

Analyte Pulse Perturbation Technique for the Determination of 6-*O*-Acetylmorphine in Seized Street Drug Samples

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Received January 29, 2007; E-mail: bimesel@eunet.yu

A pulse perturbation technique was applied to the Bray–Liebhafsky (BL) oscillatory reaction system in a stable nonequilibrium stationary state close to the bifurcation point, to determine micro-quantitatively 6-*O*-acetylmorphine (6-*O*-AM). The proposed method, in optimized reaction conditions ($[KIO_3]_0 = 5.9 \times 10^{-2}$ M, $[H_2SO_4]_0 = 5.5 \times 10^{-2}$ M, and $[H_2O_2]_0 = 2.0 \times 10^{-1}$ M, $j_o = 2.95 \times 10^{-2}$ min^{-1}, and $T = 42.9$ °C), relied on the linear relationship between maximal potential shift, ΔE_m, caused by perturbation, and the logarithm of the amount of 6-*O*-acetylmorphine. The method had a rather good sample throughput of 40 samples h^{-1} with the sensitivity determined to be 0.9 μg mL^{-1} and the precision RSD = 1.8%. The proposed method was successfully applied to the quantitative determination of 6-*O*-AM in a real seized street drug sample. The obtained result agrees with those obtained by HPLC. There was no interference from structurally related and associated compounds, such as papaverin, noscapin, and heroin.

It is well known that some nonlinear reactions with a feedback being far from equilibrium can exhibit oscillatory behavior. Particularly, during the course of these complex dynamic systems that involve a large number of chemical species, the concentrations of intermediates or catalysts oscillate (sometime over several orders of magnitude), whereas the decomposition of reactants and evolution of products form stepwise.[1] The oldest known and studied oscillating system is the Bray–Liebhafsky (BL) reaction[2,3] that involves the catalytic decomposition of hydrogen peroxide in the presence of hydrogen and iodate ions (Eq. 1):

$$2H_2O_2 \xrightarrow{H^+, IO_3^-} O_2 + 2H_2O \quad (1)$$

It is a net result of two main kinetic pathways, in which hydrogen peroxide acts as either a reducing (Eq. 2) or an oxidizing (Eq. 3) agent:

$$5H_2O_2 + 2IO_3^- + 2H^+ \rightarrow I_2 + 6H_2O + 5O_2 \quad (2)$$

$$5H_2O_2 + I_2 \rightarrow 2IO_3^- + 4H_2O + 2H^+ \quad (3)$$

Eq. 2 and Eq. 3 pathways consist of numerous elementary steps, during which iodine-containing intermediates, such as I^-, I_2, HIO, and HIO_2, form and decompose. The elementary steps are intertwined and form a complex self-regulating network of molecular interactions.[4–14] Thus, when driven under the conditions far from thermodynamic equilibrium, the BL reaction exhibits self-organized temporal dynamic structures typical of nonlinear systems, including nonequilibrium stationary states, simple periodic oscillations, as well as complex oscillations, bursts and deterministic chaos.[15–17]

Among the most interesting studies of chemical oscillating reactions is their application to analytical chemistry, since they are extremely sensitive to perturbations. After the first paper about the quantitative use of oscillatory reaction by Tichonova et al.,[18] numerous analytical applications of chemical oscillators have been published regarding various species.[19–23] Moreover, the application of oscillating chemical reactions to analytical determination over a period from 1986 to 2004 has been summarized in two papers.[24,25]

So far, two distinct analytical methods have been proposed. The first method[26] is based on the relationship between the concentrations of analyte and the response of the system being in the oscillatory state with respect to the main characters of oscillations, such as amplitude, period, and others. The second method[27–32] relies on perturbing the matrix system that is in a stable stationary state in the vicinity of bifurcation point, and it is based on the relationship between the potential displacement (ΔE_m) in the moment of perturbation and the logarithm of analyte concentrations.

More recently, the application of an analyte pulse perturbation technique to determine the presence of morphine (MH) in bulk drug as well as pharmaceutical dosage form[32] has opened up prospects for quantitative analytical determination of forensic material constituents based on the BL oscillatory chemical system. It is well known that, in toxicology and illicit drugs analysis, the identification and trace quantification of forensic material constituents are very important for understanding their probable origin and the possible route of drug trafficking. On the other hand, the seized samples are very complex, and therefore, some very sensitive and selective techniques need to be established for its accurate analysis. The aim of this work was to develop and optimize a method for quantitative determination of 6-*O*-acetylmorphine (6-*O*-AM) by perturbing a stable stationary state that is found in the vicinity of a bifurca-

Published on the web October 12, 2007; doi:10.1246/bcsj.80.1942

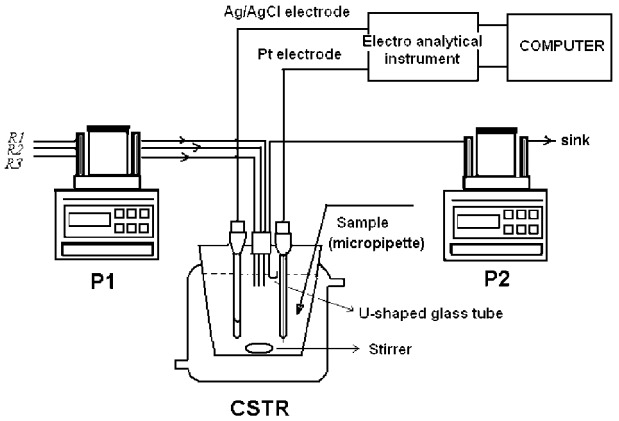

Fig. 1. A schematic diagram of the instrumental assembly. P1 and P2 stands for peristaltic pumps. $R1 = 5.9 \times 10^{-2}$ M KIO_3; $R2 = 5.5 \times 10^{-2}$ M H_2SO_4; $R3 = 2.2 \times 10^{-1}$ M H_2O_2.

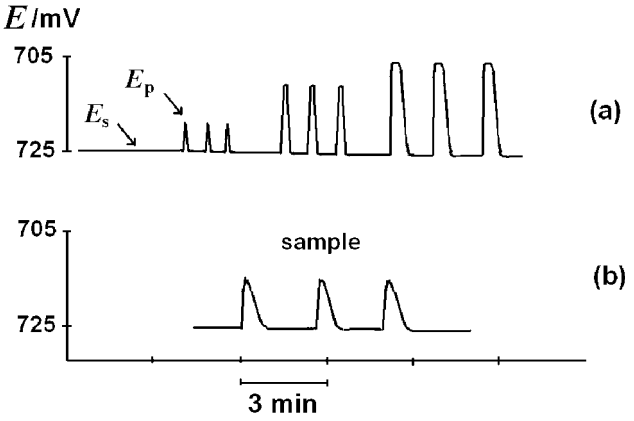

Fig. 2. Potentiometric signals obtained for 6-*O*-AM standards and sample solution of illicit powder. (**a**) From left to right: triplicate signals for three perturbation reference solutions, $c_{6\text{-}O\text{-AM}} = 3.2\,\mu g\,mL^{-1}$, $c_{6\text{-}O\text{-AM}} = 14.0\,\mu g\,mL^{-1}$, and $c_{6\text{-}O\text{-AM}} = 33.2\,\mu g\,mL^{-1}$; (**b**) three consecutive signals for sample.

tion point in the BL reaction matrix and to demonstrate that it can be successfully applied to the determination of 6-*O*-AM in bulk drugs and in complex matrices, such as seized street drug samples. To this end, for quantitative determination of 6-*O*-AM, we used conditions of the BL reaction similar to those for the determination of MH.[32] However, detailed optimization of the proposed method had to be performed. Finally, having the proposed method, the detection limit has reduced to $0.9\,\mu g\,mL^{-1}$ (0.00014 μmol) and the linear range of the concentrations for 6-*O*-AM determination was expanded.

Experimental

Chemicals. Only analytical grade reagents without further purification were used for preparing the solutions. Potassium iodate, sulfuric acid, and ethanol were obtained from Merck, and hydrogen peroxide from Fluka. Pure 6-*O*-AM was provided through the Ministry of Interior Administration of Police Forensic Science, Belgrade and used without further purification. To prepare the solutions of KIO_3, H_2SO_4, and H_2O_2, deionized water ($\rho = 18$ MΩ cm, Milli-Q, Millipore, Bedford) was used. Standard stock solution of 6-*O*-AM was prepared with a concentration of 2.0×10^{-3} M in ethanol and was stored in a refrigerator in the dark. Prior to injection, stock solutions were appropriately diluted with ethanol before being used as working solutions.

Apparatus. The BL reaction, used as the matrix system, was conduced in an open reactor, i.e., in the continuously fed well stirred tank reactor (CSTR). A simplified schematic diagram of the apparatus is shown in Fig. 1. It consisted of about 50-mL glass CSTR vessel (Metrohm Model 876-20) wrapped in a water recirculation jacket connected to a thermostat (Series U8, MLW Freital) with an accuracy ±0.1 °C and equipped with a magnetic stirrer (IKA-COMBIMAG RET). Temporal evolution of the system was recorded by means of a Pt electrode (Metrohm model 6.0301.100) and double junction Ag/AgCl electrode (Metrohm model 6.0726.100) interfaced to a PC-AT 12 MHz compatible computer via a PC-Multilab EH4 16-bit ADC. The flows (inflow and outflow) of chemical species through CSTR were driven by peristaltic pumps (P1 and P2) (Manuel/RS 232 Controlled Peristaltic Pumps, Type 110). Viton tubes (Deutch & Neuman) were used to transport the aqueous solutions of potassium iodate and sulfuric acid, whereas tygon tubes (Ismatec) were used to transport hydrogen peroxide from their reservoirs to the reaction vessel. These tubes were connected to teflon tubes (Varian), and the reagents were introduced to the reaction vessel through them. In all experiments, the feed substances were kept in reservoirs at room temperature and were introduced into the reaction vessel separately without being previously thermostated. The volume of the reaction mixture was kept constant at 22.2 ± 0.2 mL by removing the surplus volume of the reaction mixture.

Procedures for Determination of 6-*O*-AM. The experimental setup for CSTR was similar to that reported earlier.[27–29] First, thermostated (water bath set at $T = 60.0$ °C) and shielded from light, reaction vessel was filled with three separate inflows of the reactants ($[KIO_3] = 5.9 \times 10^{-2}$ M, $[H_2SO_4] = 5.5 \times 10^{-2}$ M, and $[H_2O_2] = 2.0 \times 10^{-1}$ M) at a maximum flow rate of 12 mL min^{-1}. Under these conditions, over 3.5 min, a nearly double reaction mixture volume was charged. Then, the inflows were stopped, the stirrer was turned on ($r = 900$ rpm), and the excess reaction mixture was sucked out through a U-shaped glass tube to achieve an actual reaction mixture volume of 22.2 ± 0.2 mL. Hence, the reaction commenced under the bath conditions. After two bath oscillations (30 min), the inflows were turned on at the required specific flow rate (2.95×10^{-2} min^{-1}), and the temperature was adjusted to the working temperatures in the range from 30.0 to 48.5 °C.

Under the above-described experimental conditions, the matrix reaction system was found take in different stable stationary states depending on selected temperature. After adding the 6-*O*-AM solutions to the matrix, being in a stationary state, the responses of the BL matrix were recorded. Typical responses of the BL matrix to perturbations with 6-*O*-AM are presented in Fig. 2a. As can be seen, injection of 6-*O*-AM caused an abrupt decrease in the potential to the value denoted by E_p, which is the minimum potential that can be reached. After achieving this value, the potential returned to a value that is equal or only slightly different from the potential of the initial nonequilibrium stationary state, E_s.

Perturbations were performed by adding 50 μL of both the 6-*O*-AM standard stock solutions and ethanol samples solutions with micropipettes (Transfepette, Brand). We applied manual injections with an approximate duration of 0.5 s. As a quantitative measure of the intensity of the perturbation corresponding to the

analyte concentration, we chose the analytical signals, that is, the potential displacement, $\Delta E_m = E_p - E_s$. When 6-*O*-AM is introduced into the BL matrix, it takes part in a very complex mechanism, changing the ratio between $[HIO]_{ss}$ and $[I^-]_{ss}$, which are established in the stationary state before perturbations. The potential shift due to changes in their concentrations reflects the systems response to the applied perturbation.[27,29,32]

The effects of different variables, such as temperature and injected volume of analyte, were studied in order to establish the optimum working conditions for determination of the examined analyte.

Results and Discussion

Dynamics of Matrix Reaction System. In this work, as the matrix reaction system suitable for the determination of 6-*O*-AM, the Bray–Liebhafsky (BL) oscillatory system[2,3] was chosen based on extensive knowledge of its mechanism[9,11,12,14,33] and previous positive experience.[27–32]

With aim to locate different stable nonequilibrium stationary states of BL matrix in the vicinity to bifurcation point necessary for analytical purposes, we examined the dynamics of the BL matrix as a function of temperature, which can vary slightly for different setups in different laboratories. As we have already described in detail earlier,[30–32] in order to find a bifurcation point between sustained oscillations and a stationary state, the temperature, which was the control parameter, was gradually reduced from 48.5 to 30.0 °C, while the other parameters (specific flow rate and mixed inflow concentration of the fed substances) were kept constant. From the obtained dynamic structures observed under the above described conditions at different temperatures, we obtained a diagram of all stationary points as a function of the control parameter, i.e., a bifurcation diagram (Fig. 3a).

The bifurcation point was found to be at $T = 43.2$ °C by linear extrapolation of a plot of the square of the amplitude of the limit cycle oscillations versus temperature (Fig. 3b).

Optimization of Procedure. Around the found bifurcation point, we analyzed several stable stationary states (indicated by arrows in Fig. 3a) and tested their sensitivity to perturbations with 6-*O*-AM. Five different temperatures, $T = 30.0$, 35.0, 37.0, 40.0, and 42.9 °C were selected for perturbation analysis. As a rule, we found that the sensitivity to 6-*O*-AM decreased with an increase in the distance from the bifurcation point (Fig. 4). Hence, the highest sensitivity was achieved at $T = 42.9$ °C.

The procedure was also optimized with respect to the injected volume of the analyte. For this purpose, the response of the matrix system was studied by varying the injection volume. In particular, the following volumes were tested: 10, 30, 50, 100, 200, 300, and 500 μL. We found that under the current set-up, the perturbations with volumes equal to or larger than 500 μL were not reliable, because the dynamic pattern also changed due to dilution. The impact of dilution effects were also observed for 200 and 300 μL samples, resulting in a larger standard deviation of the potential displacement and somewhat prolonged decay times. Injection volumes ranging from 30–100 μL were applicable for our current setup, and 50 μL injection volumes were selected as being optimal.

Quantitative Determination of 6-*O*-AM. Under the above-described optimal experimental conditions, the BL matrix was perturbed with various concentrations of 6-*O*-AM. Typical response curves, obtained after perturbations of the chosen stable nonequilibrium stationary state for $T = 42.9$ °C, are given in Fig. 2a. The response to the perturbations was measured as a function of the change in potential.

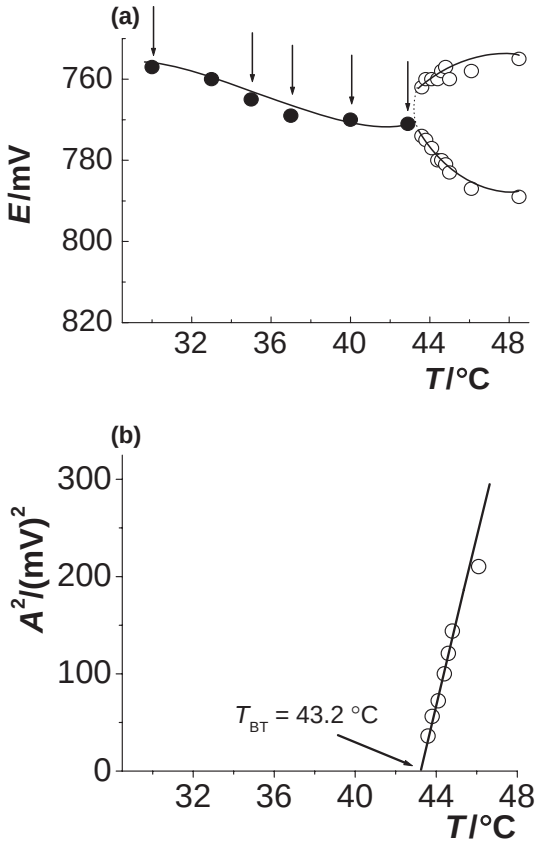

Fig. 3. (**a**) Bifurcation diagram showing transition from the stable stationary state (solid circles) to the sustained periodic oscillations (open circles) that denote minimal and maximal potential in an oscillation. The operations points are indicated by arrows. (**b**) Plot of the square of the oscillation amplitudes as a function of temperature. The bifurcation point is determined from the intercept on the abscissa.

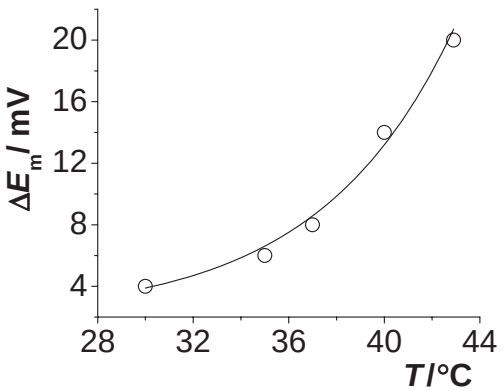

Fig. 4. Influence of temperature on the analytical signal. Concentration of 6-*O*-AM is $c_{6\text{-}O\text{-AM}} = 31.7\,\mu g\,mL^{-1}$.

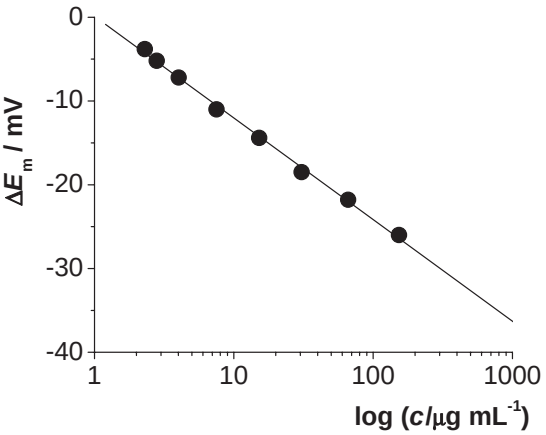

Fig. 5. Calibration curve of the maximal potential shift versus the logarithm of 6-*O*-AM.

It was found that the maximal potential displacement, $\Delta E_m = E_p - E_s$, was proportional to the intensity of the perturbation. A plot of the ΔE_m against the logarithm of the injected 6-*O*-AM concentrations provided a calibration graph that was fitted by the least-square method.

The proposed method was validated according to the International Conference on Harmonization guidelines. In accordance with these guidelines, linearity, precision, limit of detection (LOD), limit of quantitation (LOQ), and sample throughput were analyzed. The LOD corresponds to amount of analyte, which produces an instrumental response that is three times as large as the standard deviation of the instrumental noise level, whereas LOQ is assessed at a minimum signal-to-noise ratio of 10. LOD and LOQ were experimentally verified by eight injections of 6-*O*-AM at the LOD and LOQ amounts, which gave acceptable precision and accuracy under the ICH guidelines. Precision was expressed by the relative standard deviation ($RSD^* = (t \times RSD)/\sqrt{n}$ with a theoretical *t*-value at 95% confidence limit for seven degrees of freedom), and it was checked for eight samples containing three different analyte concentrations within the calibration curve. The accuracy was measured as the "recovery" value (RCV) i.e., (concentration found/known concentration) × 100. Sample throughput (sample h^{-1}) was based on the time needed for the system to recover to the initial stationary state after each perturbation.

Under the optimal experimental conditions described above, the calibration curve (Fig. 5) over the concentration range 2.3–153.0 μg mL^{-1} obeyed the following linear regression equation:

$$\Delta E_m/\text{mV} = 0.1\ (\pm 0.3) - 12.2\ (\pm 0.2) \log(c_{6\text{-}O\text{-AM}}/\mu\text{g mL}^{-1}), \quad (n = 8, R = 0.9988) \qquad (4)$$

However, no linearity was observed when the concentration of 6-*O*-AM was outside the range 2.3–153.0 μg mL^{-1}. LOD and LOQ were found to be 0.9 and 2.5 μg mL^{-1}, respectively. The precision of the method was established by the repeated assays ($n = 8$) using of 2.5, 25.5, and 100.0 μg mL^{-1} of 6-*O*-AM. The average RSD^* for the determination of concentration of 6-*O*-AM was 1.8%, and the obtained average RCV was 97.9%.

Table 1. Tolerance to External Species and Ions in the Determination of 6-*O*-AM in Relation to the Maximal Potential Displacement, ΔE_m

Species and ions added	Tolerable [interferent species] /[6-*O*-AM] ratio (M/M)[a)]
Papaverin, noscapine, heroin	>300[b)] [>300[b)]][c)]
Carbamide	>50[b)] [>30[b)]]
Glucose, sucrose, starch, fructose	>40[b)] [>40[b)]]
Cocaine	40 [15]
Codeine	40 [1]
Acetylcodeine	40 [0.7]
Caffeine	20 [0.8]
Morphine, Acetylsalicylic acid	15 [9]
Paracetamol	11 [11]
IO_4^-	1.5 [1]
I^-, Br^-, Cl^-	1 [1]
$CaCO_3$	0.2 [0.1]

a) 6-*O*-AM concentration was equal to 33.2 μg mL^{-1}. b) Maximum ratio tested. c) In square brackets is tolerable ratio obtained by using method employing the characteristic period, t_p (see Appendix).

As can be seen, the calibration curve exhibited excellent linear behavior over the concentration range of about two orders of magnitude, acceptable precision (1.8% as RSD^*), quite good exceptional sensitivity (detection limit of 0.9 μg mL^{-1}) and excellent sample throughput (40 samples h^{-1}).

Selectivity. We investigated the selectivity of the proposed method by observing potential interference between 6-*O*-AM and 16 foreign species that may exist together with 6-*O*-AM in real samples. In addition, we tested the selectivity towards 6-*O*-AM in the presence of some ions that were components of the oscillatory reactions itself. Selectivity was examined by perturbing the matrix with solutions containing a fixed amount of 6-*O*-acetylmorphine, $c_{6\text{-}O\text{-AM}} = 33.2\ \mu\text{g mL}^{-1}$, in the presence of increasing amounts of possible interfering agents. We decided that a species does not influence the determination of 6-*O*-AM if they affect the analytical signal by less than ±5%. The obtained results are summarized in Table 1. The sign ">" denotes maximum ratio tested. The values without any sign denote the last one that is insensitive to the interferent.

Based on Table 1, large amounts of certain structurally related compounds, such as papaverin, noscapine, and heroin, had no effect on the determination, but ions that are known to perturb strongly the dynamics of the BL reaction, such as I^-, Br^-, or Cl^-, had strong adverse effects on 6-*O*-AM determinations. The halogen ions cause a decrease in the analytical signal due to their interaction with the matrix reaction system.[27] A typical signal profile for stock solutions of 6-*O*-AM in the presence of some interferents are shown in Fig. 6. Thus, in the case of paracetamol, cocaine, acetylcodeine, and iodide with a further increase in [interferent species]/[6-*O*-AM] ratio, a decrease in potential shift occurred, whereas in the case of substances like caffeine, an increase in potential shift was obtained. At the same time, we should note that compounds that structurally similar to 6-*O*-AM, such as morphine, interfere with the determination of 6-*O*-AM with a tolerance limit of

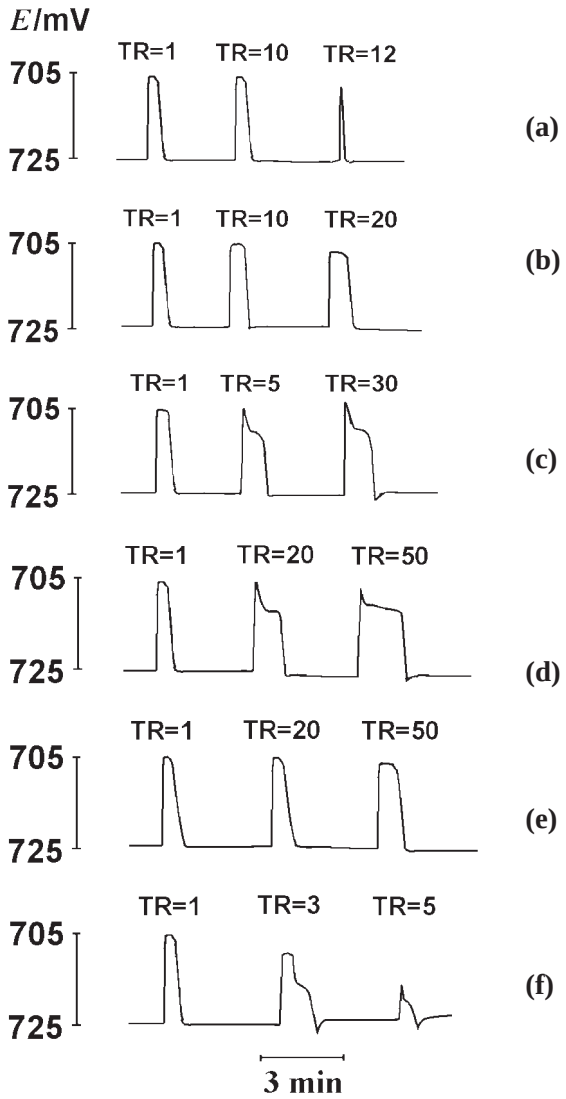

Fig. 6. Typical signal profile provided by addition of 33.2 $\mu g\,mL^{-1}$ 6-*O*-AM in the presence of different interferents: (a) paracetamol, (b) morphine, (c) caffeine, (d) cocaine, (e) acetylcodeine, and (f) iodide ion. The tolerable [interferent species]/[6-*O*-AM] ratios, denoted as TR, increase from left to right.

15-fold, which is the main limitation of the proposed method. Therefore, it may be applicable for 6-*O*-AM determinations in real samples containing sufficiently low concentrations of this alkaloid.

The interference was analyzed above with respect to maximal potential shift. However, in accordance with the previous discussion on interference, we would like to underline that sometimes the forms of signal profile can change in the presence of the other species (Figs. 6c and 6d) without changing the maximal potential shift. This is particularly the case in the presence of higher amounts of interfering species. Namely, it is evident that changes in the chemical mechanism occur owing to the addition of the different analytes to the matrix system. In such a way, addition of different analytes that are not originaly present in the matrix will inevitably introduce different new chemical reactions as well as different species; in fact, new chemical dynamic systems are generated. This could be the starting point for understanding the appearance of the selectivity in the proposed method.

Table 2. Results Obtained by Using the Proposed Method and HPLC Method for the Assay of 6-*O*-AM in a Seized Street Drug Sample

Sample	Composition[a)]	Determination of 6-*O*-AM		
		Proposed method		HPLC
		Found $\pm$ SD[b)] /$\mu g\,mL^{-1}$	RCV[c)] $\pm$ RSD[d)] /%	Found $\pm$ SD[b)] /$\mu g\,mL^{-1}$
	Heroin	—	—	260.0
	6-*O*-AM	9.6 ± 0.5	98.1 ± 1.8	10.0 ± 0.3
	Acetylcodeine	—	—	10.0

a) Indicated by HPLC. b) Found values are the average of two independent analysis $\pm$ the corresponding standard deviation ($n = 3$). c) Performed as accurate addition of 5.0 $\mu g\,mL^{-1}$ of 6-*O*-AM to the diluted samples. The RCV values are mean recoveries ($n = 3$). d) Relative standard deviations of recovery.

Sample Analysis. Applicability of the developed method for the analysis of real sample was examined by assaying for 6-*O*-AM in an illicit drug sample. As indicated by high-performance liquid chromatography, this sample contained heroin and acetylcodeine at concentrations levels of 260.0, and 10.0 $\mu g\,mL^{-1}$, respectively. To determine 6-*O*-AM from the drug sample, a sample solution was prepared by dissolving a small amount of the powder sample (0.0105 g) in a 10 mL volumetric flask with ethanol. Perturbations of the matrix system were performed using 50 µL aliquot of this solution.

Response curves obtained from the perturbations of the chosen stable stationary state are shown in the Fig. 2b. The average concentrations were calculated from three measurements. In order to test the accuracy of the procedure, additional recovery experiments were carried out with the examined sample from those listed in Table 1. In all instances, the standard addition method was performed by accurately adding 5.0 $\mu g\,mL^{-1}$ of 6-*O*-AM to the dilute samples. Table 2 shows the obtained results; it can be seen that the average recovery was 98.1% indicating that the developed method is free from interference and provides accurate results. Also, the obtained results correspond to those reported for high-performance liquid chromatography (average RCV was 96.0%).

Conclusion

Our results demonstrated that the proposed method for quantitative determination of 6-*O*-AM based on perturbing the dynamics of the matrix Bray–Liebhafsky reaction in a stable nonequilibrium stationary state in the vicinity of a bifurcation point has rather good analytical attributes (limit of detection, precision and accuracy are 0.9 $\mu g\,mL^{-1}$, 1.8 and 97.9%, respectively) and an excellent sample throughput (40 samples h^{-1}). Moreover, the proposed method had acceptable selectivity and was applicable to 6-*O*-AM determination in a seized drug sample, as reflected by RCV = 98.1%. In order words, in this sample, the 6-*O*-AM was determined successfully in the presence of a lot of sample compounds, including some structurally related compounds, such as heroine and ace-

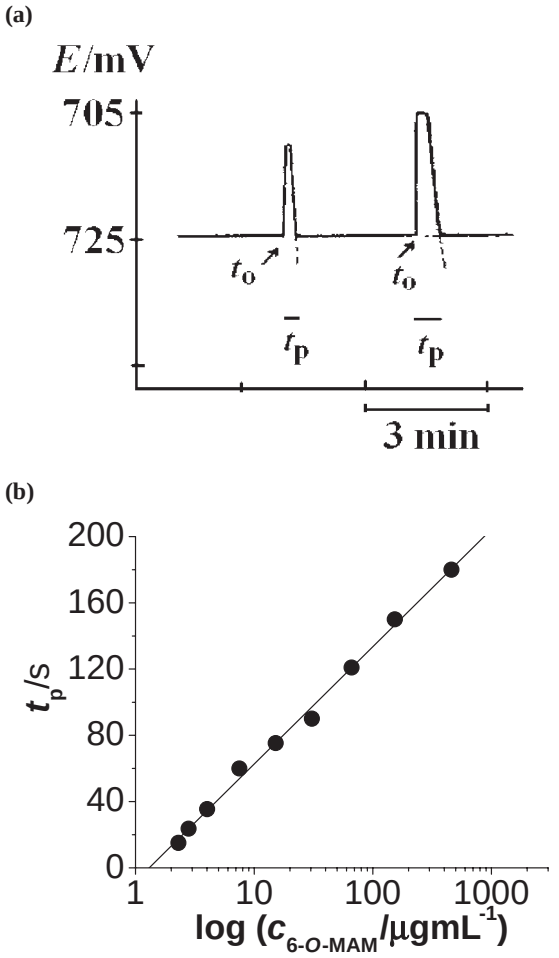

Fig. 7. (**a**) The typical period (t_p) in the system BL-6-*O*-acetylmorphine. The perturbation strengths are (from left to right): $c_{6\text{-}O\text{-AM}} = 14.0\,\mu g\,mL^{-1}$ and $c_{6\text{-}O\text{-AM}} = 33.2\,\mu g\,mL^{-1}$. (**b**) Plot of the characteristic time (t_p) as a function of logarithm of the 6-*O*-AM concentration.

tyl codeine. In addition, the proposed method involved neither expensive equipment nor any time-consuming extraction procedures.

The present investigations are partially supported by The Ministry of Sciences and Environmental Protection of Serbia, under the Project 142025.

Appendix

The dynamic behavior of the BL matrix, after perturbations by 6-*O*-AM, depends on the intensity of the applied perturbation. For the examined concentrations of 6-*O*-AM, we usually observed the type of behavior shown in Fig. 7a. After introducing the 6-*O*-AM, an abrupt change in potential was observed. This sudden response was followed by relatively fast return to the initial stationary state. Both the amplitude of perturbation and period for which the matrix system reverts to the initial stationary state, denoted by t_p (Figs. 2a and 7a), depended on perturbation intensity. Perturbing the matrix system by injecting a microvolume of 6-*O*-AM caused a change in the characteristic period that was quantitatively related to the analyte concentration. As it was found that the characteristic period t_p was proportional to the intensity of the perturbation, their relationship could also be used to prepare a calibration curve. Particularly, a plot of the t_p against the logarithm of the injected 6-*O*-AM concentrations provided a calibration graph that was fitted by the least squares. Under the optimal experimental conditions described above, the calibration curve (Fig. 7b) over the concentration range of 2.3–460.2 $\mu g\,mL^{-1}$ obeyed the following linear regression equation:

$$t_p/s = -8.2\ (\pm 2.6) + 70.9\ (\pm 1.7)\log(c_{6\text{-}O\text{-AM}}/\mu g\,mL^{-1}) \quad (n = 9, R = 0.9979) \quad \text{(A-1)}$$

The proposed method was tested for the precision, sensitivity and selectivity. The precision of the method was established by the repeated assays ($n = 8$) using the 6-*O*-AM amounts of 2.5, 100.0, and 325.0 $\mu g\,mL^{-1}$. The average RSD and RCV for 6-*O*-AM determinations were 4.2 and 96.4%, respectively. Experimentally derived LOD and LOQ for 6-*O*-AM were determined to be 1.5 and 4.2 $\mu g\,mL^{-1}$. Related to the above-described method employing the change in maximal potential displacement, ΔE_m, this method has a wider dynamic linear range, lower precision and a higher detection limit.

The selectivity of the method was also examined in a manner similar to that described in above Section. The same experimental results were analyzed with respect to the characteristic period, t_p. We decided that a species does not influence the determination of 6-*O*-AM if they affect t_p by less than ±10%. The obtained results are summarized in Table 2 where they are given in square brackets.

As in the first method, large amounts of some structurally related compounds, such as papaverin, noscapine, and heroin, had no effect on the determination of 6-*O*-AM. However, in the very last case, only lower levels of some interferents, such as acetyl codeine, codeine, caffeine, and cocaine, were tolerated (Table 1). Thus, acetyl codeine was found to interfere above a 6-*O*-AM interferent ratio 1:0.7. In the chosen real sample, the ratio of concentration of 6-*O*-AM and acetylcodeine was 1, which is more than experimentally obtained tolerable ratio (Table 1) which can enplane the problems encountered previously. In general, since the maximal potential displacement, ΔE_m is less sensitive to interference than t_p, the calibration curve ΔE_m versus time is more appropriate for such kind of analysis than corresponding curve t_p versus time.

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