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Sub-micromolar Determination of Codeine in Biofluides Using Kinetic Spectrophotometric Method

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ABSTRCT

In this study, a new kinetic spectrophotometric method for the determination of codeine was proposed using Orange G as an analytical reagent. The present method was developed based on the inhibitory effect of codeine on the oxidation of Orange G by bromate. The change in absorbance as a criterion of the oxidation reaction progress was followed spectrophotometrically at λ_{max}. To obtain maximum sensitivity, the effective reaction variables were optimized. Under optimum experimental conditions, calibration graph was linear over the range 0.8 – 397.9 µmol L^{-1} of codeine including two linear segments. The calculated detection limit ($3s_b/m$) was 0.22 µmol L^{-1} for eight replicate determinations of blank signal. The relative standard deviations 1.03, 0.99 and 0.98% for 2.5, 10.0 and 100.0 µmol L^{-1} of codeine confirm the repeatability of the developed method. The interfering effect of various species commonly associated with codeine in real samples was also studied. The present method was successfully applied for the determination of codeine in human serum and urine samples. The method's validity was checked by comparing the obtained results with high peformance liquid chromatography and also by evaluation of results using *F*-test.

Keywords*:* Orange G; Codeine; Biofluids; Kinetic spectrophotometry.

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INTRODUCTION

Codeine (3-methylmorphine) is an alkaloid that found in the opium poppy and has pharmacological and toxicological activity. Opium poppy has been used throughout the human history for hypnotic and a variety of medicinal properties (analgesic, anti-tussive and anti-diarrheal). The name of codeine was derived from the Greek word "kodeia" for poppy head. Later in 1832 in France, codeine was isolated during the morphine extraction by Pierre Robiquet [1,2]. Codeine is currently the most widely-used opiate in the world and is one of the most commonly used drugs overall according to numerous reports by WHO [2,3]. It is one of the most effective orally administered opioid analgesics and has a wide safety margin. Also, it can be used as a recreational drug. A heroin addict may use codeine to ward off the effects of a withdrawal [4,5]. Codeine and its major metabolites can be quantitated in biofluids including blood, plasma or urine in order to monitoring the therapy, confirming a diagnosis of poisoning or assistting in a medicolegal death investigations. Moreover, drug abuse screening programs generally test urine, hair and sweat. Blood or plasma codeine concentrations are typically in concentration range 0.17 – 1.0 µmol L^{-1} (50 – 300 µg L^{-1}) in persons taking the drug therapeutically, 2.34 – 23.4 µmol L^{-1} (700 – 7000 µg L^{-1}) in chronic users and 3.34 – 33.40 µmol L^{-1} (1000–10,000 µg L^{-1}) in cases of acute fatal overdosage [6,7]. Therefore, the quantitation of codeine in biofluides can be attractive for clinical, forensic and biochemical aims.

The survey of literature reveals that several procedures have been reported for the determination of codeine in various matrices. Chromatographic methods including thin layer chromatography[8], high performance liquid chromatography [9,10], solid phase extraction-high performance liquid chromatography [11], high performance liquid chromatography-mass spectrometry [12] and gas chromatography-mass spectrometry [13] are the most common methods. Along with the low detection limit, shortages such as set up cost and hard operation were found in their application. In recent years, electroanalytical chemists have shown great interest for the determination of codeine, and various modified electrodes using different modifiers [14,15] have been constructed. The main drawbacks of electrochemical methods are hard operation and poor selectivity of the measurement. Other methods are electrophoresis [16] spectrophotometry [17] and flow injection [18]. Thus, highly sensitive and selective methods are still required for tracing codeine determination.

Catalytic spectrophotometric method as a relatively easy handling and low cost technique that has sufficient accuracy is considered as an effective and appropriate method for quantitative determination of different species such as vanadate [19] in foods and vitamin B_9 [20] and major metabolite of heroine [21] in pharmaceutical and biological samples. To the best of our knowledge, there was not found any report for kinetic spectrophotomeric determination of codeine.

In continuing of our research interest for the determination of drugs, the authors developed a simple, rapid, sensitive and selective kinetic spectrophotometric method. The method is based on inhibitory effect of codeine on the Orange G – bromate reaction system.

Applying the proposed method for the determination of codeine in real samples without any purification or preconcentration is the most advantage of the developed procedure.

EXPERIMENTAL

Reagents and apparatus:

Chemicals with analytical reagent grade and doubly distilled water were used throughout in the experiments. 50 mL standard working codeine solution of 1.0 mmol L^{-1} was prepared by dissolving 0.0185 g of codeine phosphate.1/2 H_2O (Sigma, USA) in water daily. Orange G solution (6.6×10^{-4} mol L^{-1}) was prepared by dissolving 0.2985 g of Orange G (Merck, Germany) in appropriate amount of water and diluted to 1 L in a volumetric flask. Potassium bromate stock solution of 0.05 mol L^{-1} was prepared by dissolving 4.1752 g of $KBrO_3$ (Merck, Germany) in 500 mL volumetric flask. Sulfuric acid solution 4.0 mol L^{-1} was prepared by appropriate dilution of concentrated sulfuric acid solution (Merck, Germany) in water.

Kinetic data and all spectra were obtained by using a single beam UV-vis spectrophotometer Agilent (8453, USA) with a 10-mm glass cell. All solutions were kept in a thermostatic water-bath at 25.0 ± 0.1 °C. A stop-watch was used for recording the reaction time.

A HPLC system consisting of a Waters model 515 solvent-delivery system, a Waters model 996 photodiode-array detector (MILFORD, MA, USA) and a Waters 717 plus autosampler using a 20 mL sample loop. The Millennium 2010 software was applied for controlling and data analyzing. Separation was carried out at ambient temperature using a µBondapak C_8 (5 µm, 250×4.6 mm i.d.; Waters, Milford, MA, USA) column. A guard column (10 µm Bondapak C_{18} in disposable plastic inserts and Waters Guard-Pak holder) was used to safeguard the analytical column. All of the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

Chromatographic conditions:

HPLC analysis was performed by isocratic elution with flow rate 1.0 mL min^{-1}. The mobile phase composition was 0.01 mol L^{-1} KH_2PO_4, methanol, acetonitrile and isopropyl alcohol 420/20/30/30 (v/v/v/v). All solvents were degassed in an ultrasonic bath. Volumes of 10 µL each prepared solutions and samples were injected into the column. Quantification was effected by measuring at the 215 nm as established from the three dimensional chromatogram. The chromatographic run time was 10 min and the column void volume was 1.735 min.

Recommended procedure:

After initial kinetic spectrophotometric studies of the reaction system, the reagents concentration (except the codeine) were judiciously chosen for the analytical procedure. For this purpose, thermal equilibrium was established at 25.0 ± 0.1 ºC for 30 min in thermostated

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water bath. Then, to a 10 mL volumetric flask, 1.2 mL of 6.6×10^{-4} mol L^{-1} of Orange G solution, 1.0 mL of 4.0 mol L^{-1} of sulfuric acid and 25.0 µmol L^{-1} of codeine solution were placed. The solution was diluted to ca. 8 mL. Then, 0.5 mL of bromate solution 0.05 mol L^{-1} was added to the reaction mixture, followed by dilution to 10 mL with water. Time measurement was just started after adding the last drop of bromate solution. After thorough mixing, a portion of the solution was transferred to the cell and the absorbance was measured against water at 478 nm and 25 °C for fixed times of 30 and 450 s. The measurements were repeated in the absence of codeine to obtain the value for the uninhibitored reaction as the absorbance of blank (Fig. 1 and its inset). The absorbance changes of the inhibitored and uninhibitored reactions were labeled ΔA_s and ΔA_b, respectively. The difference in the absorbance ($\Delta A = \Delta A_b - \Delta A_s$) was considered as the response. Under optimum experimental conditions, a calibration graph was constructed by plotting the response (ΔA) against codeine concentration.

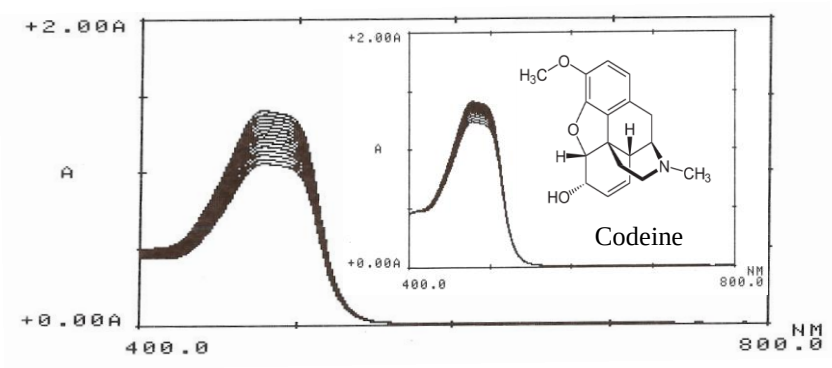

Fig. 1: Absorption spectra of the uninhibitored reaction. (Conditions:Orange G, 1.2 mL of 6.6×10^{-4} mol L^{-1}; sulfuric acid, 1.0 mL of 4.0 mol L^{-1}; bromate, 0.5 mL of 0.05 mol L^{-1}; 25 °C and 7.5 min). Inset shows the absorption spectra of the inhibitored reaction in persence of 25.0 µmol L^{-1} of codeine.

Biological sample preparation:

Human urine and serum were selected as biological samples for the determination of codeine. Urine samples were analysed using proposed method after filtration through a 0.45 µm Millipore filter. The human serums that collected from males and females were centrifuged for 5 min at 5000 rpm and diluted properly. It was then treated according to the given procedure.

RESULTS AND DISCUSSION

Orange G (also as known orange gelb), an orange powder azo based dye, useful to stain keratin in histology, color marker to following the electrophoresis process, pH indicator, dyeing of textiles, paper and leather and preparing of coloring inks. Orange G can be oxidized to a colorless product by oxidizing agents [22].

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The aim of this study is to establish a new strategy for quantitative determination of codeine. The method is based up on the inhibitory effect of codeine on the oxidation of orange G by bromate in acidic media. The possible mechanism describes as follow:

1. Orange G in reduced form oxidized by bromate in acidic media that resulted to produce bromide and Orange G in reduced form as a colorless product. The reaction is quite slow (Reaction 1).

$$\text{Orange G}_{(\text{Red})} + BrO_3^- + 6H^+ \rightarrow \text{Orange G}_{(\text{Ox})} + Br^- + 3H_2O \qquad (1)$$

2. Bromine generation down by a well known reaction between bromide and bromate in acidic media (Reaction 2).

$$BrO_3^- + 6H^+ + 5Br^- \rightarrow 3Br_2 + 3H_2O \qquad (2)$$

3. Orange G can be oxidized by generated bromine faster than bromate (Reaction 3).

$$\text{Orange G}_{(\text{Red})} + Br_2 + H^+ \rightarrow \text{Orange G}_{(\text{Ox})} + 2Br- \qquad (3)$$

4. A reducing agent (in this case codeine) reacts faster than Orange G with bromine. Therefore, decolorizing of Orange G was retarded. (Reaction 4).

$$\text{Codeine}_{(\text{Red})} + Br_2 + H^+ \rightarrow 2Br^- + \text{Codeine}_{(\text{Ox})} \qquad (4)$$

Since the level of inhibitation depends on the codeine amount, developing an analytical method for the determination of it is possible.

In order to take the maximum sensitivity of the proposed procedure, the reagents concentration and reaction conditions must be optimized. The parameters were optimized by one at the time method. The maximum difference between blank and sample signal (ΔA) was considered to obtain the most sensitive results.

Optimization of reaction variables:

The influence of Orange G concentration on the inhibitored and uninhibitored reactions was studied over the range 26.9 to 79.2 µmol L^{-1}. The response was increased up to 66.0 µmol L^{-1} whereas at higher concentrations was decreased (Fig. 2). Therefore, 66.0 µmol L^{-1} of Orange G was selected for further study. Aggregation of Orange G at higher concentrations can be resulted to this behavior.

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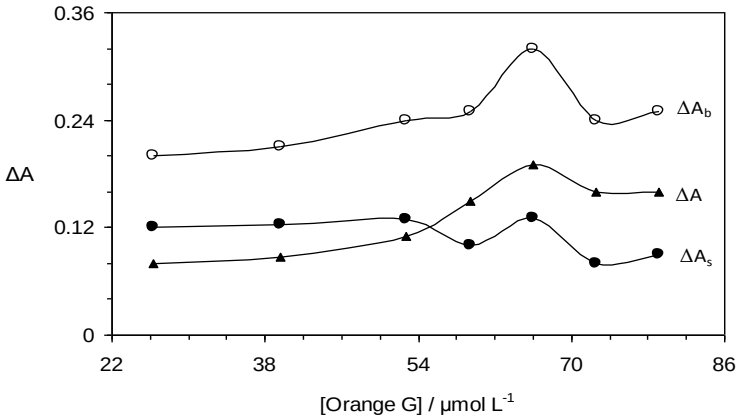

Fig. 2: Effect of Orange G concentration on the rate of uninhibitored (ΔA_b), inhibitored (ΔA_s) reactions and response (ΔA). (Conditions: sulfuric acid, 0.4 mol L^{-1}; codeine, 25.0 µmol L^{-1}; bromate, 2.5 mmol L^{-1}; 25 °C and 450 s).

The influence of sulfuric acid concentration on the response was investigated over the range 0.18 to 0.52 mol L^{-1}. The response was increased along with the sulfuric acid concentration up to 0.44 mol L^{-1} (Fig. 3). Therefore, 0.44 mol L^{-1} of sulfuric acid concentration was selected as optimum. The decrease of response at higher acidic media may be attributed to protonation of Orange G.

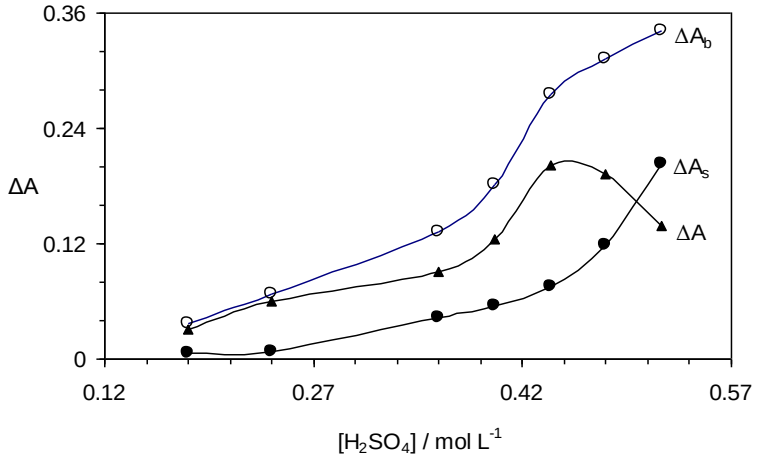

Fig. 3: Effect of sulfuric acid concentration on the rate of uninhibitored (ΔA_b), inhibitored (ΔA_s) reactions and response (ΔA). (Conditions: Orange G, 66.0 µmol L^{-1}; codeine, 25.0 µmol L^{-1}; bromate, 2.5 mmol L^{-1}; 25 °C and 450 s).

Under optimum concentration of Orange G and sulfuric acid, the influence of bromate concentration on the sensitivity was investigated in the range of 1.5 to 3.0 mmol L^{-1} (Fig. 4). The maximum sensitivity was obtained at 2.5 mmol L^{-1}. Since the absorbance change of uninhibitored is more than inhibitored reaction, the sensitivity was decreased.

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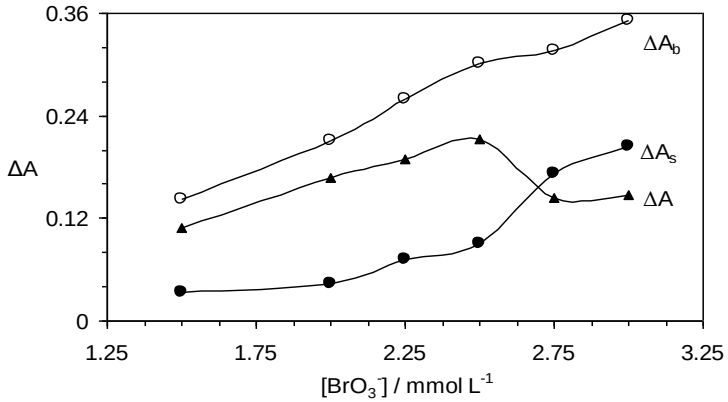

Fig. 4: Effect of bromate concentration on the rate of uninhibitored (ΔA_b), inhibitored (ΔA_s) reaction and response (ΔA). (Conditions: Orange G, 66.0 µmol L^{-1}; sulfuric acid; 0.44 mol L^{-1}; codeine, 25.0 µmol L^{-1}; 25 °C and 450 s).

The effect of temperature on the reaction rate was studied in the range 15 to 35 °C with obtained optimum conditions. The maximum sensitivity was obtained at 25 °C. Therefore, 25 °C was selected as the optimum temperature.

The optimum time was found by measuring the change in absorbance during 30 to 540 s (Fig. 5). The reaction rate was increased up to 390 s, and for longer times it was almost constant. Therefore, 390 s was selected as the optimum value.

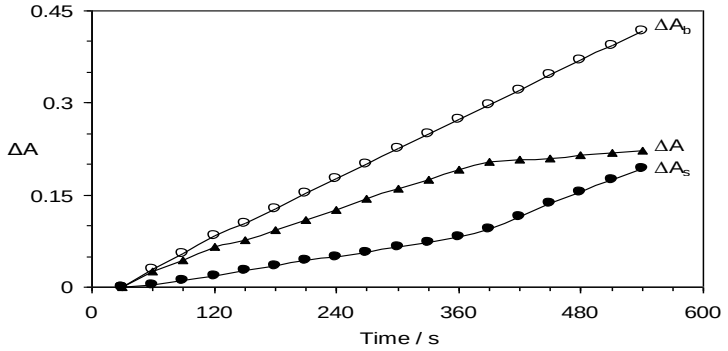

Fig. 5: Effect of time on the rate of uninhibitored (ΔA_b), inhibitored (ΔA_s) reactions and response (ΔA). (Conditions: Conditions: Orange G, 66.0 µmol L^{-1}; sulfuric acid, 0.44 mol L^{-1}; codeine, 25.0 µmol L^{-1}; bromate, 2.5 mmol L^{-1} and 25 °C).

Analytical parameters:

Calibration graph was constructed by plotting the response against codeine concentration. Under optimized experimental conditions that outlined above, calibration graph was linear over the range 0.8 – 397.9 µmol L^{-1} of codeine including two linear segments of 0.8 – 37.6 and 37.6 – 397.9 µmol L^{-1}. The regression equation of the two segments gave as equations 5 and 6, respectively.

$\Delta A = 0.0049$ [Codeine] + 0.0227 ($R^2 = 0.9988$) (5)

$\Delta A = 0.0002$ [Codeine] + 0.2023 ($R^2 = 0.9992$) (6)

Where ΔA is the difference in the absorbance of blank and sample signals, [Codeine] is the codeine concentration in µmol L^{-1} and R^2 is the correlation coefficient.

The limit of detection ($3s_b/m$; s_b is the standard deviation of the blank signal and m is the slope of calibration curve) was 0.22 µmol L^{-1} of codeine for eight replicate determinations. The relative standard deviations (n = 8) were 1.03, 0.99 and 0.98% for 2.5, 10.0 and 100.0 µmol L^{-1} of codeine, respectively.

In order to asses the possible analytical applications of the proposed method, the influence of concomitant species on the determination of codeine in real samples was studied. The tolerance limit was taken as the concentration which caused an error of 5% in the rate of the inhibitored reaction with 10.0 µmol L^{-1} of codeine. The obtaind results and normal concentration range of interferences in blood [23] are presented in Table 1. According to the results, interferences from various species commonly associated in real samples matrix were not found.

Table 1. Tolerance concentration of foreign species on the determination of 10.0 µmol L^{-1} of codeine.

Species	Tolerance concentration [a]	Normal range in blood [23]	
		Male	Female
Phosphate	2.1 mmol L^{-1}	0.8-1.4 mmol L^{-1}	0.8-1.4 mmol L^{-1}
Glucose	46.0 mmol L^{-1}	3.9-6.4 mmol L^{-1}	3.9-6.4 mmol L^{-1}
Urea	11.2 mmol L^{-1}	3.0-9.2 mmol L^{-1}	2.5-7.2 mmol L^{-1}
Creatinine	8.3 mmol L^{-1}	0.06-0.12 mmol L^{-1}	0.05-0.12 mmol L^{-1}
Cholestrol	6.1 mmol L^{-1}	<5.2 mmol L^{-1}	<5.2 mmol L^{-1}
Triglycerides	5.2 g L^{-1}	<2.2 g L^{-1}	<2.0 g L^{-1}

[a] Maximum concentration of the species that was used.

Application in real samples

Evaluation the reliability and analytical applicability of the developed method makes it potentially useful for the quantitative determination of codeine in real samples with different matrices. Human serum and urine samples were selected as real samples for the determination of codeine by the proposed method. After sample preparation that was discussed previously, they were spiked with different amounts of codeine and analysed by the recommended procedure and HPLC as another method. The results of four replicate determinations were given in Table 2. The obtained results indicated that codeine contents of samples by the two procedures are in good agreement together. The precision (RSD%) varies in the range 0.95-1.08% and 0.94-1.48% for codeine in urine and human serum, respectively. Also, the precision of the developed procedure and HPLC method was evaluated using *F*-test. The precision of the

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two methods is the same, as obtained results confirm it. Therefore, the developed method is free from interfering effect of matrix effect and suitable for analysis of codeine in biofluides.

Table 2. Determination of codeine in human urine and serum samples using the developed procedure and HPLC method.

Sample	Added (µmol L^{-1})	Proposed method			HPLC method			Statistical test
		Found [a] (µmol L^{-1})	RSD (%)	Recovery (%)	Found [a] (µmol L^{-1})	RSD (%)	Recovery (%)	*F* test
Urine (male)	2.0	1.96 ± 0.02	1.02	98.5	2.02 ± 0.02	0.99	101.0	1.0
	10.0	10.04 ± 0.10	0.98	100.4	10.03 ± 0.09	0.89	100.3	1.23
	20.0	19.95 ± 0.19	0.95	99.75	19.96 ± 0.19	0.95	99.8	1.08
Urine (female)	2.0	2.02 ± 0.02	0.99	101.0	1.98 ± 0.03	1.51	99.0	2.25
	10.0	10.12 ± 0.11	1.08	101.2	10.04 ± 0.10	0.99	100.4	1.21
	20.0	19.6 ± 0.20	1.02	98.0	20.15 ± 0.20	0.99	100.7	1.10
Serum (male)	2.0	2.03 ± 0.03	1.48	101.5	2.04 ± 0.02	0.98	102.0	2.25
	10.0	10.05 ± 0.12	1.19	100.5	9.96 ± 0.10	1.00	99.6	1.44
	20.0	19.94 ± 0.21	1.05	99.7	20.03 ± 0.21	1.05	100.2	1.0
Serum (female)	2.0	1.96 ± 0.02	1.02	98.0	1.98 ± 0.02	1.01	99.0	1.0
	10.0	10.02 ± 0.10	0.99	100.2	9.96 ± 0.10	1.00	99.6	1.0
	20.0	20.09 ± 0.19	0.94	100.4	20.11 ± 0.18	0.94	100.6	1.11

[a] Mean ± standard deviation (n = 4).
[b] Tabulated *F*-value for three degrees of freedom at P(0.95) is 9.28.

CONCLUSIONS

Codeine can be determined with a good precision and accuracy at levels as low as 0.22 µmol L^{-1} using Orange G-bromate reaction system. Since the developed procedure is free from interfering effect of co-exist speieses of real samples, the method was successfully applied for the determination of codeine in human serum urine samples without any purification or extraction that is the most advantage of the developed procedure. Other advantages of the developed method are simplicity, low cost, ease of operation and use of a common spectrophotometry instrument.

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