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Sensitive Kinetic Spectrophotometric method for Sub-micro molar Determination of Diazepam in Drug Formulations and Biological Samples.

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ABSTRCT

In this study, a sensitive reaction system for sub-micro molar determination of diazepam was introduced. The developed method is based on catalytic effect of diazepam on the Janus Green-bromate system. The change in absorbance was followed spectrophotometrically at 618 nm. The dependence of sensitivity on the reaction variables including reagents concentration, temperature and time was investigated. Under optimum experimental conditions, calibration curve was linear over the range 0.035 – 45.7 µmol L^{-1} of diazepam including two linear segments and the relative standard deviations (n=6) for 2.5 and 25.6 µmol L^{-1} of diazepam were 1.17 and 1.09%, respectively. The limit of detection was 0.029 µmol L^{-1} of diazepam. The effect of diverse species was also studied. The developed method was applied satisfactorilly for the determination of diazepam in pharmaceutical and biological samples.

Keywords*:* Janus Green-Bromate; Diazepam; Sub-micro molar; Kinetic spectrophotometry.

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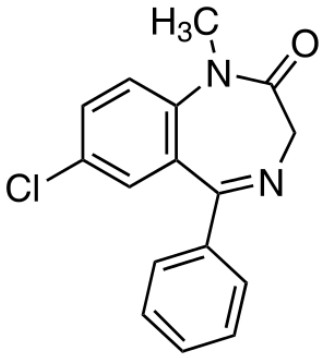
INTRODUCTION

Diazepam, see Scheme 1 for molecular structure; synthesized by Leo Sternbach and marketed as Valium by Roche, is a kind of benzodiazepine drugs [1]. It is a core medicine in the World Health Organization's Essential Drugs List, which list minimum medical needs for a basic health care system. It is used to treat a wide range of conditions, and has been one of the most frequently prescribed medications in the world since its launch in 1963 [2]. Generally, it may uses to treat insomnia, seizures, muscle spasms, alcohol withdrawal, benzodiazepine withdrawal and opiate withdrawal syndrome. In addition, it may be used before certain medical procedures to reduce tension and anxiety [1]. Anterograde amnesia and sedation as well as paradoxical effects such as excitement and depression are the adverse effects of diazepam. Long-term effects of taking the diazepam are diazepam dependence, diazepam withdrawal syndrome upon dose reduction and cognitive deficits [3]. Advantages of diazepam are a rapid onset of action and high efficacy rates, which is important for managing acute seizures, anxiety attacks and panic attacks; benzodiazepines also have a relatively low toxicity in overdose [1,4]. Given the widespread use of the drug, developing the rapid, low cost and reliable procedure for quantification of it in real samples with different matrices is necessary.

Scheme 1: Molecular structure of diazepam.



Various reports have been found for the determination of diazepam at different levels and different matrices such as chemiluminescence [5], liquid chromatography-mass spectrometry (LC/MS) [6,7], liquid chromatography-electrospray tandem mass spectrometry (LC/MS/MS) [8], adsorptive stripping voltammetry [9,10], differential pulse voltammetry [11] and flourimetry [12]. The methods have advantages such as high sensitivity and low detection limit [5-11] and shortages such as high cost [6-8,12], hard operation [6-10], low repeatability [9-11] and time consuming [9-11].

High sensitivity, sufficient accuracy, simplicity, speed and the necessity of less expensive apparatus make kinetic spectrophotometric method as an attractive method for the determination of trace elements in samples with different matrices such as foods [13], biological and pharmaceutical [14,15] samples.

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 catalytic effect of diazepam on the Janus Green–bromate reaction system. The developed method has been successfully applied for the determination of diazepam in pharmaceutical and biological samples. To the best of our knowledge, we don't have any report for the determination diazepam using kinetic spectrophotometric method.

EXPERIMENTAL

Apparatus

A double beam PG instruments UV-Vis spectrophotometer (T80+, UK) with two matched 1-cm glass cell was used to measure the absorbance-time changes at fixed wavelength. A thermostated water bath (Heidolph, Germany) was used to keep the temperature of all solutions at the working temperature of 25.0 ± 0.1°C. A stop-watch was used to record the reaction time.

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Chemicals and reagents

All chemical reagents used in this work were analar grade and were used without further purification. Diazepam (Sigma) stock solution 3.51 mmol L^{-1} was prepared just before use by dissolving 0.0025 g of diazepam in water and diluted to the mark in a 25 mL calibrated flask. An appropriate amount of the solution was used for preparing the working solution. A solution of Janus Green (4.4×10^{-4} mol L^{-1}) was prepared by dissolving 0.2270 g of Janus Green (Merck) in water and diluting to 1.0 L with water. Sulfuric acid solution (2.0 mol L^{-1}) was prepared by appropriate dilution of conc. sulfuric acid (Merck). A 0.05 mol L^{-1} of potassium bromate solution was prepared by dissolving 8.3504 g of potassium bromate (Merck) in water and diluting to 1.0 mL in a calibrated flask.

General procedure

The catlaysed reaction was studied spectrophotometrically by monitoring the change in absorbance of the reaction mixture at 618 nm (λ_{max}). For this purpose, to a series of 10 mL volumetric flasks, 1.0 mL of 2.0 mol L^{-1} sulfuric acid solution, 0.85 mL of 4.4×10^{-4} mol L^{-1} of Janus Green solution, and the sample or standard solutions containing 3.50×10^{-4} - 4.56 10^{-1} µmol of diazepam were added. The solution was mixed and diluted to 8 mL with water. Then, 0.5 mL of 0.05 mol L^{-1} bromate solution was added and diluted to the mark. A time measurement was started just after adding the last drop of the bromate solution. After thorough mixing, a portion of the solution was transferred to a glass cell. The absorbance of catalysed reaction (ΔA_s) was measured against water at λ_{max} and 15 °C for time interval 30-420 s. The measurement in the absence of diazepam was repeated to obtain the values for the uncatalysed reaction (ΔA_b). Finally, the difference in the absorbance change was considered as the response ($\Delta A = \Delta A_s - \Delta A_b$). The calibration curve was constructed by plotting the response against the diazepam concentration.

Procedure of sample preparation

Pharmaceutical samples preparation

Six diazepam tablets (in each dosage of 2 and 10 mg) were powdered and mixed thoroughly. An amount corresponding to each dosage of diazepam was weighed, dissolved with 10.0 mL of water and sonicated for 3 min. The sample was filtered through a Whatman filter paper (No. 1), transferred to a 25 mL volumetric flask and diluted to the mark with water. A suitable aliquot of the solution was used for analysis using the procedure.

Biological samples preparation

Diazepam was determined in human urine and serum as biological samples. They were spiked with diazepam and solid phase extraction technique with C_{18} cartridge (Supelco Inc., 10 mL) was used for purification and pre-concentration of diazepam from the samples. The purification and pre-concentration procedure was performed as discussed in Ref. [16]. According to the procedure, the extracted diazepam was determined.

RESULTS AND DISCUSSION

Janus Green, also named Diazin Green, for the first time was introduced by Michaelis around 1900. It is a basic dye and vital stain used in histology. It can be oxidized to a colorless product by oxidizing agents such as bromate in acidic media at a slow reaction. It was used as an indicator for the quantitative determination of different species such as opiates [15].

The poposed reaction mechanism for Janus Green-bromate system may be represented as follow:

The uncatalysed reactions that resulted to blank signal (ΔA_b) carries out in a cyclic way by the reactions 1 to 3:

$$\text{Janus Green}_{(Red)} + BrO_3^- + H^+ \rightarrow \text{Janus Green}_{(Ox)} + Br^- + 3H_2O \quad (1)$$

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

$$Janus\ Green_{(Red)} + Br_2 + H^+ \rightarrow Janus\ Green_{(Ox)} + Br^- \quad (3)$$

In the presence of diazepam, Br– generation was increased (reaction 4):

$$Diazepam_{(Red)} + Br_2 + H^+ \rightarrow Br^- + Diazepam_{(ox)} \quad (4)$$

where Red and OX are the reduced and oxidized form of reactant, respectively.

Diazepam along with the Janus Green participating on Br^- generation. Therefore, Br_2 generation was increased (reaction 2) and resulted to increasing the possibility of decolorization of Janus Green (reaction 3). Since the change in absorbance in persence of diazepam was increased seriously, the proposed reaction system has sufficient sensitivity for the determination of diazepam.

Optimization of the effective factors

To obtaining the maximum sensitivity as employing the proposed procedure, the effective factors including reagents concentration and reaction conditions must be optimized. The maximum response was considered to obtain the most sensitive results.

Optimization of the reagnts concentration

The effect of the sulfuric acid concentration on the catalyzed and uncatalyzed reactions was studied over the range of 0.12 to 0.26 mol L^{-1} (Fig. 1). The maximum sensitivity was obtained at 0.20 mol L^{-1}. At higher acid concentrations, the sensitivity was decreased. Protonation of Janus Green that makes oxidaion quite defficult resulted to the disorder. Therefore, 0.20 mol $L^{-1}$of sulfuric acid was used for further study.

The effect of Janus Green concentration on the reaction rate was studied over the range 26.6 – 44.0 µmol L^{-1}. As it an be seen in Fig. 2, the sensitivity was increased up to 37.7 µmol L^{-1} of Janus Green. At higher concentrations, the reaction rate was decreased that may be attributed to the dye aggregation. Thus, 37.7 µmol L^{-1} of Janus Green as optimum concentration was selected for further study.

The effcet of bromate concentration on the reaction rate was studied over the range 2.0 – 3.0 mmol L^{-1}. As shown in Fig. 3, the net reaction rate was increased up to 2.5 mmol L^{-1} which was selected as being the optimum concentration of oxidant.

Optimization of the reaction conditions

Under optimized reagents concentration, the effect of temperature on the reaction rate was investigated in the range of 5 to 25 °C. Increasing of temperature up to 15 °C caused an increase in the sensitivity, whereas at higher temperatures it decreased. Thus, 15 °C was selected as being the optimum temperature.

As it can be seen in Fig. 4, the optimum time was found by measuring the change in absorbance during 30 – 600 s. The reaction rate increased up to 420 s, and in longer times the reaction rate was decreased. Therefore, 420 s was selected for further study.

Analytical parameters

Calibration curve was constructed by plotting the response (ΔA) against diazepam concentration (Fig. 5). Using the developed procedure and under optimized conditions that outlined above, calibration curve was linear over the range 0.035 – 45.7 µmol $L^{-1}$of diazepam including two linear segments of 0.035 – 3.5 and 3.5 – 45.7 µmol L^{-1}. The regression equation of the two segments gaves as equations 5 and 6, respectively.

$$\Delta A = 0.0329\ [Diazepam] + 0.0076\ (R^2 = 0.9985) \quad (5)$$

$$\Delta A = 0.0097[\text{Diazepam}] + 0.0884 \quad (R^2 = 0.9989) \qquad (6)$$

where ΔA is the difference in the absorbance between the blank and the sample, [Diazepam] is the diazepam concentration in µmol L^{-1} and R^2 is the correlation coefficient.

Also, data analysis in concentration range 0.035 - 0.35 µmol $L^{-1}$of diazepam (inset of Fig. 5) gave regression equation ΔA = 0.0317 [Diazepam] + 0.0077 (R^2= 0.9976) and shows the good agreement between results in low concentrations.

Figure 1: Effect of sulfuric acid concentration on the rate of uncatalysed (ΔA_b) and catalysed (ΔA_s) reactions and response (ΔA). (Conditions: sulfuric acid 0.12 - 0.28 mol L^{-1}; Janus Green, 26.6 µmol L^{-1}; diazepam, 0.176 µmol; bromate, 2.5 mmol L^{-1}; 20 °C and 6.0 min).

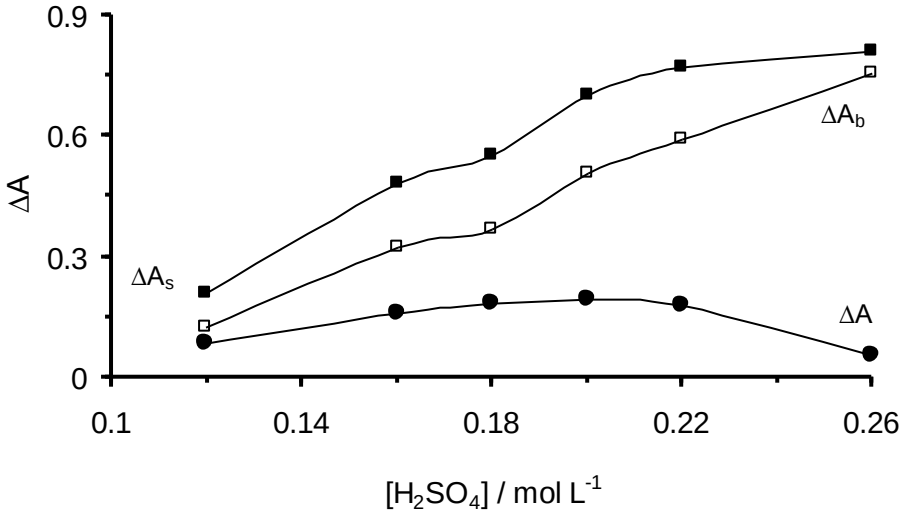

Figure 2: Effect of Janus Green concentration on the rate of uncatalysed (ΔA_b) and catalysed (ΔA_s) reactions and response (ΔA). (Conditions: sulfuric acid 0.2 mol L^{-1}; Janus Green, 26.6 – 44.0 µmol L^{-1}; diazepam, 0.176 µmol; bromate, 2.5 mmol L^{-1}; 20 °C and 6.0 min).

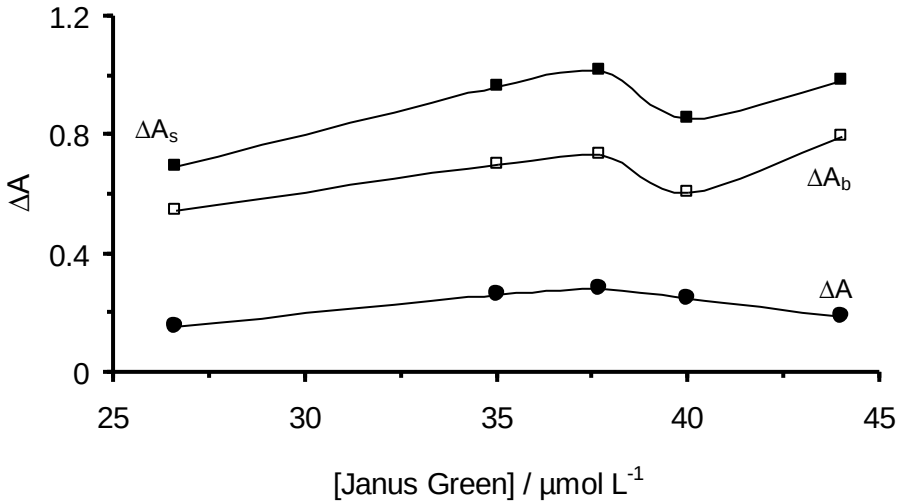

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Figure 3: Effect of bromate concentration on the rate of uncatalysed (ΔA_b) and catalysed (ΔA_s) reactions and response (ΔA). (Conditions: sulfuric acid 0.2 mol L^{-1}; Janus Green, 37.7 µmol L^{-1}; diazepam, 0.176 µmol; bromate, 2.0 – 3.0 mmol L^{-1}; 20 °C and 6.0 min).

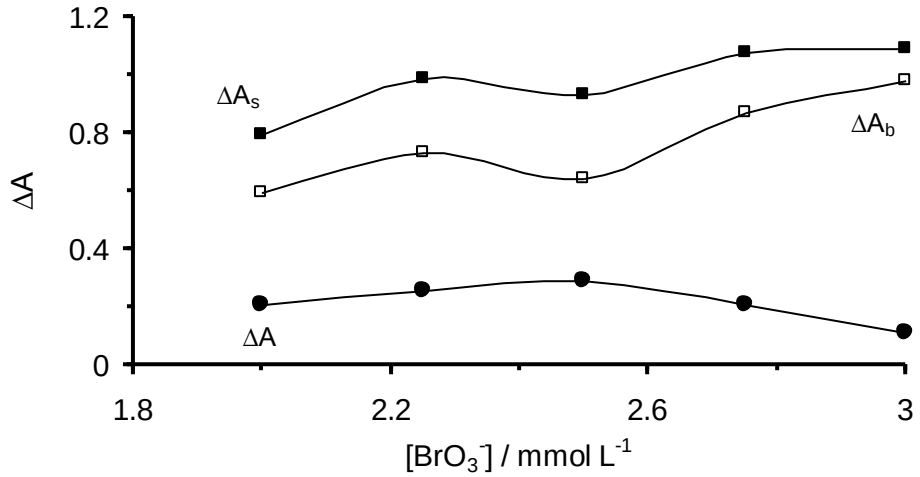


Figure 4: Effect of time on the rate of uncatalysed (ΔA_b) and catalysed (ΔA_s) reactions and response (ΔA). (Conditions: sulfuric acid 0.2 mol L^{-1}, Janus Green, 37.7 µmol L^{-1}; diazepam, 0.176 µmol; bromate, 2.0 mmol L^{-1}; 25 °C and 0.5 - 10.0 min).

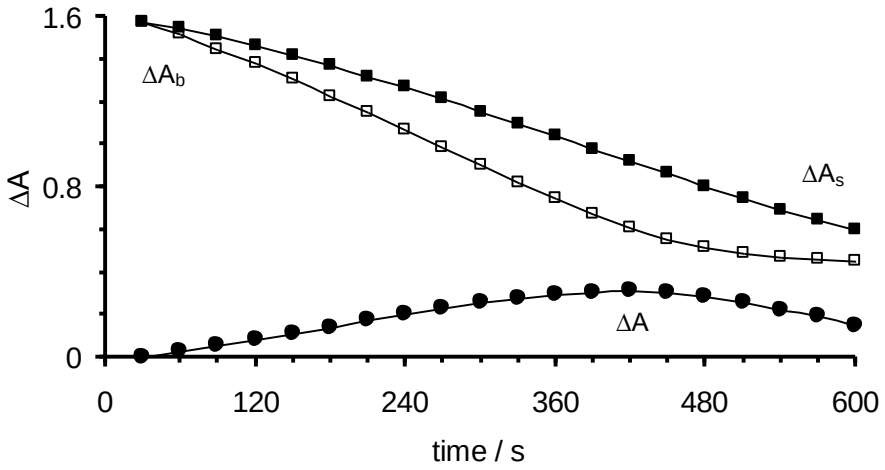
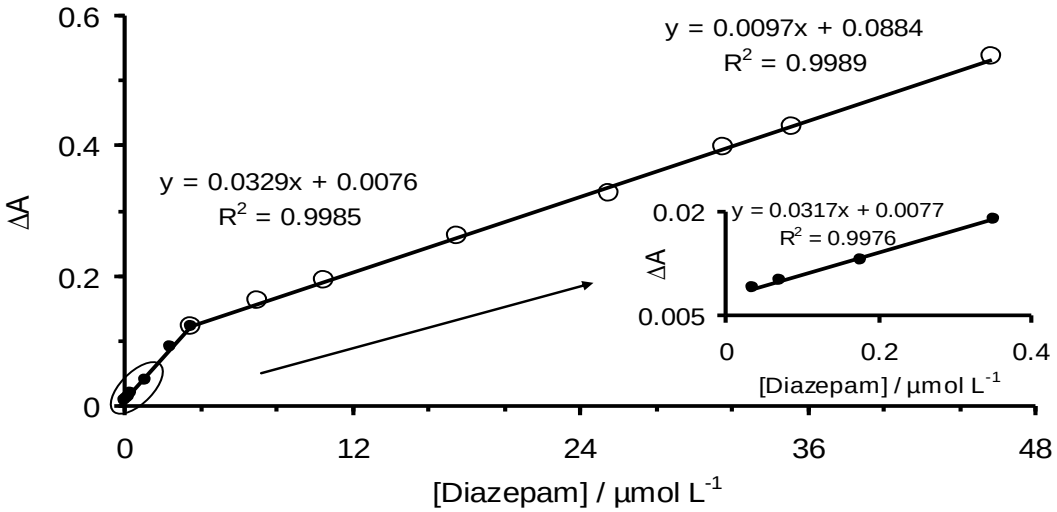


Figure 5: Calibration curve in concentration range 0.035 – 3.5 and 3.5 – 45.7 µmol L^{-1}. Inset shows a part of calibration curve in concentration range 0.035 – 0.35 µmol L^{-1}.



ISSN: 0975-8585

Table 1. Tolerance limit for foreign species on the determination of 7.0 µmol L^{-1} of diazepam.	
Foreign species	Tolerance limit ($W_{Diazepam}/W_{species}$)
Na^+, K^+, NH_4^+	1000
SO_4^{2-}	1000
HCO_3^-, CO_3^{2-}, NO_3^-	970
Saccarose, fructose, glucose	960
Ethanol	930
Urea, uric acid	895
Lysine, glycine	520
I^-, Br^-, Cl^-, NO_2^-	<1

Table 2. Determination of diazepam in diazepam tablet in dosages 2 and 10 mg/tablet using the developed procedure.					
Sample	Found[a] (mg/tablet)	RSD (%)	Labled (mg/tablet)	Statistical *t* test[b]	Pharmaceutical Co /Batch No.
Diazepam tablet					
1	2.02 ± 0.02	0.99	2	2.00	Arya-Iran/023
2	1.99 ± 0.02	1.00	2	1.00	Arya-Iran/024
Diazepam tablet					
1	9.94 ± 0.11	1.11	10	1.09	Abidi-Iran/108
2	10.04 ± 0.10	0.99	10	0.80	Abidi-Iran/110
[a]Mean±standard deviation (n=4) [b]Tabulated *t*-value for three degrees of freedom at P(0.95) is 3.18.					

Table 3. Determination of diazepam in human serum and urine samples using the developed procedure.				
Sample	Added (µmol L^{-1})	Found[a] (µmol L^{-1})	RSD (%)	Recovery (%)
Human urine				
1	—	<D.L	—	<D.L
	2.5	0.03 ± 2.44	1.23	97.6
	25.0	0.28 ± 25.21	1.11	100.8
	35.0	0.41 ± 35.42	1.16	101.2
Human serum				
1	—	<D.L	—	<D.L
	2.5	0.03 ± 2.54	1.18	101.6
	25.0	0.27 ± 24.78	1.09	99.1
	35.0	0.37 ± 35.21	1.05	100.6
[a]Mean±standard deviation (n=4)				

Limit of detection and precision

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 0.029 µmol L^{-1} of diazepam for eight replicate determinations. The relative standard deviations (n = 6) were 1.17, 1.09% for 2.5 and 25.6 µmol L^{-1} of diazepam, respectively.

Interference Studies

The interfering effect of foreign species on the determination of 7.0 µmol L^{-1} of diazepam was investigated. The tolerance limit was defined as the concentration of the added species causing an error (analytical signal) more than ± 5%. The results are given in Table 1. The obtained results show that halide ions and nitrite have seriously interfering effect, whereas not exist in real sample matrix.

Real Sample Analysis

Evaluation the reliability and analytical applicability of the developed method makes it potentially useful for the quantitative determination of diazepam in real samples with different matrices. Pharmaceutical sample preparation was performed using the mentioned procedure. An appropriate amount of the samples

were analysed by the recommended procedure. The results of four replicate determinations were given in Table 2. The precision (RSD%) varies in the range 0.99-1.00% and 0.99-1.11% for diazepam tablet in dosage 2 and 10 mg, respectively. The statistical *t*-test did not show any significant difference between the obtained results and certified value for tablets (the 95% confidence level and three degrees of freedom). Also, the procedure was used for the determination of diazepam in urine and serum samples. After sample preparation, as discussed previously, they were spiked with different amounts of diazepam and analysed using recommended procedure. The obtained results were given in Table 3. The samples were spiked with 2.5, 25.0 and 35.0 µmol L^{-1} of diazepam and accuracy of the procedure were confirmed by recovery. The recoveries for spiked urine and serum samples vary over the range 97.6-101.2%. and 99.1-101.6%, respectively Successive applications of developed method for drug determination in pharmaceutical preparations and urine samples were performed. Therefore, the developed method is free from interfering effect of matrix effect and suitable for analysis of diazepam in different samples.

CONCLUSIONS

This study reports a sensitive and relatively selective spectrophotometric method for the detrmination of diazepam using Janus Green as a sensitive and selective reagent. The developed method possesses distinct advantages over chromatographic methods in cost, simplicity, ease of operation and applicable to real samples analysis. Moreover, the reliability of this method permits the analysis of pharmaceutical and biological samples with satisfactory results.

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