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Determination of Epinephrine by Flow-Injection Analysis Using Luminol–Hexacyanoferrate(III) Chemiluminescence Detection

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A novel flow-injection method for the determination of epinephrine in pharmaceutical preparations has been proposed. Detection of the drug is based on the enhancement of chemiluminescence of the luminol–potassium hexacyanoferrate(III) system by epinephrine in the alkaline medium. Linear calibration plot is observed for epinephrine concentration ranging from 20–260 μg L^{-1}. The detection limit was 12 μg L^{-1} and the relative standard deviation was 0.9% for 140 μg L^{-1} epinephrine (n = 22). The sampling throughput of 160 samples h^{-1} was achieved. The proposed method has been successfully applied to the determination of epinephrine in pharmaceutical injections.

Opracowano nową wstrzykowo-przepływową metodę oznaczania epinefryny w preparatach farmaceutycznych. Detekcja oparta była na pomiarze chemiluminescencji układu luminol–$Fe(CN)_6^{3-}$ w środowisku zasadowym, wzmacnianej przez analit. Krzywa kalibracyjna wykazywała liniowy przebieg w zakresie stężeń epinefryny: 20–260 μg L^{-1}, granica wykrywalności wynosi 12 μg L^{-1} a częstotliwość próbkowania 160 h^{-1}. Względne odchylenie standardowe wyznaczone dla stężenia epinefryny 140 μg L^{-1} wyniosło 0.9% (n = 22). Opracowaną metodę zastosowano z dobrym rezultatem do oznaczania epinefryny w zastrzykach.

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Epinephrine is an exemplary catecholamine acting as neurotransmitters in the central nervous system. Moreover, they are important markers for several diseases and possess important pharmacological properties. Hence sensitive methods for their determination in biological fluids and pharmaceutical preparations are needed. Epinephrine (adrenaline [1-(3,4-dihydroxyphenyl)-2-methyloaminoethanol]) is extensively employed in the treatment of cardiac arrest, bronchial asthma, cardiac surgery, myocardial infraction and glaucoma [1]. In recent years, several methods for the determination of epinephrine and other catecholamines in pharmaceutical preparations have been reported. These methods utilise flow injection analysis (FIA) with spectrophotometric [1–6], spectrofluorimetric [7–9], amperometric [10], biamperometric [11–12] and piezoelectric detection [13]. Chemiluminescence (CL) detectors have been also successfully applied for this purpose. This kind of detection is highly sensitive and is easily coupled with the FIA system to provide a fast, cheap, simple and precise method for the determination of pharmaceuticals, including epinephrine. A few CL methods are based on the measurement of chemiluminescence produced by the direct oxidation of epinephrine by different oxidant reagents [14–16]. Deftereos *et al.* [14] used permanganate in acidic medium as an oxidant in the presence of for-maldehyde as a sensitiser. Matsue *et al*. [15] oxidised epinephrine using oxygen dissolved in alkaline solution using dioctadecyldimethylammonium chloride bilayer mem-brane vesicles as a sensitiser in the presence of manganese(II) as a catalyst. Also the unstable hypobromide was generated on-line by the hydrolysis of N-bromosuccinimide in alkaline medium and used for the oxidation of epinephrine [16].

Luminol CL reaction has been applied to the indirect determination of epinephrine [17–19]. Zhang *et al.* [17] proposed a method based on the inhibition of the intensity of the luminol-hypochlorite CL by epinephrine. Hypochlorite is an unstable oxidant, and it was generated electrochemically on-line in the FIA system. An sensitizing effect of epinephrine on the weak CL emission of the electrochemically-oxidised luminol was used by Zheng *et al.* [18]. Michałowski and Hałaburda described a method based on the enzymatic oxidation of epinephrine in the presence of alkaline solution of luminol [19]. They employed raw apple juice containing polyphenol oxidase as a carrier stream in the FIA system. Another procedure was based on the decomposition of epinephrine in the presence of imidazole as a catalyst. The generated hydrogen peroxide was detected in the CL reaction with luminol, catalysed by horseradish peroxidase [20]. Recently, the inhibiting effect on the chemiluminescence of $Ru(bpy)_3^{2+}$/tripropyloamine system was used to detect the former [21].

It is the well known fact that the mixture of hexacyanoferrate(III) and luminol in alkaline solution produces strong CL, which can be enhanced or inhibited by polyphenols [22–24], phenolic acids [25,26] and reducing organic agents [27]. Epinephrine was found to be another enhancing agent. In its presence, chemiluminescence signal

of the above system increases linearly as the epinephrine concentration rises. This has been used in the new flow injection CL method for the determination of epinephrine.

EXPERIMENTAL

Reagents

All the reagents were of analytical grade. Doubly distilled water was used throughout. Epinephrine and luminol were purchased from Sigma (USA). Other compounds were obtained from POCH (Poland).

Stock solution of epinephrine (1000 mg L^{-1}) was prepared in acetic buffer (pH 3.48) and stored at 4°C in a refrigerator to minimise the exposure to light and air. Working standard solutions were prepared daily by an appropriate dilution of the stock solution with water to obtain the concentrations of 20–260 μg L^{-1}.

Luminol stock solution (2×10^{-2} mol L^{-1}) was prepared by dissolving the appropriate amount of the compound in 1.8 mol L^{-1} sodium hydroxide solution. It was stored in darkness. Further dilutions were performed with 1.8 mol L^{-1} NaOH containing 2.5 mol L^{-1} potassium hexacyanoferrate(II).

Potassium hexacyanoferrate(III) and potassium hexacyanoferrate(II) solutions were prepared by dissolving pure compounds in water and 1.8 mol L^{-1} NaOH solution, respectively. More dilute solutions were obtained by appropriate dilutions of the stock solutions.

Apparatus

The scheme of the flow injection system used in this work is presented in Figure 1. The system comprises an Ismatec MS–Reglo peristaltic pump, a rotary injection valve (Model 5021, Rheodyne, Cotai, CA) and flow luminometer (KSP, Poland) with a coiled PTFE tube of 1 mm I.D. (length of 25 cm in 6 windings) as the flow cell. Photomultiplier was operated at 750 V, and detector response was computer-recorded using software provided by the manufacturer of the luminometer. The flow system was made of 0.8 mm-in-I.D. PTFE tube. Reagent and carrier streams were merged in a Perspex T–piece.

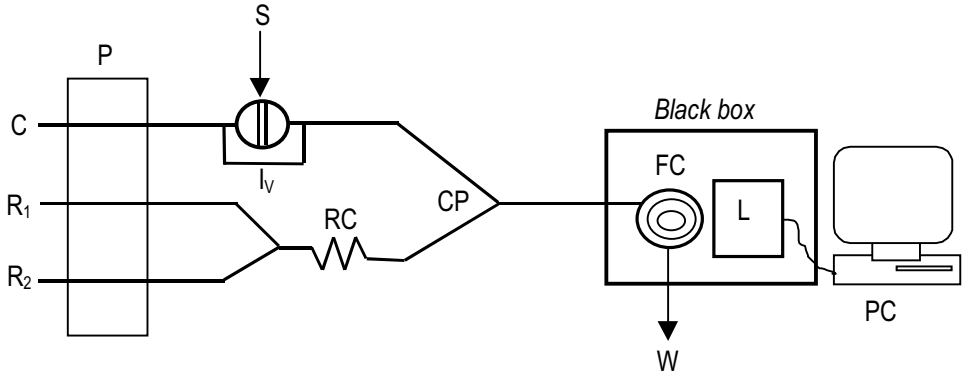

Figure 1. Schematic diagram of the flow-injection system for epinephrine determination; P: peristaltic pump; C: water carrier stream; R_1: potassium hexacyanoferrate(III) solution; R_2: luminol and potassium hexacyanoferrate(II) in sodium hydroxide solution; RC: mixing coil; S: sample; I_V: injection valve; L: luminometer; FC: flow cell; PC: computer; CP: confluence point; W: waste

Absorption spectra were obtained using a diode array spectrophotometer, Model 8452A (Hewlett–Packard, Germany).

RESULTS AND DISCUSSION

The optimisation of chemical variables (the concentration of luminol, sodium hydroxide, potassium hexacyanoferrate(III) and potassium hexacyanoferrate(II)) and instrumental variables (volume of injected sample, length of mixing coil, flow rate of solutions) was performed using the univariate optimisation procedure (changing one variable in every turn and keeping the others at their optimum values). All these parameters were optimised for 70, 130 and 190 μg L^{-1} of epinephrine concentration with respect to the sensitivity and reproducibility on the basis of the peak height of CL signal and peak height to noise ratio.

The chemiluminescence reaction of luminol occurs in the alkaline solution. The effect of luminol concentration on its chemiluminescence reaction was examined within the range of 5.0×10^{-4}–3.5×10^{-3} mol L^{-1} with the NaOH concentration fixed at 1.8 mol L^{-1}.

The maximum CL intensity was observed at 2.0×10^{-3} mol L^{-1} (Fig. 2A) and this value was selected for further studies. Next, the effect of NaOH concentration on the chemiluminescence of luminol was examined in the range of 1.2–2.0 mol L^{-1}. As shown in Figure 2A, the strongest CL signal was observed at 1.8 mol L^{-1} and this concentration of NaOH was finally chosen.

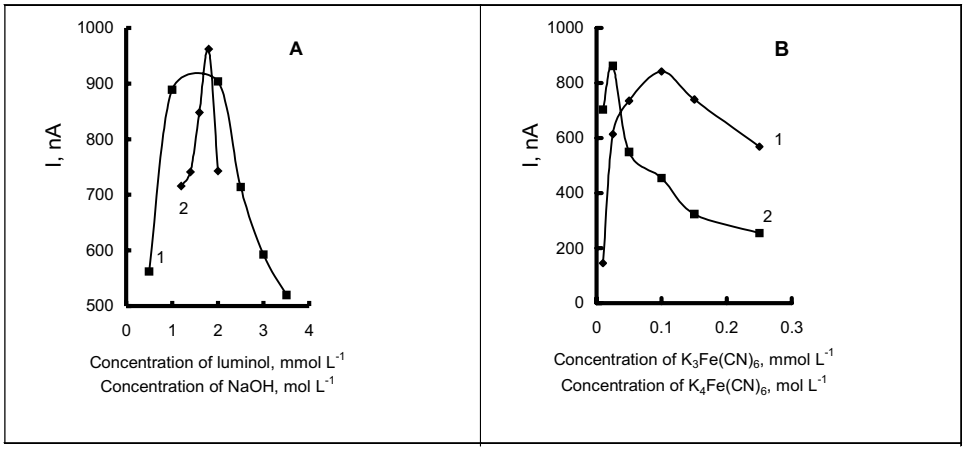

Figure 2. Optimisation of the flow-injection system for epinephrine determination; A: concentration of luminol (1) and sodium hydroxide (2); B: concentration of potassium hexacyanoferrate(III) (1) and potassium hexacyanoferrate(II) (2); C: the effect of the length of the reaction coil RC; D, E: the effect of the flow rate of R_1, R_2 (1) and carrier (2) streams; F: the effect of sample volume; epinephrine concentration: 130 μg L^{-1}. (Continuation on the next page)

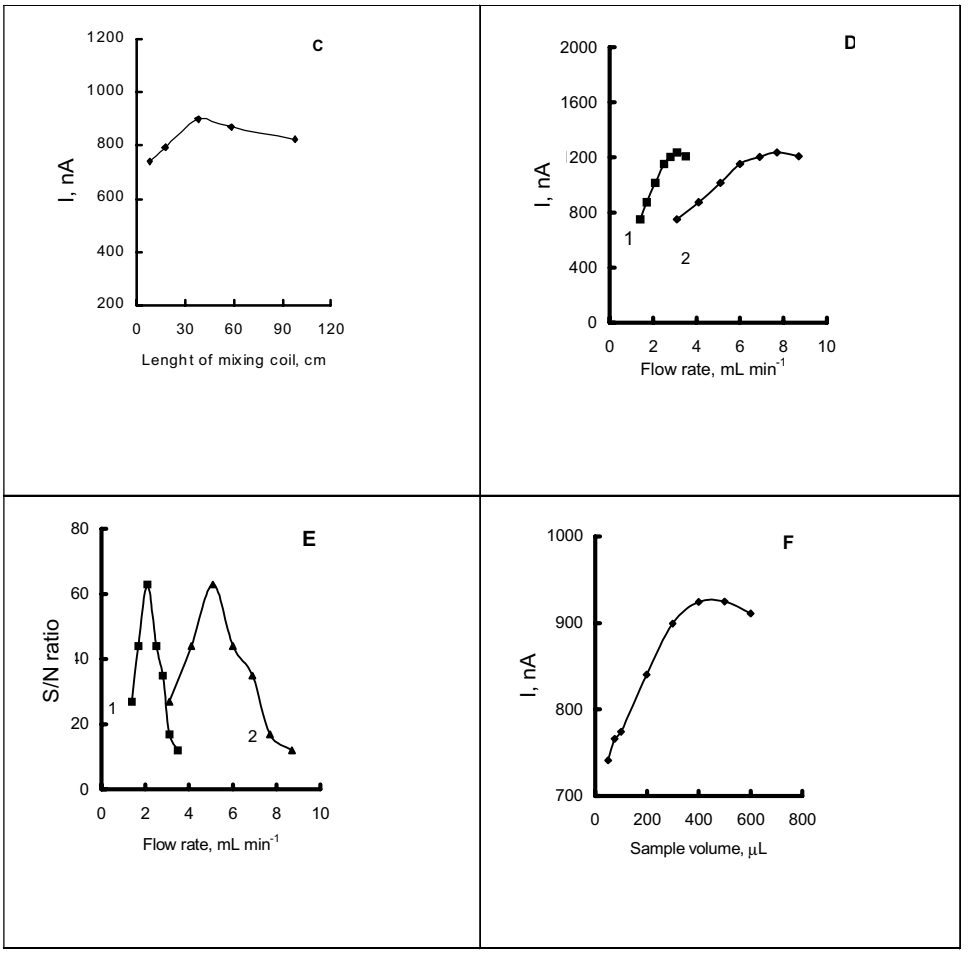

Figure 2. (Continuation)

Luminol oxidation in the alkaline medium in the presence of hexacyanoferrate(III) is accompanied by strong chemiluminescence, however affected by high noise. The background signal intensity is strongly decreased by addition of potassium hexacyanoferrate(II) to the system [24,27]. Therefore the influence of potassium hexacyanoferrate(III) and potassium hexacyanoferrate(II) concentrations on the measured CL signals was studied. The obtained results are presented in Figure 2B. The maximum CL intensity was observed in the presence of 0.1 mmol L^{-1} and 2.5×10^{-2} mol L^{-1} potassium hexacyanoferrate(III) and potassium hexacyanoferrate(II), respectively. The background signal was decreased about six-fold in the presence of potassium hexacyanoferrate(II) in the luminol stream.

To ensure the efficient chemiluminescence reaction between luminol, potassium hexacyanoferrate(III) and epinephrine, the flow injection system was equipped with a mixing coil. As a result, the CL intensity remarkably increased for the mixing coil length ranging between 7.5–38 cm, and above this range decreased slightly (Fig. 2C). Thus, the length of 38 cm was selected for further investigations. Due to the short time reaction, the distance between the confluence point (CP) and the detector cell had to be minimised. To obtain the best results, the distance should be less than 8 cm. The reaction between the reagents and the analyte occurred in the planar coil placed near the photomultiplier.

Flow rates of the solutions were critical parameters significantly influencing the CL intensity. Optimum flow rate allows the reaction to proceed for a suitable time, before the reagents enter the cell. Flow rates of the carrier, R1 and R2 streams were being examined in the ranges of 3.1–9.7 and 1.4–3.9 mL min^{-1}, respectively. Flow rates of R1 and R2 were kept equal. As the flow rate of the streams was increased, both the CL intensity of epinephrine (Fig. 2D) and the background noise increased. The signal-to-noise ratio reached its maximum for 5.1 and 2.1 mL min^{-1} flow rates of the carrier and both streams, respectively (Fig. 2E) and these values were maintained during further studies.

The sample injection volume was varied from 50 to 600 μL by changing the length of the sample loop in the injection valve. It was found that the peak height increased up to the sample volume of 400 μL. For larger samples a decrease in the peak height was observed (Fig 2F). A 400 μL sample volume was chosen as optimal.

Analytical parameters of the optimised system

Under the optimum conditions given above, the CL intensity (I, nA) was plotted *vs* epinephrine concentration (C, μg L^{-1}). The obtained calibration curve (regression equation: $I = 6.19C + 87.9$, $R^2 = 0.9921$) was linear in the range of 20–260 μg L^{-1}. The detection limit was assigned to the analyte's concentration, which produces the signal equal to that of the blank plus three times the standard deviations of the blank. It was found to be 12 μg L^{-1} [28]. The precision of the method was estimated for 22 injections of epinephrine at the concentration of 140 μg L^{-1}. Relative standard deviation (RSD) was 0.9%. In order to check the day-to-day reproducibility, three calibration graphs were obtained on different days, and their slopes were averaged. The mean slope was 6.44 with RSD = 4.9%. The sample throughput was 160 samples per hour.

Interference study

The influence of foreign compounds commonly accompanying epinephrine in pharmaceutical preparations was investigated. For this purpose a standard solution of epinephrine (0.14 μg mL^{-1}) containing different amounts of the added interfering spe-

cies was analysed. The obtained signals were compared to those of the epinephrine solution without any foreign substances. The effect of foreign compound was assumed to be tolerable if its presence caused a relative error less than 5%. The results presented in Table 1 indicate norepinephrine, dopamine and ascorbic acid as the only severe interferents. Usually only catecholamine is present in pharmaceutical preparations.

Table 1. Tolerable concentration ratios for some interfering compounds (with respect to the concentration of epinephrine)

Compound	Tolerable concentration ratio
EDTA, NaCl, $Na_2S_2O_3 \cdot 5H_2O$	>10 000
Sodium citrate	5 500
$CaCl_2$	7000
Lactose	1000
Glucose	850
$Na_2B_4O_7$, $NaHSO_3$	700
Formaldehyde	70
Ascorbic acid	1.0
Norepinephrine	0.1
Dopamine	0.004

Application to commercial product

The applicability of the developed method to the analyses of real samples was investigated. In the study a commercially available preparation Injec. Adrenalini 0.1% (POLFA, Warsaw) was utilised. Each ampoule of the injection solution contains 1 mg of epinephrine and 0.5 mg of $NaHSO_3$ in 1 mL NaCl solution of physiological concentration. The sample solution was appropriately diluted with acetic buffer in order to adjust the analyte's concentration to the linear calibration range. The samples were triply injected into the FIA system. No or very little interference from the antioxidant $NaHSO_3$ was observed. The obtained results (Tab. 2) were in excellent agreement with nominal contents, as well as with the values obtained by spectrophotometric method with sodium metaperiodate and m-aminophenol [29]. Recovery studies were performed by adding known amounts of epinephrine standards to the pharmaceutical preparation. Recovery values were calculated by comparing the results obtained before and after the addition of standard solutions. The results are given in Table 3.

Table 2. The results of epinephrine determination in pharmaceutical injection preparations

Sample[a]	1 ampoule content mg	FIA method found[b] mg	Error %	Spectrophotometric method found[b] mg	Error %
1.	1.0000	1.0007 ± 0.0208	0.07	0.9914 ± 0.0025	0.94
2.	1.0000	1.0226 ± 0.0041	2.26	0.9961 ± 0.0134	2.66
3.	1.0000	1.0253 ± 0.0032	2.53	0.9943 ± 0.0082	3.12

[a] Three different batches of the preparation.

[b] Averaged from three determinations ± standard deviation.

Table 3. Recoveries of epinephrine from pharmaceutical preparations

Sample	Added mg/ampoule	Found* mg/ampoule	Recovery %
Injec. Adrenalini	0.7500	0.7593 ± 0.0295	101.24
	1.0000	1.0081 ± 0.0065	100.81
	1.2500	1.1928 ± 0.0403	95.42

* Averaged from three determinations ± standard deviation.

Discussion of the possible chemiluminescence mechanism

It is well known that fast oxidation of luminol in the presence of hexacyanoferrate(III) in alkaline medium produces strong chemiluminescence. The reaction product, 3-aminophthalate, has been confirmed to produce chemiluminescence in the excited state. Some organic compounds (*e.g.* polyhydroxy phenols, phenolic acids) have been found to strongly enhance or inhibit chemiluminescence of the luminol – hexacyanoferrate(III) system [23–25,30]. CL and UV–VIS spectra of the „enhanced" and „unenhanced" reactions are independent of the organic compound used, which reveals that 3-aminophthalate being a luminophor of the luminol–hexacyanoferrate(III)–organic system.

In order to explain the possible reaction mechanism the spectra of luminol–hexacyanoferrate(III) and luminol–hexacyanoferrate(III)–epinephrine systems (Fig. 3) were recorded. The luminol–hexacyanoferrate(III) system exhibits two absorption peaks at 300 and 350 nm, similarly as the luminol–hexacyanoferrate(III)–epinephrine system, however in the latter case the absorbance is slightly larger. The absorption peak at 350 nm is characteristic of 3-aminophthalate, which is the luminophor of both chemiluminescence systems.

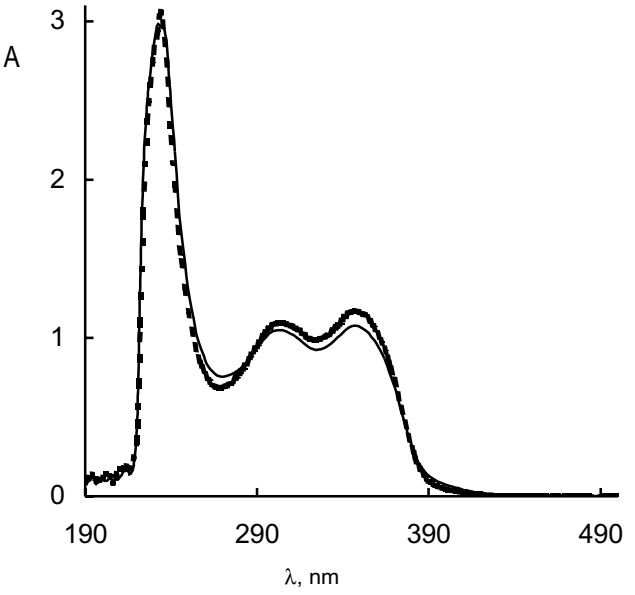

Figure 3. UV–VIS absorption spectra; 1: luminol–potassium hexacyanoferrate(III) system (thin solid line); 2: luminol–potassium hexacyanoferrate(III)–epinephrine system (dashed line). Luminol: 2×10^{-4} mol L^{-1} in 1.8 mol L^{-1} NaOH; potassium hexacyanoferrate(III): 2×10^{-4} mol L^{-1}; epinephrine 2.7×10^{-6} mol L^{-1}; blank: 1.8 mol L^{-1} NaOH

The CL intensity of the investigated system decreased by about 15% after deoxygenating of all the reagent solutions with the flow of nitrogen. The previous CL intensity was restored after saturation of the solutions with oxygen. Moreover, CL signal decreased by about 10% after the addition of sodium benzoate, which acts as a scavenger of reactive oxygen species. One may conclude that dissolved oxygen and reactive oxygen species participate in the CL reaction.

The formation of superoxide radical is an important intermediate process accompanying the reaction of luminol with hexacyanoferrate(III) in the alkaline medium [25]. The mechanism of „unenhanced" reaction involves the formation of luminol radical, which reacts with oxygen to produce superoxide anion ($O_2^{\cdot-}$), and subsequently to form luminol endoperoxide with superoxide anion. In the next stage, luminol endoperoxide displaces molecular nitrogen, producing the stable aminophthalate dianion in the excited state. The latter produces CL emission. Catecholamines, including epinephrine, can be oxidised to produce the superoxide anion radical by dissolved oxygen in alkaline solution [31]. Thus, in the studied system the enhancing effect of epinephrine on the CL emission can be attributed to the formation of additional quantities of $O_2^{\cdot-}$.

CONCLUSION

The flow-injection method for the determination of epinephrine has been proposed. It utilises the enhancement of CL signal of the luminol–potassium hexacyanoferrate(III) system by epinephrine. Compared with the already known CL FIA methods for the epinephrine determination in pharmaceutical preparations, the proposed procedure is more advantageous in respect of its rapidity, simplicity, low costs, high sensitivity, and selectivity over commonly used sulfite as antioxidant. The possible mechanism of the CL reaction has been also proposed.

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