ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 1017–1025

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Short communication

Oxidation of adrenaline and noradrenaline by solved molecular oxygen in a FIA assembly

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Received 28 May 2001; received in revised form 7 August 2001; accepted 28 August 2001

Abstract

A simple and effective procedure is proposed for the study and simultaneous determination of adrenaline and noradrenaline. The fluorimetric determination of both substances is performed in a flow injection assembly and by oxidation of both drugs with the solved molecular oxygen. The influence of different parameters is empirically studied and the interpretation of the reaction mechanism is also added. The determination of adrenaline is monitored at 450 nm and the outputs at 520 nm correspond to the adrenaline and noradrenaline global amount; for both lectures λ_{exc} 329 nm. The influence of temperature is relevant and analytical determination occurred at 55 °C by immersing the sample loop in a water bath. The linear range for adrenaline is over 0.5–20 μg ml^{-1}, limit of detection for both compounds is 0.2 μg ml^{-1}: the influence of foreign compounds as potential interferents is also tested; and, finally the procedure is applied to determination of both chatecolamines in synthetic samples.

Keywords: FIA; Adrenaline; Noradrenaline; Pharmaceuticals; Fluorimetry

1. Introduction

Epinephrine and norepinephrine are chatecolamines of clinical interest in the investigation of tumours of neurological origin. The oxidation of aqueous solutions of adrenaline (epinephrine) and noradrenaline (norepinephrine) in a basic environment results in a relevant change on the fluorescence intensity of drugs. The native and derivative fluorescence spectra of the molecules or its products overlap interfering. In analytical literature appears some examples of simultaneous determination of these compounds. In one of them [1], the determination of adrenaline and noradrenaline ratio was done by calculations using first and second derivative fluorimetric spectra. Other methods [2–4] involves second derivative synchronous fluorescence spectra after oxidation

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0731-7085/02/$ - see front matter
PII: S0731-7085(01)00610-0

of drugs to trihydroxyindoles. Two simultaneous kinetic determinations of adrenaline and noradrenaline by the stopped-flow technique were carried out [5,6]. The oxidation of both chatecolamines was monitored by measuring the initial rate of change in absorbance or fluorescence, respectively. A flow injection analysis (FIA) method for simultaneous determination of adrenaline and noradrenaline by first derivative spectrophotometry has been published [7]. Usually the determination is accomplished by a separation procedure, mainly chromatographic; there are a certain number of published works that make use of chromatographic techniques [8–11].

Binary mixtures of chatecolamines adrenaline and dopamine have been also analysed in different kind of samples and detectors like the piezoelectric detection of ion pairs [12], amperometry [13], derivative voltammetry [14] and liquid chromatography [15–19]. Ternary mixtures (noradrenaline, adrenaline and dopamine or mixtures of its derivatives) have been also subject of interesting publications, most of them dealing on chromatographic techniques [20–22]. Recently have been published papers dealing on the resolution of this mixture, adrenaline and noradrenaline, or with other compounds; all of them are dealing with in liquid chromatography methods [23–28] or in capillary electrophoresis [29,30].

In this work, we propose molecular oxygen as oxidant of those drugs maintaining a close control of pH and temperature and in a continuous-flow assembly. An earlier article from this team exploited for first time the oxidation of adrenaline by the solved molecular oxygen; the procedure was applied to fluorimetric determination of adrenaline in formulations containing not parent compounds; and, without studying the kinetics of the reaction. The present works studies the analytical behaviour versus the molecular oxygen of adrenaline and one very similar compound, the noradrenaline. The study revealed a very different kinetic behaviour which allowed to propose the simultaneous determination of both parent compounds. In the reported earlier work the noradrenaline was a serious interfering agent in the adrenaline determination.

The present paper put together two different kinetics for very similar compounds and describes the simultaneous determination of adrenaline and noradrenaline in mixtures without using oxidative chemicals (clean chemistry) and by exploiting the kinetic differences observed in the formation of two emission bands.

For this purpose, FIA is very useful mainly due to eminently kinetic character of this technique. In our case, the emission of one of products (an intermediate unstable species) occur very quickly and its intensity is transitory. A flow injection manifold permit us rapid and reproducible monitoring in a very short fixed time. The large number of papers dealing with FIA and pharmaceuticals demonstrated the excellent results [31] specially referred to reproducibility, simplicity, quickness and low cost.

2. Experimental

2.1. Reagents and apparatus

Adrenaline (Guinama, pure), noradrenaline (Sigma, pure), hydrochloric acid and sodium hydroxide (Panreac, a.r.); for interference study, lidocaine, novocain, tetracaine, benzocaine, tartaric acid, picric acid, salicylic acid, Peru balsam, hydroxyquinoleine-8-sulphate (all from Guinama, pure), boric acid, phenol, $Na_2S_2O_5$, $ZnSO_4$ (all from Panreac, a.r.) were used.

The stability of aqueous solutions of adrenaline and noradrenaline in hydrochloride media was tested by periodic recordings of the UV absorption spectra. Results are according to the reported elsewhere for adrenaline [32].

The assembly includes a Model 5041 sample injector (Rheodyne) and a Minipuls 2 peristaltic pump (Gilson). Fluorimetric measurements were made with a Model F-4500 Fluorescence Spectrophotometer (Hitachi) provided with a quartz flow-cell of 18 μl internal volume and 1 cm pathlength (Hellma). PTFE tube coils were of 0.5 mm internal diameter.

2.2. Continuous-flow procedure

Fig. 1b shows the manifold used. In a carrier of distilled water heated at 55 °C were inserted 333.6 μl of solution formed by the confluence of adrenaline and noradrenaline (in 0.1 mol l^{-1} hydrochloric acid) and 2.0 mol l^{-1} sodium hydroxide solutions. The mixture was heated at 55 °C in the sample loop immersed in a water-bath. The product of oxidation was led to the fluorimeter and the emission intensity read at 450 nm for determination of adrenaline concentration, and at 520 nm for determination of [noradrenaline]/[adrenaline] ratio and subsequently, noradrenaline concentration (in both cases, $\lambda_{ex} = 329$ nm).

2.3. Optimisation of experimental parameters

Optimisation of experimental parameters was carried out by the univariate method; the preliminary optimisation was based on the results obtained from an assembly previously proposed to determination of adrenaline in pharmaceutical formulations [32]. Two independent optimisation processes were required, each one to make the system suitable for measurements at two emission wavelengths employed.

The selection of optimum values was made mainly according to the selectivity, and the reproducibility.

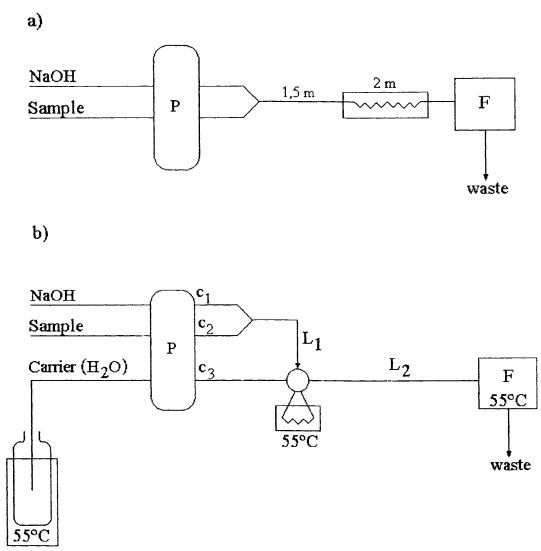

Fig. 1. Continuous-flow assemblies: (a) Continuous-flow (no injection of sample solution) for preliminary assays; (b) Proposed FIA assembly for the simultaneous determination of adrenaline and noradrenaline. Sample volume, 333.6 μl; heating time of the sample in the sample-loop, 10 min; flow–rate ratio NaOH/sample solution, 0.8 and 0.5 at 450 nm and 520 nm, respectively.

3. Results and discussion

3.1. Preliminary investigations

Preliminary experiments were carried out to study the evolution of emission intensity of adrenaline and noradrenaline when oxidised. For this purpose, a solution of drug (25 ml, 10 μg ml^{-1}) and NaOH (25 ml, 1.5 mol l^{-1}) was prepared and heated at 59 °C. The reaction was monitored by recording different spectra from this solution every 6 s for 4 min. The results for adrenaline are depicted in Fig. 2. Adrenaline showed a very fast appearance of a band at 520 nm that decreased quickly (in 60 s). Simultaneously, a new band of emission with the maximum at 450 nm appeared; it was raising in intensity until reached a constant value from 3.5 min. For noradrenaline, the emission band at 450 nm was not observed at all and the other one at 520 nm was 20 times smaller than the adrenaline (Fig. 3).

At a subsequent stage, we prepared mixtures of adrenaline (10 μg ml^{-1}) and noradrenaline in different concentrations (from 5 to 20 μg ml^{-1}) and recorded the evolution of reaction in the same way. We confirmed that emission intensity at 450 nm only depends on adrenaline concentration being independent of noradrenaline amount, up to a ratio of concentrations 1:1. However, emission intensity of adrenaline at 520 nm was seriously decreased in presence of increasing amounts of noradrenaline. A linear behaviour between emission intensity and [noradrenaline]/[adrenaline] concentrations ratio was observed.

After, we studied the influence of the amount of dissolved oxygen on reaction. For this purpose, solutions were treated as follows: in order to increase the amount of oxygen present, com-

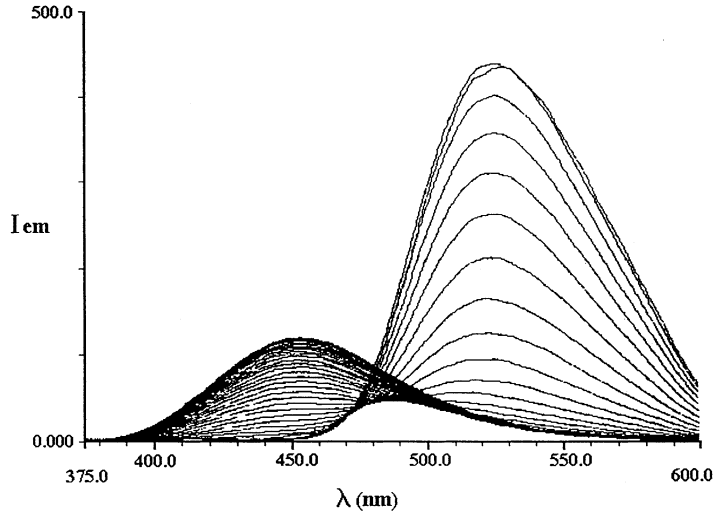

Fig. 2. Fluorimetric spectra of adrenaline versus time. Spectra obtained each 6 s up to 4 min. Temperature 59 °C. The tested solution was prepared by mixing 25 ml containing 10 μg ml^{-1} of adrenaline with 25 ml 1.5 mol l^{-1} of sodium hydroxide.

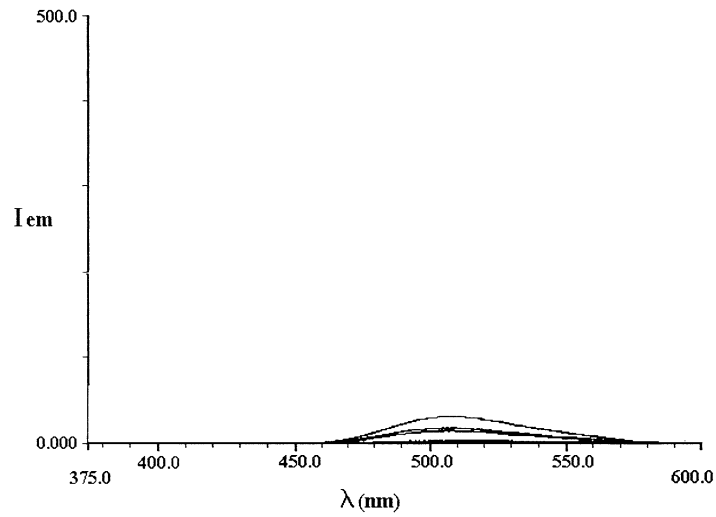

Fig. 3. Fluorimetric spectra of noradrenaline versus time. Id. Fig. 2.

pressed air was bubbled through solutions for 30 min, with stirring. A different procedure was used for decreased oxygen concentrations. Thus, solutions were nitrogen bubbled through them for 30 min, with stirring too.

In the former case (with increasing the amount of solved oxygen) the same evolution on the fluorimetric spectra and their intensity was observed for adrenaline, noradrenaline solutions and mixtures; only this evolution was faster.

A drastic decrease on reaction rate was observed when oxygen was removed by solutions. Specifically, the emission band at 450 nm did not appear for adrenaline after 5 min of reaction and the band at 520 nm appeared very slowly. The same behaviour was observed for mixtures. In the case of noradrenaline, the small band at 520 nm disappeared.

These results show the significance of the oxygen amount with respect to the oxidation rate of

adrenaline and noradrenaline, but not respect to the emission intensity obtained from solutions aerated and untreated.

Based on them, we decided to continue using solutions prepared in the normal way, without reducing or increasing the concentration of dissolved oxygen present in solutions, because this concentration is enough to complete the oxidation of drugs.

We carried out some preliminary tests involving merging solutions containing 10 μg ml^{-1} adrenaline and 5, 10 or 20 μg ml^{-1} noradrenaline in 0.1 mol l^{-1} HCl within an 1.5 mol l^{-1} NaOH solution (see Fig. 1a), both flowing at 0.5 ml min^{-1}. The resulting solution was driven to a 2 m long piece of PTFE tubing immersed in a water bath at 48 °C and after to the flow-cell, kept at 48 °C too; then we stopped the flow and recorded the spectra of oxidised products for 15 min. A stable and reproducible value of emission intensity (with R.S.D. $< 3\%$) was obtained after 10 min of reaction at 450 nm from all solutions: we choose this time of reaction for determination of adrenaline in mixtures.

The effect of temperature was studied by heating the reaction coil and the flow-cell from 38 up to 70 °C. The highest fluorescence at 450 nm was observed at 55 °C.

Finally, we assayed different concentrations for NaOH solution; the values tested were 1.5, 2.0, 2.2, 2.5, 2.7 and 3.0 mol l^{-1}. The best results were obtained with a NaOH concentration of 2.0 mol l^{-1}.

3.2. Unsegmented continuous-flow experiments

Fig. 1b shows the manifold used. We carried out the optimisation from the next initial conditions: NaOH flow-rate (c_1), 1.2 ml min^{-1}; sample flow-rate (c_2), 1.5 ml min^{-1}; carrier flow-rate (c_3), 4.0 ml min^{-1}; sample volume, 313 μl; L_1 and L_2, 47.7 and 77.4 cm, respectively; and temperature, 55 °C.

Due at the irreproducibility of transient signals at 520 nm, we tested warm up the carrier as well as the sample loop and the flow-cell: we obtained a great improvement of reproducibility.

We started with the optimisation of manifold for measures at 520 nm. The first parameter tested was the carrier flow-rate. The values assayed were comprised between 0.4 and 4.1 ml min^{-1}. We obtained calibration graphs (emission intensity — [noradrenaline]/[adrenaline] ratio) for each value of carrier flow-rate from solutions containing 10 μg ml^{-1} of adrenaline and 0, 1, 5, 10 or 15 μg ml^{-1} of noradrenaline. The highest value of slope was obtained with 1.1 ml min^{-1}.

The influence of sample volume was studied by calibration at five different values, from 270.6 to 355.3 μl (six points each calibration graph) and the obtained slopes were increasing when increased the sample volume up to about 330–340 μl; then diminished. The calculated slopes varied from 258.4 to 296.8 and was selected a volume of 333.6 μl because it provided the highest slope of linear graphs.

Finally, the best flow-rate ratio between NaOH and sample streams was chosen. In this case there were not significant differences between slopes of graphs; then the regression coefficient of them dictated the optimum value: this was $c_1/c_2 = 0.5$. The interval studied was from 0.3 to 1.0. Maintaining this ratio $c_1/c_2 = 0.5$, we also studied the influence of flow-rate of both (NaOH and sample) streams, from 0.3 to 1.1 ml min^{-1} for the NaOH and from 0.6 to 2.2 ml min^{-1} for the sample stream. The optimum values were $c_1 = 0.8$ ml min^{-1} and $c_2 = 1.6$ ml min^{-1}.

The next step was the optimisation of manifold for measures at 450 nm. At first, we tested the flow-rate ratio between NaOH and sample streams; we obtained the highest transient signals with a ratio of 0.8.

The influence of carrier flow-rate on fluorescence was minimal in this case; then we choose the fastest that the manifold permits: 3.8 ml min^{-1}.

Table 1 shows the characteristics of manifold after the optimisation process. At this moment was tested the repeatability of the FIA system by injecting a solution containing 20 mg l^{-1} of adrenaline; the calculated relative standard deviation (in %) was 0.6.

3.3. Oxidation by molecular oxygen

Since adrenaline (also the noradrenaline), is an o-diphenol (it contains a hydroxyl group in the α-position) it is a strong reducing reagent [14] This means it is easily oxidised by different oxidants like iodine, potassium hexacyanoferrate(III), potassium persulfate, manganese dioxide and molecular oxygen. Oxidation of adrenaline is to occur through the transient formation of adrenaline quinone with formation of adenochrome. In certain conditions and according to different authors, the oxidation of the adrenaline by molecular oxygen results in the formation of a brownish insoluble stuff of indefinite structure. The reaction adrenaline–molecular oxygen is also extremely complex; it can occur in absence of heavy metal ions and it involves free radical sequences [14].

As was observed adrenaline produces a sequence of fluorescent products when oxidised in a basic aqueous solution (see the reactions in Fig. 4), being probably the 3,5,6-trihydroxy-1methylindole (or adrenolutine) and the 3,4-dihydroxyacetophenone the products responsible for the fluorescence at 520 and 450 nm, respectively. See the reproduction of the schematic mechanism in Fig. 4.

Table 1
Results of optimisation (in both cases, $\lambda_{ex} = 329$ nm)

	Measures at $\lambda_{em} = 450$ nm	Measures at $\lambda_{em} = 520$ nm
NaOH flow-rate (c_1)	1.2 ml min^{-1}	0.8 ml min^{-1}
Sample flow-rate (c_2)	1.5 ml min^{-1}	1.6 ml min^{-1}
Ratio c_1/c_2	0.8	0.5
Carrier flow-rate (c_3)	3.8 ml min^{-1}	1.1 ml min^{-1}
L_1	47.7 cm	47.7 cm
L_2	77.4 cm	77.4 cm
Sample volume	333.6 μl	333.6 μl
Heating time of sample in the sample-loop	10 min	–
Temperature of carrier, sample-loop and flow-cell	55 °C	55 °C

3.4. Analytical figures of merit

The analytical determination of adrenaline and noradrenaline in mixtures is based in two consecutive determinations. The first step is to determine adrenaline by recording outputs at 450 nm; then the outputs are recorded at 520 nm; those outputs are the result of the adrenaline and noradrenaline emission sum. With the manifold optimised for measuring at 450 nm, the linear range for adrenaline was over 0.5–20 ug ml^{-1} of the drug (LOD, 0.2 ug ml^{-1}), fitting with the equation $I = 6.527 + 17.414\ C$, where I represents the intensity emission obtained at 450 nm ($\lambda_{ex} = 320$ nm) and C is the adrenaline concentration in μg ml^{-1}; correlation coefficient 0.998. Then was tested the between day reproducibility by testing different freshly prepared solutions and repeating the calibration graph; the average of five slopes gives 17.408 with a rsd (%) of 1.7.

Then we continued with the study of the linear range for determination of the [noradrenaline]/[adrenaline] ratio with the suitable manifold and the obtained results are depicted in Table 2. Minor tested noradrenaline concentration 0.2 ug ml^{-1}.

The influence of foreign compounds that usually can be found in pharmaceutical formulations of those drugs was studied by preparing solutions containing 10 mg ml^{-1} of adrenaline (measures at 450 nm) or solutions containing 10 mg ml^{-1} and 10 mg ml^{-1} of noradrenaline (for determinations at 520 nm) and increasing concentration of the foreign compound up to 1000 mg ml^{-1} or up to the concentration resulting in a relative error not surpassing the 3%. Relative errors were calculated by comparing the obtained outputs with the resulting from the solutions containing the same amount of the drug without the interference. Empirical results are depicted in Table 3.

The analytical results observed with synthetic samples [25] prepared in this laboratory by mixing adrenaline (10 mg l^{-1}) and noradrenaline (4 or 12 mg l^{-1}) resulted in the following figures (average of three replicates and the relative errors were calculated by comparing the obtained output with the added amount): (a) adrenaline; added, 10.0 mg l^{-1}, Relative Error −1.40%; and (b) nora-

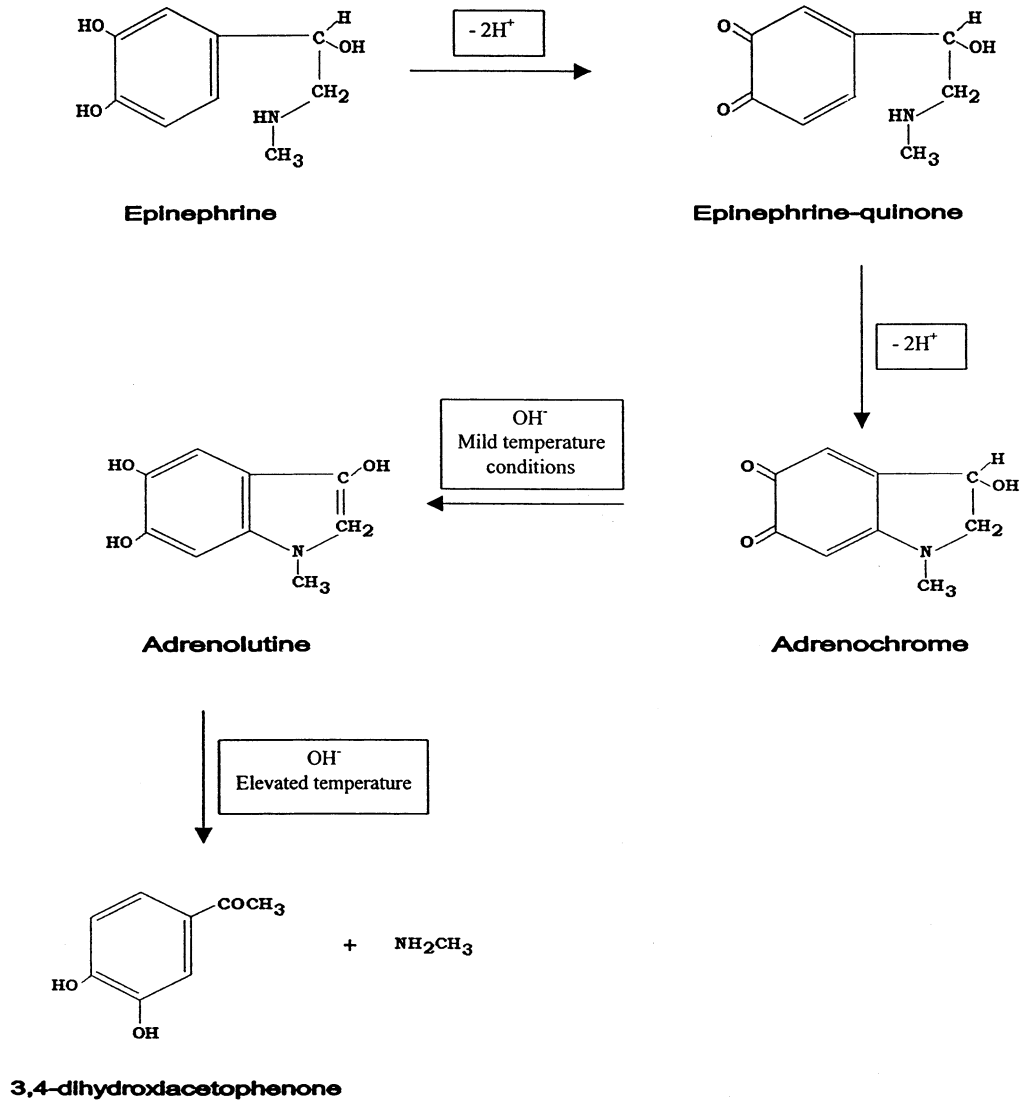

Fig. 4. Schematic representation of the proposed mechanism for oxidation of adrenaline.

drenaline; added 4.0, Relative Error 0.25; added 12, Relative Error 0.90.

4. Conclusions

A simple and inexpensive procedure for determination of the mixture adrenaline–noradrenaline is proposed. The method is performed on a FIA manifold and based on the oxidation of both compounds by solved molecular oxygen. No chemical oxidants (clean chemistry) are required and no expensive separation instrumentation.

The kinetic behaviour of both catecholamines is different enough to propose a differentiated procedure for their determination in a binary mixture. The kinetic behaviour is explained. Other published procedures for their determination are based on very small differences or in HPLC or Capillary Electrophoresis instrumentation.

Table 2
Obtained calibration equations for noradrenaline in presence of different amounts of adrenaline

Adrenaline concentration (μg ml^{-1})	Concentration range of noradrenaline (μg ml^{-1})	Linear equation (average of three replicates) (Nr/Ad)	Correlation coefficient
1	0.2–2.0	$I = 65.66–11.24$	0.9923
3	0.6–6.0	$I = 189.49–67.39$	0.9910
5	1.0–10	$I = 336.43–110.55$	0.9950
7	1.4–14	$I = 422.95–206.89$	0.9936
10	2.0–15	$I = 569.65–323.16$	0.9972
12	2.4–18	$I = 611.45–365.21$	0.9994
15	3.0–22.5	$I = 698.79–464.41$	0.9973
17	3.4–20.4	$I = 811.21–598.22$	0.9989
20	4.0–24	$I = 845.54–622.63$	0.9982

Table 3
Influence of foreign compounds

Compound	Amount (Relative error, %) Adrenaline, 100 ppm	Amount (Relative error, %) Adrenaline + noradrenaline, 100 ppm each
Lidocaine	700 (1.1)	100 (2.3)
Tetracaine	7 (2.1)	1 (5.7)
Borax	1000 (2.3)	1000 (1.1)
Tartaric acid	10 (2.3)	10 (6.1)
Phenol	50 (0.0)	50 (2.3)
Novocaine	5 (2.3)	5 (7.5)
NaCl	1000 (0.2)	900 (2.6)
Sodium metabisulphite	1000 (1.2)	100 (0.5)
$ZnSO_4$	50 (3.2)	10 (3.2)

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