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Determination of catecholamines by flow-injection analysis and high-performance liquid chromatography with chemiluminescence detection

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Abstract

A chemiluminescence (CL) detection of catecholamines [norepinephrine (NE), epinephrine (E), dopamine (DA) and L-dopa (LD)] is described for the flow-injection (FI) and high-performance liquid chromatographic (HPLC) determination of these compounds. The detection method is based on the inhibition effect of catecholamines (CAs) on the CL reaction of luminol with iodine in the alkaline medium. The proposed FI method allows the determination of CAs in pharmaceutical preparations for the purpose of drug quality control. The calibration curves show good linearity in the concentration range of: 1.1–20.0 $\mu g\,l^{-1}$ (NE), 0.5–5.0 $\mu g\,l^{-1}$ (E), 0.6–9.0 $\mu g\,l^{-1}$ (DA) and 0.6–10.0 $\mu g\,l^{-1}$ (LD). The limits of detection (defined as a signal-to-noise ratio of 3) are: 0.34 $\mu g\,l^{-1}$ (NE), 0.15 $\mu g\,l^{-1}$ (E) and 0.18 $\mu g\,l^{-1}$ (DA, LD). The HPLC procedure was successfully applied for the determination of catecholamines (NE, E, DA) in human urine after solid-phase extraction (SPE). In a simple run time CAs can be determined in 20 min. The chromatographic linear ranges are: 5.0–72.0 $\mu g\,l^{-1}$ (NE), 5.0–48.0 $\mu g\,l^{-1}$ (E) and 5.0–96.0 $\mu g\,l^{-1}$ (DA). The limits of detection for three urinary CAs are: 0.71 $\mu g\,l^{-1}$ (NE), 0.26 $\mu g\,l^{-1}$ (E) and 0.73 $\mu g\,l^{-1}$ (DA).
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1. Introduction

Catecholamines represent a group of biogenic amines, among which epinephrine, norepinephrine and dopamine act as neurotransmitters or hormones and L-dopa is a dopamine physiological precursor. They are important markers for the diagnosis of many diseases and are also used in the treatment of bronchial asthma, Parkinson's disease, myocardial infarction and cardiac surgery. This has prompted the development of many methods for the determination of catecholamines in blood plasma, urine and pharmaceutical preparations. Among them a large number of flow-injection methodologies have been proposed employing spectrophotometry [1,2], spectrofluorimetry [3,4], chemiluminometry [5–10], and electrochemistry [11–13] as detection techniques. FI systems allow to be achieved high measurement throughput, low reagent consumption and high precision and have found widespread application in the automation of analyses.

Luminol CL reaction utilizing different oxidants such as hydrogen peroxide, potassium hexacyanoferrate(III), chlorate [14], hypochlorite [15], periodate [16,17] has been used for the indirect detection of CAs based on their CL inhibition or enhancement especially in flow-injection analysis but also in capillary electrophoresis. However, to our knowledge, there are no reports using CL of luminol–I_2 system for quantitation of CAs. Moreover, there are only few examples of applying this detection system quoted in the literature [18–20].

In this work, the inhibiting effect of CAs on the CL reaction of luminol oxidized by iodine in alkaline solution has been studied for the first time. The principal advantages of the method described here is its high sample throughput (over 80 samples h^{-1}) and low detection limit, which is lower than that of majority of previous FI methods for the determination of catecholamines based on the luminescent properties of luminol [14–17]. The method was applied to the determination of cat-

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echolamines in pharmaceutical formulations with satisfactory results.

Since catecholamines in biological fluids occur in only small quantities, the analyses method for their determination should be both selective and sensitive. It usually requires the use of a combination of effective separation techniques such as high-performance liquid chromatography with electrochemical, fluorescence, and chemiluminescence detection, capillary electrophoresis [14]; microbore HPLC [21]. Chemiluminescence as a detection technique used with HPLC seems very attractive due to its high sensitivity and selectivity. As far, in trace analysis of catecholamines HPLC was usually combined with peroxyoxalate chemiluminescence detection system [14]. To the best of our knowledge, no publications dealing with CL of luminol, the most often used CL reagent, as the post-column detection for the determination of catecholamines in biological samples have been published so far. Despite previously reported methods [14] offered higher sensitivity and enable the measurement of CAs in plasma samples, our developed method is suitable for the determination of norepinephrine, epinephrine and dopamine in urine samples.

Separation of catecholamines in urine samples was carried out by HPLC using the proposed detection method. Using post-column reaction detection enabled catecholamines to be separated in their native form. It allowed the separation procedure quoted in the literature to be used [22]. To assess the applicability of this method catecholamines were determined in real urine samples.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and solutions were prepared using ultra-pure water obtained by a Milli-Q Plus water purification system (Millipore S.A., Molsheim, France). Diphenylborinic acid (DPBA) ethanolamine ester was obtained from Sigma–Aldrich (Steinheim, Germany). HPLC grade methanol and acetonitrile were from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, EDTA disodium salt, ammonium chloride, concentrated ammonia and phosphoric and acetic acids were obtained from POCH (Gliwice, Poland). Sodium dodecyl sulphate (SDS) was purchased from Sigma–Aldrich Chemie (Steinheim, Germany).

Luminol was supplied by Fluka (Steinheim, Germany), I_2, KI, NaOH by POCH (Gliwice, Poland). A 2.5×10^{-2} mol l^{-1} stock luminol solution was prepared in 1.0 mol l^{-1} NaOH and was kept in the dark at +4 °C. The 5×10^{-2} mol l^{-1} stock I_2 solution was prepared in 100 ml of water containing 4 g of KI. The catecholamine standards (norepinephrine, epinephrine, dopamine, L-dopa) were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Stock solutions of catecholamines (1000 mg l^{-1}) were prepared in the acetic buffer (pH 3.48) and stored at +4 °C in dark bottles. The stock solutions were diluted with water to obtain an appropriate concentrations of working solutions.

Urine endocrine controls (normal range) were obtained from Chromsystems (München, Germany). The lyophilised control urines based on human urine were reconstituted in 8.0 ml of ultra-pure water according to the manufacturers' instructions and stored tightly capped at +4 °C.

2.2. Apparatus and procedure

The configuration of the FI system is illustrated in Fig. 1A. Reagent and carrier solutions were propelled using an Ismatec MS-Reglo peristaltic pump with an appropriate flow rate and merged in a Perspex T-piece. Analytes were injected with a sample loop of 600 μl directly into an H_2O carrier stream using a four-way rotary injection valve (Model 5041, Rheodyne, USA) and merged with the combined stream of luminol (prepared in 1.0 mol l^{-1} NaOH) and I_2 (prepared in 4.82×10^{-4} mol l^{-1} KI) solution at a mixing tee. The detection system comprised a flow-through luminometer (KSP, Poland) which consisted of a coiled PTFE flow cell of 1 mm i.d. (length of 25 cm in six windings) positioned in front of the photomultiplier in a light-tight box. The control of the system and the data acquisition were performed through special software provided by the manufacturer of the luminometer.

The chromatographic system (Fig. 1B) (Thermo Separation) consisted of the CL detection system, low-gradient pump P2000, vacuum membrane degasser SCM Thermo Separation, and Rheodyne loop injector (200 μl loop). The measurement was carried out using the reversed-phase analytical column Lichrosorb LC-8, 150 mm × 4.6 mm (5 μm) (Supelco, USA). The isocratic mobile phase used was a modification of that applied by Grossi et al. [22]. The mobile phase was a buffer solution containing 50 mmol l^{-1} of potassium dihydrogen phosphate, 100 ml l^{-1} of methanol, 200 ml l^{-1} of acetonitrile, 500 mg l^{-1} of sodium dodecyl sulphate and 250 mg l^{-1} of EDTA. The pH of the mobile phase was adjusted to 3.5 with orthophosphoric acid and passed through a filter. The flow rate was 1.0 ml min^{-1}. The compounds studied were separated within 13 min with retention times for NE, E and DA (6.8, 7.6 and 11.3 min, respectively). The total run time was 20 min.

The SPE clean-ups were performed using a SPE-12G vacuum manifold (J.T. Baker, NJ). The 3 ml solid-phase extraction columns, packed with reversed-phase octadecylsilane (C_{18}), purchased from J.T. Baker, were used for the purification of urine samples [22].

Absorption spectra were performed on a model 8452A diode array spectrophotometer (Hewlett-Packard, Germany). Chemiluminescence spectra were monitored using a Hitachi F-2500 fluorescence spectrophotometer (Hitachi Ltd., Japan) with the light source switched off. pH measurements were carried out on a pH-meter pX-processor PM-600 (TMS Electronics, Poland) with a combination glass electrode (Eurosensor, Poland).

2.3. Sample preparation for pharmaceuticals

The pharmaceutical formulations analysed in this work were: Injec. Adrenalini 0.1% ampoules (Polfa, Warsaw) containing 1.0 mg of epinephrine, Dopaminum Hydrochloricum

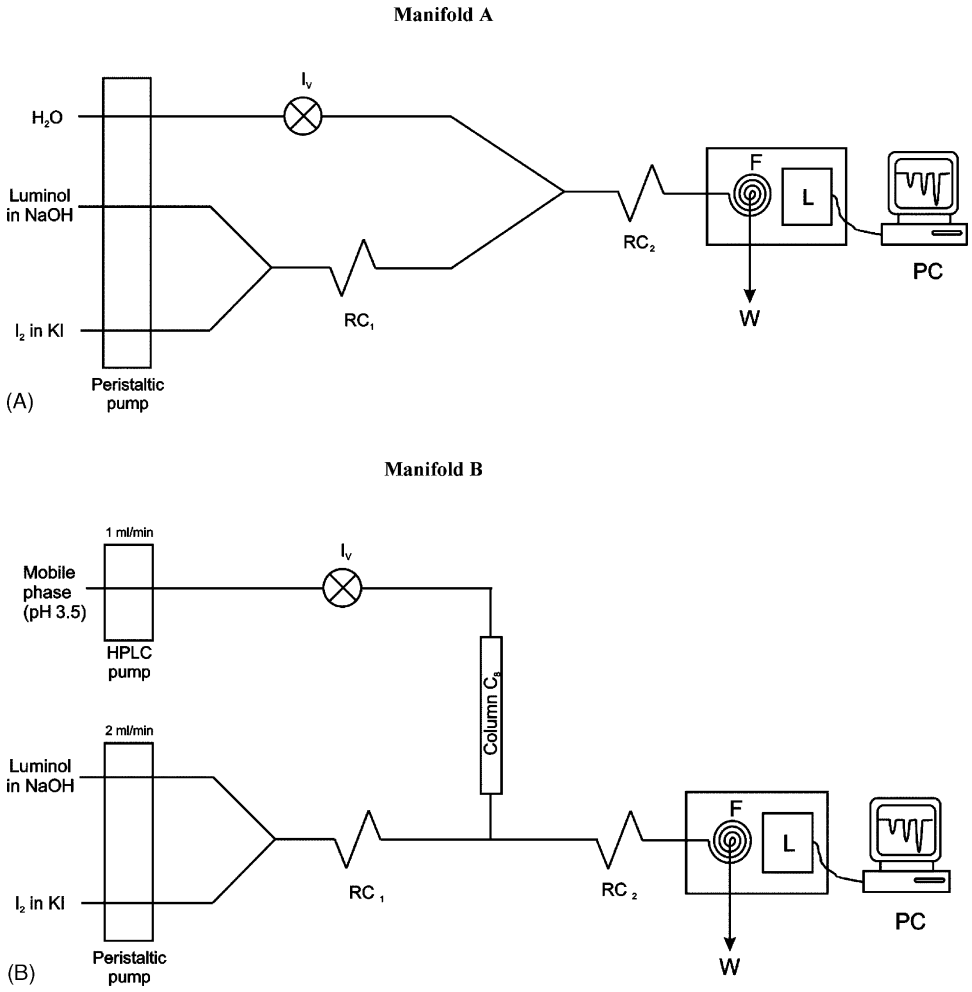

Fig. 1. (A) Schematic diagram of the flow-injection system with CL detection for determination of CAs in pharmaceutical preparation and (B) schematic diagram of FIA unit with CL detection coupled to the HPLC system for determination of CAs in urine. RC_1, RC_2: mixing coils; I_V: injection valve; L: luminometer; F: flow cell; PC: computer; W: waste.

ampoules (Polfa, Warsaw) with a certified amount of 200 mg of dopamine hydrochloride and Madopar capsules (Roche, Switzerland) containing 50 mg of L-dopa and 12.5 mg of benserazide hydrochloride. Each ampoule of the injection preparation of epinephrine or dopamine hydrochloride was diluted with water to fit the concentration of analyte within the range of a calibration curve. The contents of several capsules of Madopar were weighed and a suitable amount of this powder (equivalent to 200 mg of L-dopa) was weighed accurately, transferred into a 100-ml calibrated flask and made up to a volume with acetic buffer (pH 3.48). After filtering, aliquots of the solution were diluted with water to adjust the concentration of analyte to the linear calibration range.

2.4. Urine sample pre-treatment

Drug-free urine specimens used in this study were obtained from healthy donors (here: the investigators). The urine samples were collected during 6 h in plastic containers, acidified with 6.0 mol l^{-1} of hydrochloric acid as a preservative (final pH of the samples was between 1 and 3) and stored in a dark place at +4 °C until analysis [23].

2.5. Clean-up procedure for urine samples

Simultaneous extraction of norepinephrine, epinephrine and dopamine from urine using octadecylsilane resin was based on the method of Grossi et al. [22]. To 1 ml of urine, 2 ml of buffer containing a complexing agent (0.2% DPBA and 5 g l^{-1} EDTA in 2 mol l^{-1} of NH_4Cl–NH_4OH, pH 8.5) was added. After mixing, the pH of the complexed urine sample was checked with a pH probe and was adjusted with concentrated ammonia to obtain pH 8.5 if necessary.

The following procedure was performed for the sample purification: 1.5 ml of complexed samples were applied to the SPE columns which were first activated and equilibrated with 2 ml of methanol followed by 2 ml of wash buffer (0.2 mol l^{-1} NH_4Cl–NH_4OH, pH 8.5). The sample was sucked into a −200 kbar vacuum. The impurities were rinsed with 2 ml of wash buffer followed by 2 ml of 20% methanol in wash buffer (pH 8.5), the column was dried for 1 min at −350 kbar of vacuum, and catecholamines were eluted with 1.5 ml of 1 mol l^{-1} acetic acid. The eluate was directly injected into the column.

Catecholamines eluted in acetic acid were stable for at least 24 h at room temperature [23].

3. Results and discussion

3.1. Discussion of the inhibition mechanism

It is known that oxidation of luminol in alkaline aqueous solution generates chemiluminescence. The luminophore of this system is 3-aminophtalate (3-APA), an excited product which results from this chemical reaction [18]. The maximum emission of the CL reaction is at 425 nm. In this work, luminol was oxidized by iodine, and catecholamines strongly inhibited the CL of the luminol–I_2 system. In order to elucidate the CL mechanism and find the reaction product generating the CL, the emission spectra of luminol–I_2 CL reaction system in the absence and in the presence of catecholamines were examined (dopamine was chosen as an example) (Fig. 2). The results showed that the maximum emission appeared at 425 nm for the two reactions and the relative CL intensity was lower when catecholamine was presented. It indicated that the CL spectrum is independent of catecholamines, which revealed that the luminophore of luminol–I_2–catecholamine system is still 3-APA.

The inhibition mechanism of catecholamines on luminol–I_2 system was suggested based on the analysis of UV–vis absorption spectra (Fig. 3) of each catecholamine (in this case dopamine) (a), I_2 (b) and catecholamine–I_2 system (c). It could be seen that light absorption of the mixed system (c) was different from the algebraic sum of the light absorption of the individual compounds (d), which reveals that there is a chemical reaction between iodine and catecholamines. Moreover, a new absorption band appeared in the range 340–450 nm which probably corresponds to an oxidation product of dopamine. The consumption of iodine, the oxidant of luminol–I_2 system, led to the decrement of the CL intensity of luminol.

3.2. Flow-injection chemiluminescent determination

3.2.1. Effect of chemical and instrumental variables

In the alkaline medium, luminol reacted with iodine to generate chemiluminescence, due to the emission of excited

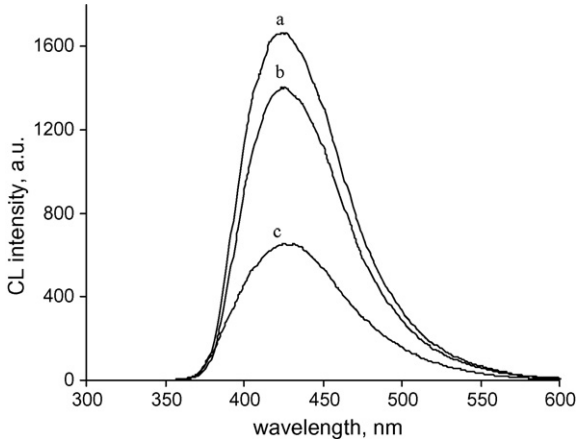

Fig. 2. Chemiluminescence spectra: (a) luminol–I_2; (b) luminol–I_2–dopamine ($5\,\mu g\,ml^{-1}$); (c) luminol–I_2–dopamine ($10\,\mu g\,ml^{-1}$). Luminol: 5×10^{-4} $mol\,l^{-1}$; I_2: 5×10^{-3} $mol\,l^{-1}$; NaOH: 3.3×10^{-1} $mol\,l^{-1}$.

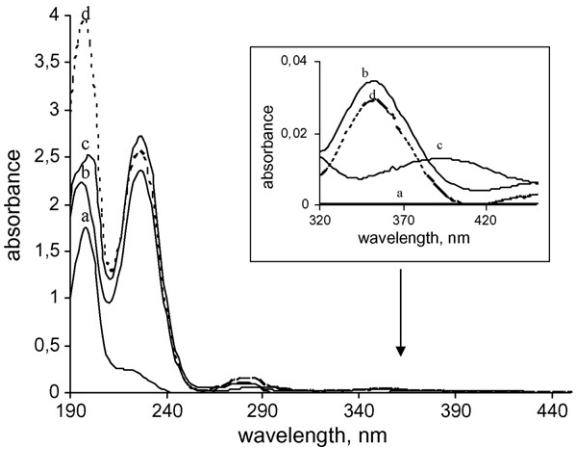

Fig. 3. UV–vis absorption spectra: (a) dopamine; (b) I_2; (c) dopamine + I_2; (d) algebraic sum of individual absorption spectra a and b. Dopamine: $5\,\mu g\,ml^{-1}$; I_2 (in 4.82×10^{-4} $mol\,l^{-1}$ KI): 9×10^{-6} $mol\,l^{-1}$; blank: water.

3-aminophtalate formed in the reaction [24]. Norepinephrine, epinephrine, dopamine and L-dopa were observed to inhibit the CL from the luminol–I_2 system. To determine the reaction parameters that gave the optimum signal for each catecholamine, a series of univariate searches were performed on the flow rate, volume of injected sample, length of mixing coils and the concentration of reagents and sodium hydroxide. All these parameters were optimized for three different concentrations of catecholamines in the linear range of the calibration curve with respect to the sensitivity on the basis of the signal height and the ratio of the signal height to the noise (S/N).

The influence of the concentration of luminol on the chemiluminescent reaction was tested over the range 1.0×10^{-4} to 8.0×10^{-4} $mol\,l^{-1}$ (Fig. 4A). The optimal concentration of luminol was slightly different for different catecholamines. A concentration of 5.0×10^{-4} $mol\,l^{-1}$ of luminol was found to be suitable for dopamine, 7.0×10^{-4} $mol\,l^{-1}$ for L-dopa, epinephrine and norepinephrine.

The chemiluminescent reaction of luminol with iodine takes place in the alkaline solution. The concentration of sodium hydroxide in the luminol solution has a strong influence on the efficiency of chemiluminescence of luminol and therefore the inhibiting signal of CAs (ΔI). The effect of sodium hydroxide concentration on the chemiluminescence intensity and the signal-to-noise ratio was studied in the range 2.5×10^{-2} to $2.5\,mol\,l^{-1}$. With the decrease in the sodium hydroxide concentration, both the chemiluminescence of luminol and the background noise value increased. However, when the concentration was too low (in the range from 2.5×10^{-2} to 10^{-1} $mol\,l^{-1}$) L-dopa and epinephrine exhibit no signal. When the concentration of sodium hydroxide was $1.0\,mol\,l^{-1}$, the signal-to-noise ratio reached a maximum value for all the compounds tested (Fig. 4B). Therefore, this concentration of sodium hydroxide was chosen as the optimal one.

The inhibition efficiency appears to be strongly dependent on the concentration of oxidant. The influence of the concentration of iodine prepared in 4.8×10^{-4} $mol\,l^{-1}$ of KI was investigated in the range 5×10^{-7} to 3×10^{-5} $mol\,l^{-1}$.

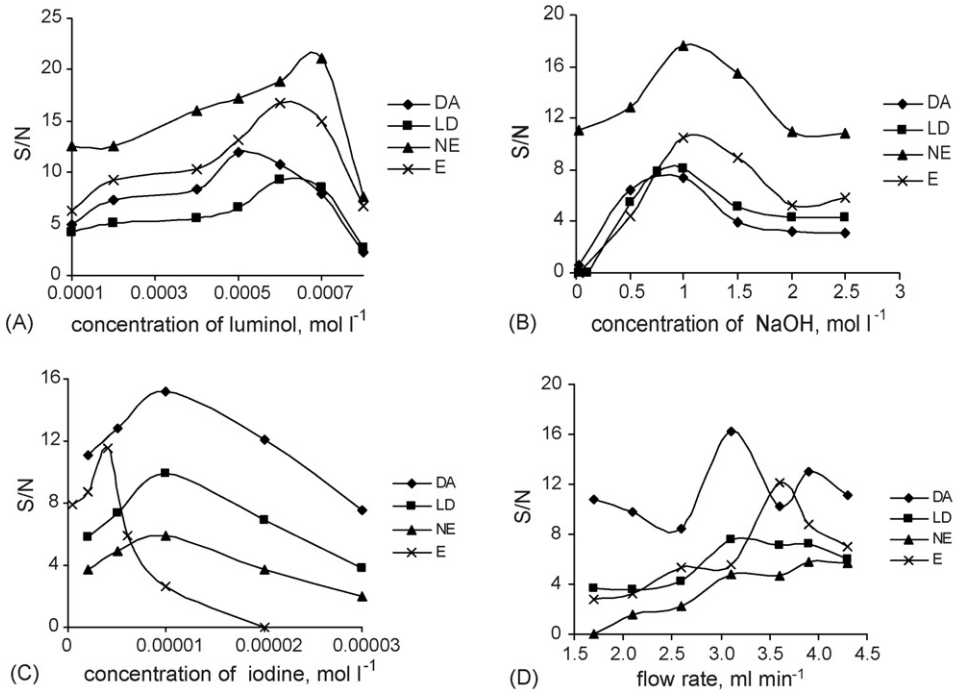

Fig. 4. Optimization of the flow-injection system for CAs determination: (A) concentration of luminol; (B) concentration of sodium hydroxide; (C) concentration of iodine; (D) flow rate. DA: dopamine; LD: L-dopa; NE: norepinephrine; E: epinephrine. Concentration of each catecholamine was 3.5 $\mu g\,l^{-1}$.

Although ΔI increased apparently with the increase in the concentration of oxidant, the background noises also increased significantly. The signal-to-noise ratio reached its maximum when 4×10^{-6} mol l^{-1} of iodine was used for the determination of epinephrine and 9×10^{-6} mol l^{-1} for the remaining three catecholamines (Fig. 4C).

Furthermore, the effect of the flow rate varied from 1.7 to 4.3 ml min^{-1}. The result showed that the negative peak ΔI increased with the increase of the flow rate from 1.7 to 3.1 ml min^{-1}; beyond 3.1 ml min^{-1} the differences were not relevant. However, the highest signal-to-noise ratio was observed when the flow rate was kept at 3.1 ml min^{-1} in the case of dopamine and L-dopa, 3.6 ml min^{-1} for epinephrine and 3.9 ml min^{-1} for norepinephrine (Fig. 4D).

The response of the system was studied by varying the volume of sample injected from 200 to 900 μl. The negative peak height increased slightly with an increase in the sample volume and reached plateau at 600 μl. Thus, 600 μl volume was selected as an optimal sample loop for all the catecholamines tested in this work.

As a final step, the influence of the length of two mixing coils (RC_1 and RC_2) was investigated. The effect of the length of RC_1 coil, which evidently improves the efficiency of the chemiluminescent reaction between luminol and iodine, was examined in the range 0.08–11.5 m. Shortening the reactor length below 1.0 m resulted in the lower inhibition effect of the compounds studied (except epinephrine). Considering the maximum value of ΔI and S/N ratio, a 2 m length of RC_1 coil was established as optimal for the determination of dopamine, L-dopa, epinephrine and 4 m for norepinephrine. The influence of the length of mixing coil RC_2 was examined in the range of 19–150 cm. With the increase in the length, the negative peak height decreased remarkably in the case of epinephrine and norepinephrine and ΔI reached a maximum when the distance to the CL detector was as short as possible. Thus 19 cm was chosen as the optimal value. The results obtained for dopamine and L-dopa showed that the negative peak height was almost stable in the range of 19–70 cm and the decrease of ΔI was observed at values higher than 70 cm. Because of the highest value of ΔI, 48 and 58 cm was judged to be the optimal length of the RC_2 coil for the detection of L-dopa and dopamine, respectively.

3.2.2. Analytical performance characteristics

The performance method was evaluated under optimal detection conditions by the determination of the linearity, detection limits, precision, reproducibility and sample throughput. The

Table 1
Analytical data for determination of norepinephrine, epinephrine, dopamine and L-dopa in flow unit with CL detection

Sample	Linear range ($\mu g\,l^{-1}$)	Slope ± S.D.	Intercept ± S.D.	Correlation coefficient (r)
Norepinephrine	1.1–20.0	−18.00 ± 0.01	−17.05 ± 0.07	0.9983
Epinephrine	0.5–5.0	−7.37 ± 0.05	−0.26 ± 0.01	0.9973
Dopamine	0.6–9.0	−42.68 ± 0.08	−21.95 ± 0.07	0.9963
L-Dopa	0.6–10.0	−28.19 ± 0.17	−7.58 ± 0.27	0.9970

Table 2
The detection limits, sampling rate and precision of the flow-injection method with CL detection

Sample	Detection limit ($\mu g\,l^{-1}$)	Sampling rate (samples h^{-1})	Precision ($n = 15$), R.S.D. (%)
Norepinephrine	0.34	113	1.26
Epinephrine	0.15	93	3.03
Dopamine	0.18	82	2.21
L-Dopa	0.18	87	0.65

calibration curves were constructed by plotting the negative peak height (ΔI, nA) against the CAs concentration (C, $\mu g\,l^{-1}$) ($n = 6$). Linear correlation coefficients (r) were higher than 0.9963 for all the target compounds (Table 1). The detection limits of the investigated compounds (600 μl injection volume), defined as a signal-to-noise ratio of 3, are listed in Table 2. The precision of the method was evaluated by the analysis of 15 replicates of samples containing 5 $\mu g\,l^{-1}$ of each catecholamine (Table 2). The sampling rate was between 82 and 113 samples h^{-1}. The reproducibility obtained by preparing three independent calibration graphs in three different days resulted in an average slope of −17.17, −7.41, −43.90 and −29.26 and its reproducibility was (as R.S.D. in %) of 5.15, 7.24, 5.87 and 5.48 for NE, E, DA and LD, respectively. These results indicate that calibration graph should be prepared the same day the real sample is analysed.

In order to assess the selectivity of the method developed, the influence of the usual excipients and active principles used in pharmaceutical formulations containing catecholamines was investigated. Moreover, the effect of other catecholamines was also studied. The procedure consists of preparing synthetic solutions, each one containing a single catecholamine (4 $\mu g\,l^{-1}$) and different concentrations of only one interferent. Later, the CL signal of these solutions using the conditions fixed in Manifold A was measured. The criterion for interferences was a relative error of less than ±5% of the average CL signal corresponding to the standard solution of catecholamine (4 $\mu g\,l^{-1}$) containing no foreign substances. Table 3 shows the maximum concentrations of each interferent tested which causes no serious interference. Only catecholamines showed significant interference but they are not present together in pharmaceutical preparations.

3.2.3. Method application

Following the procedure described in Section 2.4, the proposed method was successfully applied to the analysis of epinephrine, dopamine and L-dopa in pharmaceutical dosage forms. The results given in Table 4 are in good agreement with the nominal contents. The results obtained using the proposed

Table 3
Acceptable concentrations of the interfering species in epinephrine, dopamine, norepinephrine and L-dopa determination

Interferent	Dopamine[a]		L-Dopa[a]		Norepinephrine[a]		Epinephrine[a]	
	$\mu g\,l^{-1}$	Interference effect (%)	$\mu g\,l^{-1}$	Interference effect (%)	$\mu g\,l^{-1}$	Interference effect (%)	$\mu g\,l^{-1}$	Interference effect (%)
NaCl	10,000	2.0	10,000	1.4	10,000	2.8	10,000	4.1
$CaCl_2{\cdot}6H_2O$	10,000	3.2	10,000	1.4	10,000	1.4	10,000	3.9
$Na_2B_4O_7{\cdot}10H_2O$	10,000	1.0	10,000	0.2	10,000	0.9	10,000	2.1
Lactose	10,000	1.9	10,000	1.3	10,000	0.3	10,000	3.2
EDTA	10,000	0.9	10,000	1.2	10,000	4.6	7,000	3.4
Sodium citrate	10,000	2.2	10,000	1.2	10,000	2.2	4,000	2.1
Formaldehyde	10,000	2.0	10,000	3.8	10,000	2.8	3,000	3.6
HCl	10,000	3.8	10,000	2.2	10,000	0.4	3,000	3.5
Glucose	10,000	4.9	10,000	3.8	10,000	3.1	2,000	3.2
$NaHSO_3$	70	3.8	30	3.8	30	4.8	5	4.5
Ascorbic acid	15	4.9	15	4.4	15	3.8	1	4.2
Benserazide hydrochloride	–	–	4	4.2	–	–	–	–
$Na_2S_2O_3{\cdot}5H_2O$	12	4.9	2	0.7	4	4.8	7	3.9
Norepinephrine	4	4.3	0.5	4.5	–	–	1	3.5
Dopamine	–	–	0.3	4.9	0.2	4.2	0.3	4.1
Epinephrine	9	4.7	4	4.0	5	3.5	–	–
L-Dopa	4	4.2	–	–	0.5	4.8	2	4.8

[a] Concentration of each catecholamine was 4 $\mu g\,l^{-1}$.

Table 4
Determination of epinephrine, dopamine and L-dopa in pharmaceutical preparation samples by the proposed method and the official method

Pharmaceutical preparation	Analyte	Labeled value (mg)	Found ± S.D. (mg) ($n = 3$)		Relative error ± S.D. (%) ($n = 3$)	
			Official method	Proposed method	RE_1	RE_2
Injec. Adrenalini 0.1%	Epinephrine	1	0.975 ± 0.002	1.020 ± 0.026	2.00 ± 1.58	4.08 ± 0.20
Dopaminum Hydrochloricum	Dopamine	200	199.5 ± 3.9	198.0 ± 3.6	1.00 ± 1.78	−0.75 ± 1.79
Madopar	L-Dopa	50	50.69 ± 0.88	49.99 ± 1.75	−0.04 ± 3.52	−1.38 ± 3.45

RE_1: proposed method vs. labeled value and RE_2: proposed method vs. official method.

Table 5
Recovery of epinephrine, dopamine and L-dopa from pharmaceutical preparation samples

Pharmaceutical preparation	Standard catecholamine	mg per ampoule/capsule		Recovery ± S.D. (%) ($n = 3$)
		Added	Found ± S.D. ($n = 3$)	
Injec. Adrenalini 0.1%	Epinephrine	0.50	0.50 ± 0.01	100.0 ± 2.6
		1.00	1.04 ± 0.02	104.0 ± 2.0
		1.50	1.51 ± 0.05	100.7 ± 3.1
Dopaminum Hydrochloricum	Dopamine	200	201.7 ± 4.5	100.9 ± 2.3
		300	299.7 ± 10.5	99.9 ± 3.5
		400	398.8 ± 8.4	99.7 ± 2.1
Madopar	L-Dopa	50	50.5 ± 1.4	101.0 ± 2.7
		100	100.8 ± 2.0	100.8 ± 2.0
		150	151.3 ± 4.4	100.9 ± 2.9

method were also compared with those obtained from the reference method recommended by the USP (epinephrine, dopamine) [25] and BP (L-dopa) [26] Pharmacopoeia. It was observed that the differences among the methods are insignificant at the 95% probability level (F- and t-test) [27]. However, the proposed method is faster and allows the analysis of each catecholamine in a very short time. Recovery studies were also performed on each of the pharmaceutical samples analysed by adding a known amount of standard catecholamine to the sample before the recommended treatment. The obtained mean recoveries were in the range of 99.7–104.0% (Table 5), indicating that the proposed FI method is accurate.

3.3. Flow unit with CL detection coupled to the HPLC system

The use of CL detection for the analysis of complex biological samples, is connected with the lack of selectivity and requires isolation and separation procedures to be applied for the assay of CAs in urine samples. For this purpose, the method of Grossi et al. [22] for the HPLC determination of CAs was adopted. The Manifold A (Fig. 1A) used for the determination of dopamine was employed for optimizing the reaction conditions for the most efficient post-column CL detection. In order to check if the selected mobile phase is compatible with the post-column reaction, it was used as a carrier stream instead of water in the FI system. Changing the carrier stream resulted in a decrease of CL intensity of the luminol reaction system and the height of inhibiting signals. Introduction of an acidified mobile phase containing modifiers which strongly reduced CL emission of luminol made it necessary to decrease the flow rate of the carrier stream to 1 ml min^{-1} which in consequence diminished this effect. It was also found that decreasing the flow rates of reagents to 2 ml min^{-1} resulted in a better signal-to-noise level and better precision of the method performed. The study to select the suitable configuration of the FI system consisted also in testing the influence of the length of reaction coils (RC_1 and RC_2). Considering the maximum quenching effect and low noise level, two spirals, one with a length of 2 m (RC_1) and the second of 48 cm (RC_2) were chosen as an optimal. The studies also showed that changing the concentrations of reagents did not improve the efficiency of the CL intensity. Such a modified set-up was connected with the HPLC column (Fig. 1B) and a further optimization procedure was performed. A rapid and complete separation of the mixture of CAs was achieved by the use of RP-C_8 column. Compared to the RP-C_{18} column used by Grossi et al. [22], this one offered better reproducibility of the retention times of CAs with R.S.D. in the range 0.3–0.5%. The final step of the optimization for the post-column CL determination was to adjust the volume of the injected sample to obtain maximum inhibiting signals of the CAs eluted from the column. A 200 μl sample loop was chosen as an optimal. This set of the optimized parameters was applied for the post-column determination of all investigated CAs.

3.3.1. Analytical performance characteristics

Under the optimum conditions described above, working curves of the three CAs were obtained. It was noted that in the case of DA plotting the peak area instead of peak height versus the DA concentration of the standards (C, μg l^{-1}) ($n = 6$) resulted in better accuracy (confirmed by analysing the control urine sample) and precision (as R.S.D. in %) of the method developed. It is probably due to the fact that DA gave much broader peaks than other CAs. In the case of NE and E the calibration curves

Table 6
Analytical data for determination of norepinephrine, epinephrine and dopamine in flow unit with CL detection coupled to the HPLC system

Sample	Linear range (μg l^{-1})	Slope ± S.D.	Intercept ± S.D.	Correlation coefficient (r)
Norepinephrine	5.0–72.0	−0.56 ± 0.01	−1.41 ± 0.04	0.9989
Epinephrine	5.0–48.0	−0.37 ± 0.01	−1.83 ± 0.07	0.9968
Dopamine	5.0–96.0	−12.3 ± 0.1	−26.8 ± 0.6	0.9981

Table 7
Determination of catecholamines in control urine samples

Sample	Declared value ($\mu g\,l^{-1}$)		Determined value ($\mu g\,l^{-1}$)	
	Minimum–maximum	Mean	Minimum–maximum	Mean ± S.D. ($n=5$)
Norepinephrine	50.9–76.3	63.6	60.6–67.2	64.5 ± 2.7
Epinephrine	9.9–14.9	12.4	11.4–13.9	13.0 ± 1.1
Dopamine	165.0–247.0	206.0	207.2–218.7	214.0 ± 4.5

Table 8
The contents of catecholamines in urine samples

Sample	Mean ± S.D. ($\mu g\,l^{-1}$) ($n=3$)		R.S.D. (%)	
	Sample 1	Sample 2	Sample 1	Sample 2
Norepinephrine	45.2 ± 1.9	67.4 ± 1.8	4.22	2.66
Epinephrine	23.3 ± 0.1	23.3 ± 1.3	0.35	5.63
Dopamine	195.8 ± 7.9	208.4 ± 4.1	3.89	1.95

obtained by plotting the peak height (NE, E) versus the CA concentrations of the standards (C, $\mu g\,l^{-1}$) ($n=6$) gave satisfactory results. Linearity was obtained with r values higher than 0.9968 for all the tested CAs (Table 6). The detection limits for NE, E and DA were 0.71, 0.26 and 0.73 $\mu g\,l^{-1}$, respectively, per 200 μl injection volume at signal-to-noise ratio of 3. Precision of the method was established by repeated determinations ($n=4$) using normal human urine. The relative standard deviation values for NE, E and DA (44, 24 and 207 $\mu g\,l^{-1}$) in urine were 4.61, 4.68 and 2.05%, respectively. The reproducibility of the method was evaluated by the analysis of three independent calibration graphs of each CA in three different days. An average slope was −0.54, −0.36 and −11.56 with reproducibility (expressed as R.S.D. in %) of 5.82, 6.63 and 5.61 for NE, E and DA, respectively. Due to the fact the relative standard deviation is higher than 5% the calibration curve should be prepared the same day that the real sample is analysed.

The accuracy of the method was verified by the analysis of a lyophilised control sample based on human urine obtained from Chromsystems. The concentrations of NE, E and DA in the control urine samples were within the physiological range: 63.6, 12.4 and 206 $\mu g\,l^{-1}$, respectively. The results obtained by the proposed method are satisfactory and the mean value corresponds well with the declared amounts (Table 7).

3.3.2. Application to human urine samples

Under optimal separation conditions, the determination of catecholamines in the samples of urine from the young investigators (two females) was demonstrated. A representative chromatogram of the standard mixture of CAs and normal urine sample is shown in Fig. 5. The peaks were identified by comparing the retention times (6.8 min NE, 7.6 min E and 11.3 min DA) and addition of CAs standard solutions under exactly the same conditions. Combination of HPLC with CL detection enables to determine catecholamines on the $\mu g\,l^{-1}$ level in the presence of big excess of other constituents of complex urine sample. The results obtained through our newly developed HPLC-CL method are shown in Table 8.

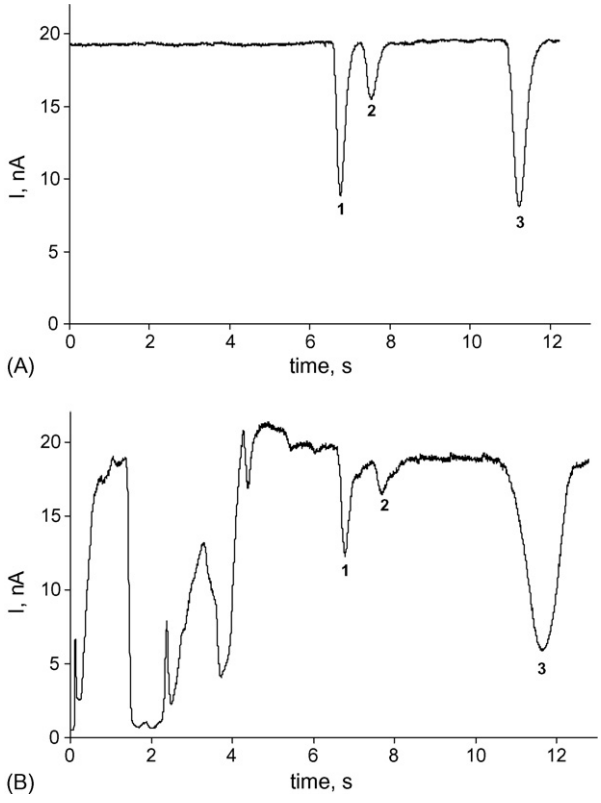

Fig. 5. (A) Chromatogram of the standard mixture of CAs (96 $\mu g\,l^{-1}$ NE, 84 $\mu g\,l^{-1}$ E, 96 $\mu g\,l^{-1}$ DA) and (B) chromatogram of normal urine sample after solid-phase extraction pre-treatment. Peaks—1: norepinephrine; 2: epinephrine; 3: dopamine.

4. Conclusion

The inhibition effect of catecholamines on the CL reaction of luminol with iodine in alkaline solution is the basis of these new FI and HPLC procedures. As far as we know, the proposed detection system has not been exploited in the determination of catecholamines. The principal advantage of the proposed method, among those already existing methods employing lumi-

nol as an luminescent reagent, is its low detection limit. What is more, the proposed procedures are simple, rapid and sufficiently sensitive for the determination of reported catecholamines in the pharmaceutical preparations and urine samples. According to the low concentrations of catecholamines in plasma samples they cannot be detected by the proposed method.

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