for 30 s. This procedure was repeated for each standard solution and sample, varying only the t_W and t_S values.

For each standard solution, the total volume that was added into MC was the same in all points of the calibration. Thus, t_{LU} and t_{HE} always were the same and, while t_S increased, t_W decreased (and vice-versa).

In Table 1 the schedule of procedures is summarized and the respective ON switching times of the valves to carry on the complete analysis are presented.

Table 1. System operation schedule

Valves	V_W	V_S	V_{LU}	V_{HE}	$V_{W/MC}$
Flow rate (mL min^{-1})	0.14	0.14	0.12	0.20	0.20
Valve switching time intervals (s) for:					
Filling channels	2	2	2	2	2
Wash system					
a- MC filling	30	–	–	–	–
b- MC emptying	–	–	–	–	35
Blank	10	–	7	4	–
Standard solutions	0–10	0–10	7	4	–
Samples	5	5	7	4	–
MC emptying	–	–	–	–	35

Chromatographic Procedure

The chromatographic procedure was carried out according to the established reference method (United States Pharmacopoeia 2005). The peaks of analytes were well resolved and tailless, with retention times of 7.1 min for DOP, 5.5 min for NOR, and 2.9 min for EPI. Each sample solution was injected by triplicate and the concentrations were calculated using a calibration curve.

Optimization of Chemical Variables

In order to calculate the effects that produce changes on the variables and their possible interactions, to describe the nature of the response surface in the experimental region, and to elucidate the optimal concentrations of the independent variables, a Box-Behnken design was applied. Three variables were studied: concentration of luminol (LU), concentration of potassium hexacyanoferrate (III) (HE), and concentration of potassium hydroxide (KOH). Each variable was studied in three levels: low (2.0×10^{-4} mol L^{-1} to LU, 2.0×10^{-7} mol L^{-1} to HE and 0.6 mol L^{-1} to KOH), basal (4.0×10^{-4} mol L^{-1} to LU, 4.0×10^{-7} mol L^{-1} to HE and 0.8 mol L^{-1} to KOH), and high (6.0×10^{-4} mol L^{-1} to LU, 6.0×10^{-7} mol L^{-1} to HE and 1.0 mol L^{-1} to KOH). Fifteen experiments, that included three central point replicates, were carried out in duplicate ($n = 30$). The studied ranges were selected based on the influence of each variable in the chemiluminescence signal. The optimization criterion was the maximum signal and highest reproducibility using lowest oxidant concentration.

Sample Preparation

The dopamine (Northia® and Fabra®), norepinephrine (Biol® and Fioritina®), and epinephrine (Lavimar® and Larjan®) were purchased in a local pharmacy. These compounds are presented in the form of injectables, with a nominal content of 100 and 200 mg of dopamine, 4 mg of norepinephrine, and 1 mg of epinephrine per injectable, respectively. In each case, the content of four ampoules was mixed and an accurate volume was measured and adequately diluted with water (1,250,000 times to DOP of 200 mg, 625,000 times to DOP of 100 mg, 62,500 times to NOR, and 20,000 times to EPI).

RESULTS AND DISCUSSION

Flow-batch Parameters

The optimization of the volumes added to the MC during the flow-batch procedure was performed by using the univariate method and selected as a compromise between sensitivity and reproducibility of the analytical signal. The total volume of reaction was 3.0 mL, which included 0.70 mL of sample, 0.70 mL of water, 0.80 mL of LU, and 0.80 mL of HE.

The lengths of tubing-connections of the solution containers to the solenoid valves-MC were approximately 100 mm. These connections were implemented as

short as possible using 0.8 mm i.d. Teflon® tubing did not impact these volumes sizes. The fill channel procedure for water, LU and HE, was carried out only once. For the sample, the fill channel procedure was carried out for each new sample, but the necessary volume for this procedure was only 50 μL, a small volume compared to the volume sample added to the MC (0.70 mL).

A critical variable was the order of the reagents added into the MC. Three different combinations were tested: LU/HE, HE/LU, and LU-HE, simultaneously. The optimum was LU/HE.

A rapid reaction between luminol and the oxidant improved the analytical signal (height and width of the peaks); thus, the optimal flow rate to HE channel was higher (0.20 mL s^{-1}) than to LU channel (0.12 mL s^{-1}). On the other hand, the mixing in MC was also analyzed. Due to the significant effect of this variable in the intensity and reproducibility of the analytical signal, it was necessary to maintain a very high and stable speed of stirring, respectively. Thus, the maximum speed of the magnetic stirrer was selected (500 rpm). In this way, an excellent mixing in the MC was always achieved with good sensitivity and reproducibility. This is one more favorable characteristic of FBA when compared to other flow analyzers that do not use MC and mechanized stirring.

Optimization of Chemical Variables

The responses to each experiment corresponding to the applied experimental design were fitted to the following first order polynomial model:

$$\begin{aligned} R_{(LU,HE,KOH)} = {} & 13.55\ [LU] + 21.58\ [HE] + 7.10\ [KOH] \\ & + 6.44[LU^{*}HE] + 4.26\ [HE^{*}KOH] + 43.84 \\ & (+/-0.049)\ (+/-0.049)\ (+/-0.049) \\ & (+/-0.70)\ \ (+/-0.70)\ \ \ (+/-0.81) \end{aligned}$$

where R is the dependent variable (response) and LU, HE, and KOH are the independent variables as mentioned previously. In this model, non-significant effects have been removed. An analysis of variance (ANOVA) was carried out to evaluate the quality of fit of the polynomial model. For the proposed linear model, there was to evidence of significant lack of fit at the 95% level: the MS_{lof}/MS_{pe} ratio was 2.91, less than the 95% $F_{3,17}$ critical value of 3.20. The MS_R/MS_r ratio was 332.9, much larger than the 95% $F_{9,20}$ critical value of 2.39, indicating a significant regression.

The corresponding surface responses (Fig. 2) shows that the optimum values for LU, HE and KOH concentrations are out of the experimental region. Nalewajko, Ramírez, and Kojlo (2004) proposed a possible mechanism to the inhibitory effect of dopamine on a luminol-potassium hexacyanoferrate (III) chemiluminescence system. Dopamine reacts with potassium hexacyanoferrate (III) consuming part of the oxidant; it makes that chemiluminescence intensity of the luminol-potassium hexacyanoferrate (III) system decreases. In agreement with our experiences, an oxidant concentration of approximately 10^{-7} mol L^{-1} can be partially consumed (and consequently the signal intensity partially inhibited) by a

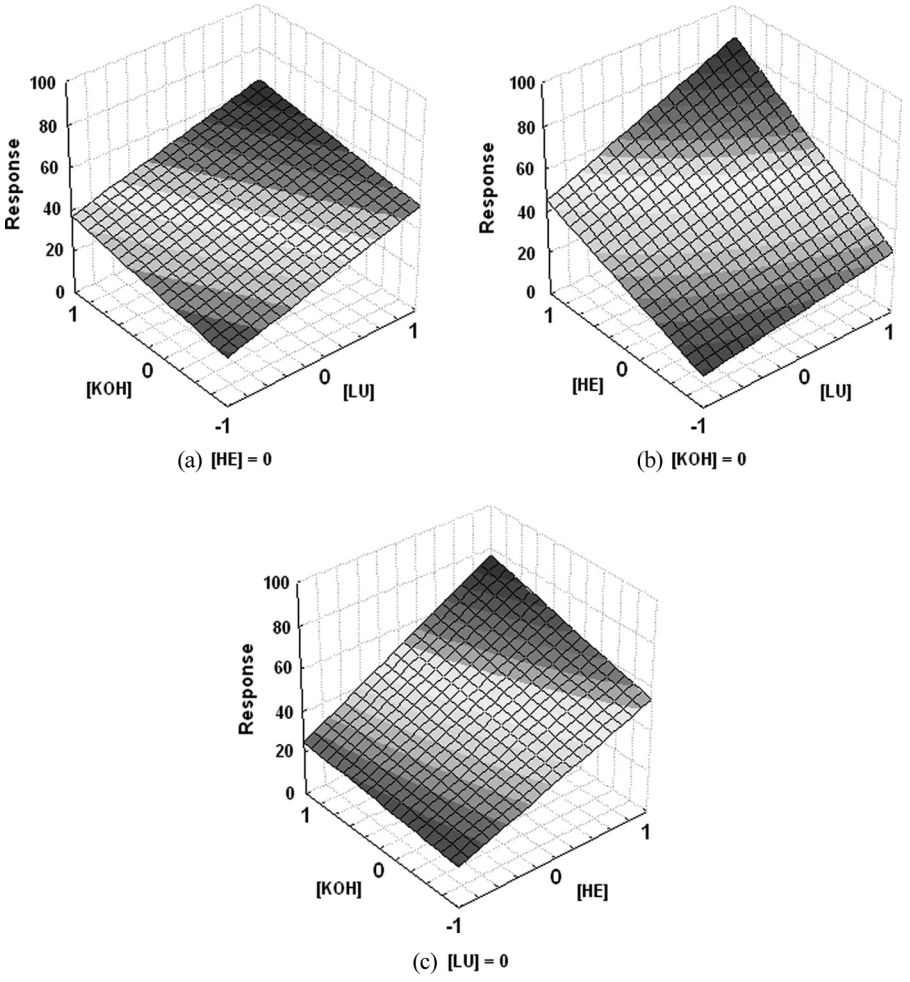

Figure 2. Response surface for (a) [HE] = 0; (b) [KOH] = 0 and (c) [LU] = 0.

sample concentration of approximately 10^{-8} mol L^{-1} of DOP, 10^{-8} mol L^{-1} of NOR and 10^{-7} mol L^{-1} of EPI. Therefore, a low limit of detection (Table 2) can be obtained working with a low oxidant concentration. Otherwise, when higher sample concentrations than those were used, all the oxidant present was consumed, resulting in the total inhibition of the analytical signal. On the other hand, lower HE concentrations than 6.0×10^{-7} mol L^{-1}, resulted in a significantly decrease of the signal intensity. In conclusion, the optimal concentration selected for HE was 6.0×10^{-7} mol L^{-1}; it was a compromise between a low limit of detection and an appropriate analytical signal. With regard to LU and KOH, higher concentrations than 6.0×10^{-4} mol L^{-1} and 1.0 mol L^{-1} respectively, presented signals with worse reproducibility. Therefore, concentrations of 6.0×10^{-4} mol L^{-1} to LU and 1.0 mol L^{-1} to KOH were chosen as suitable for the purposes of this study.

Table 2. Statistic values and analytical performance

	DOP	NOR	EPI
Statistic values			
n^a		5	
Intercept	116.7 ± 1.3	112.8 ± 1.2	126.9 ± 1.2
Slope	-3.615 ± 0.096	-5.966 ± 0.153	-2.810 ± 0.045
Coefficient of regression	0.9979	0.9980	0.9995
Analytical performance			
Linearity interval (ng mL^{-1})	8.0–18.8	4.5–10.6	14.7–34.3
Limit of detection (ng mL^{-1})	2.1	1.1	3.3
Limit of quantification (ng mL^{-1})	6.4	3.3	9.9
Sample throughput (h^{-1})		28	
Precision			
Repeatability (RSD %, $n = 3$)	3.9	4.0	3.3
Reproducibility (RSD %, $n = 3$, $k = 3$)	5.5	3.6	3.5

[a]Levels of concentration. Each level was replicated by triplicate.

Analytical Parameters

Graphs of CL intensity versus time for a series of DOP, NOR and EPI standards and real samples are showed in Fig. 3. Under the experimental selected conditions, calibration graphs of peak height vs. DOP, NOR, and EPI concentrations were established by applying univariate linear regression. The statistic values

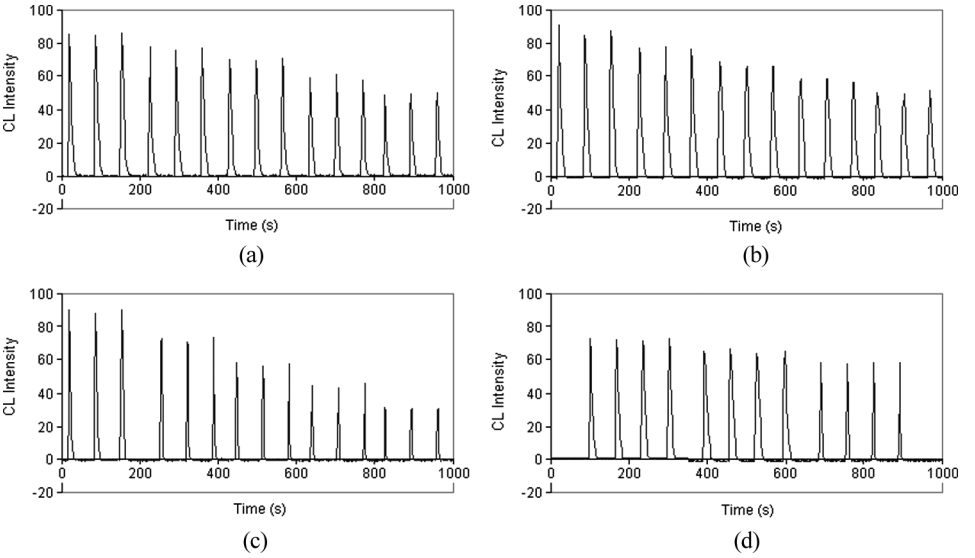

Figure 3. Graphs of CL intensity vs. time for a series of standards of: (a) Dopamine (8.0, 10.7, 13.4, 16.1, and 18.8 ng mL^{-1}); (b) Norepinephrine (4.5, 6.1, 7.6, 9.1, and 10.6 ng mL^{-1}); and (c) Epinephrine (14.7, 19.6, 24.5, 29.4, and 34.3 ng mL^{-1}). Each standard solution was carried out for triplicate. Peaks corresponding to real samples are showing in (d). To the left, four signals obtained to dopamine. In the middle, four signals obtained to norepinephrine. To the right, four signals obtained to epinephrine.

and the analytical performance are presented in Table 2. The repeatability (intra-day precision) expressed as relative standard deviations (RSD) of the peak height was calculated from three slopes (Table 2). Otherwise, RSDs lower than 3.5% were obtained from independent measures of a series of standards of DOP (10.7, 13.4, and 16.1 ng mL^{-1}), NOR (6.1, 7.6, and 9.1 ng mL^{-1}), and EPI (19.6, 24.5, and 29.4 ng mL^{-1}). The reproducibility (inter-day precision) expressed as RSD of the peak height, was calculated from three slopes corresponding to calibrations graphs, which were obtained in three different days (Table 2). Otherwise, RSDs lower than 6.5% were obtained from independent measures of a series of standards of DOP (10.7, 13.4, and 16.1 ng mL^{-1}), NOR (6.1, 7.6, and 9.1 ng mL^{-1}), and EPI (19.6, 24.5, and 29.4 ng mL^{-1}), in three different days. In all cases, the results were satisfactory, thus the precision of the proposed method is verified.

Interference Study

A frequent problem in the analyses of commercial samples is the presence of excipients. The effect of some common excipients found frequently with catecholamine was studied. Sodium chloride, sodium bisulphite (stabilizer agent) and citric acid-sodium citrate (pH regulator) in similar amounts present in the analyzed pharmaceutical preparations did not interfere significantly. The RSD was less than 5% for the peak height obtained for the standard solutions of DOP (13.4 ng mL^{-1}), NOR (7.6 ng mL^{-1}), and EPI (24.5 ng mL^{-1}). It is important to note that the three catecholamines can interfere among them, but usually only one catecholamine is present in pharmaceutical preparations.

Application to Real Samples

The proposed method was used for the determination of dopamine, norepinephrine, and epinephrine in pharmaceutical preparations. Table 3 shows the results

Table 3. Determination of DOP, NOR, and EPI in pharmaceutical preparations

	DOP				NOR		EPI	
	Northia®	Fabra®	Northia®	Fabra®	Biol®	Fioritina®	Lavimar®	Larjan®
Nominal	100	100	200	200	4.0	4.0	1.00	1.00
HPLC	100 (1)	103 (1)	205 (2)	208 (2)	4.1 (0.1)	4.2 (0.1)	0.92 (0.01)	0.92 (0.01)
FBA	94 (1)	103 (1)	204 (3)	203 (4)	4.2 (0.1)	4.2 (0.1)	0.97 (0.03)	0.93 (0.01)
Acceptance criteria[a] (%)	95–105				90–115		90–115	
t calculated[b]	1.16				2.83		2.41	
t critical[c]	2.36				3.18		3.18	
n	8				4		4	

The values are expressed in mg per ampoule. Standard deviations are in parenthesis. The samples were analyzed for triplicate.

[a]According to United States Pharmacopoeia 29.

[b]HPLC vs. proposed method.

[c]Tabulated 95% confidence limit.

using pharmacopoeia (United States Pharmacopoeia 2005) and the flow-batch proposed methods. Additionally, a significance test (test "t") for comparison between both methods is presented. As can be observed, for all analyzed samples, the obtained concentrations by the proposed method were in close agreement with those obtained by HPLC. Additionally, the samples met the requirements of the pharmacopoeia when both methods, HPLC and FBA, were applied (except to Northia 100 mg by FBA).

The viscosity of the solutions did not affect the analysis. Although, the viscosity for real samples is high, the great dilution in water of these (1,250,000 times to DOP of 200 mg, 625,000 times to DOP of 100 mg, 62,500 times to NOR and 20,000 times to EPI) before the analysis reduce this effect.

CONCLUSION

The proposed FBA with chemiluminescence detection was successfully applied to determination of DOP, NOR, and EPI in pharmaceutical preparations. The system allowed the automatic preparation of standard solutions to each analyte with minimal alterations in the physical configuration of analyzer. Moreover, all analytical process for each determination can be accomplished just by changing the operational parameters in FBA control software. The reagents consume and diluted sample are low ($\leq$ to 800 μL). The great sensitivity of the proposed method allowed the quantification of the mentioned analytes in ultratrace levels (ng mL^{-1}). The MC was fit, owing to a good and rapid mixture between the sample and reagents, which improved the quality of the analytical signal and, therefore, the sensitivity.

Precise and accurate results were obtained based on the estimation of figures of merit. The validation was done on real samples and vs. the official method of the pharmacopeia (United States Pharmacopoeia 2005).

The proposed FBA is inexpensive, rapid (28 h^{-1}), flexible, and sensitive; it can be useful as a possible alternative method for the quality control analysis of these pharmaceutical preparations.

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