## DETERMINATION OF LORAZEPAM IN DRUG FORMULATION AND BIO FLUIDS USING A SPECTROPHOTOMETRIC METHOD AND RESPONSE SURFACE METHODOLOGY[**]

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*A novel, simple, sensitive, and selective kinetic spectrophotometric method has been developed for the determination of lorazepam in pharmaceutical and bioloical samples. The procedure is based on the catalytic effect of lorazepam on the Janus Green-bromate reaction system. The change in absorbance was followed spectrophotometrically at 618 nm. To obtain the maximum sensitivity, the reagents concentration, temperature, and time were optimized by one at the time method. Under optimum experimental conditions, the calibration curve was linear over the range 0.3–19.5 μg/mL of lorazepam, including two linear segments. The relative standard deviations (n = 5) for 1.0, 5.0, and 15.0 μmol/L of lorazepam were 1.09, 1.03, and 0.97%, respectively. The limit of detection was 0.08 μg/mL of lorazepam. An experimental check under these optimal conditions confirmed good agreement in the RSM results. The developed method was successfully applied for the determination of lorazepam in real samples, and the obtained results are in a good agreement with those using HPLC.*

***Keywords:*** *lorazepam, kinetic spectrophotometry, response surface methodology, drug formulation, biofluides.*

## ОПРЕДЕЛЕНИЕ ЛОРАЗЕПАМА В ЛЕКАРСТВЕННЫХ ПРЕПАРАТАХ И БИОЛОГИЧЕСКИХ ЖИДКОСТЯХ С ИСПОЛЬЗОВАНИЕМ ЧУВСТВИТЕЛЬНОЙ СПЕКТРОФОТОМЕТРИИ И МЕТОДОЛОГИИ ПОВЕРХНОСТИ ОТКЛИКА

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*Разработан простой, чувствительный и селективный спектрофотометрический метод определения лоразепама в фармацевтических препаратах и биологических образцах. Процедура основана на каталитическом влиянии лоразепама на реакционную систему Янус Грин-бромат. Изменение оптической плотности зарегистрировано с помощью спектрофотометра на λ = 618 нм. Для получения максимальной чувствительности концентрация реагентов температура и время оптимизировались последовательно по одному параметру. При оптимальных условиях эксперимента калибровочная кривая в диапазоне концентраций 0.3–19.5 мкг/мл лоразепама состоит из двух линейных участков. Относительные стандартные отклонения (n = 5) для концентраций лоразепама 1.0, 5.0 и 15.0 мкмоль/л составили 1.09, 1.03 и 0.97 % соответственно. Предел обнаружения лоразепама 0,08 мкг/мл. Экспериментальная проверка в оптимальных условиях в соответствии со схемой спецификаций требований показала хорошие результаты. Разработанный метод успешно применен для определения лоразепама в реальных образцах. Результаты хорошо согласуются с полученными методами высокоэффективной жидкостной хроматографии.*

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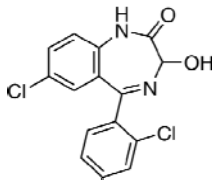
***Ключевые слова:*** *лоразепам, кинетическая спектрофотометрия, методология поверхности отклика, лекарственная форма, биожидкости.*

**Introduction.** Kinetic methods have certain advantages in pharmaceutical analysis regarding selectivity and elimination of additive interferences, which affect direct spectrophotometric methods [1, 2]. The literature is still poor in analytical assay methods based on kinetics for the determination of lorazepam in dosage forms. Some specific advantages that the kinetic methods possess are as follows: simple and fast because some experimental steps such as filtration, extraction, etc., are avoided prior to absorbance measurements; high selectivity since they involve the measurement of the absorbance as a function of reaction time instead of measuring the concrete absorbance value; other active compounds present in the commercial dosage forms may not interfere if they resist the chemical reaction conditions established for the proposed kinetic method; and colored and/or turbid sample background may not interfere with the determination process [3–6].

Lorazepam (7-chloro-5-(2-chlorophenyl)-3-hydroxy-2,3-dihydro-1H-1, 4-benzo-diazepin-2-one) is sold under the trademark Ativan; it was introduced in 1977 by D. J. Richards [7–9].




Molecular structure of lorazepam.

It is a kind of benzodiazepines that is often used as a sedative. Lorazepam has all intrinsic benzodiazepine effects such as anterograde antiemesis, amnesia, anticonvulsion, and muscle relaxation. Lorazepam is used for the short-term treatment of anxiety, insomnia, acute seizures, and sedation of hospitalized patients, as well as sedation of aggressive patients [10–13]. Also, lorazepam is the most common benzodiazepine used to decrease the likelihood of agitation and seizures in patients who have overdosed on stimulant drugs [14]. Peak effects roughly coincide with peak serum levels, which occur 10 min after intravenous injection, up to 60 min after intramuscular injection, and 90 to 120 min after oral administration. Among benzodiazepines, lorazepam has a relatively high addictive potential. Therefore, accurate and sensitive determination of lorazepam content of biological samples can be helpful for clinical and forensic purposes [15–19].

A small number of analytical techniques have been reported for the determination of lorazepam. The methods include high-performance liquid chromatography, gas chromatography, gas-liquid chromatography, gas chromatography-mass spectrometry [20–22], stripping voltammetry, and chemometrics-assisted spectrophotometry [23, 24]. Chromatographic methods have disadvantages such as high cost and difficulty of operation. Shortages such as low repeatability are the characteristic of electrochemical methods. Also, the spectrophotometric method has a small linear dynamic range [23].

Another part of the present study was to use response surface methodology (RSM) and find a suitable approximating function in order to predict and determine the future response, and to investigate the optimum operating conditions in a region for which the factors at a certain operating specification are met. RSM is essentially a particular set of mathematical and statistical methods for designing experiments, building models, evaluating the effects of variables, and searching optimum conditions of variables to predict targeted responses [25–27]. RSM is an important branch of experimental design and a critical tool in developing new processes, optimizing their performance, and improving design and formulation of new products. Its greatest applications have been in industrial research, particularly in situations where a large number of variables influence the system feature. This feature, which is termed response and normally measured on a continuous scale, represents the most important function of the system [28, 29]. One is often interested in finding a suitable approximating function for the purpose of predicting and determining the future response. Response surface procedures are not only primarily used for the purpose to understand the mechanism of the system or process; rather its purpose is to determine the optimum operating conditions or to determine a region for the factors at a certain operating specification [30–32].

The current research, for the first time, describes a kinetic spectrophotometric method, and RSM were applied to investigate the effect of process parameters for quantitative determination of lorazepam. The preliminary studies show that lorazepam has a strong catalytic effect on the oxidation of Janus Green (JG) by

bromate in acidic media. Wide linear dynamic range, low detection limit, and short analysis time make the proposed method a new prospect for the determination of lorazepam in biological and pharmaceutical samples.

**Experimental.** *Reagent and chemicals.* Redistilled water and analytical grade reagent chemicals were used; 25 mL of 100.0 μg/mL of lorazepam solution was prepared by dissolving 0.0025 g of lorazepam (Sigma) in water daily; 10.0 μg/mL of working solution was prepared by diluting appropriate amount of the solution in 50 mL volumetric flask. A solution of Janus green ($4.4\times10^{-4}$ mol/L) was prepared by dissolving 0.4540 g of it in water and then diluting to 1 L in a volumetric flask. Sulfuric acid (2.0 mol/L) was prepared by appropriate dilution of concentrated acid solution (Merck); $5.0\times10^{-2}$ mol/L of potassium bromate solution was subsequently prepared by dissolving 8.3540 g of $KBrO_3$ (Merck) in water and diluting to 1 L in volumetric flask. Lorazepam tablet was purchased from Abidi Pharmaceutical Co (Tehran, Iran).

*Apparatus.* A double beam Unique UV-Vis spectrophotometer (T80+, UK) with 1 cm matched glass cells was used to measure the absorbance. A thermostat water bath (Hieldolph, Germany) was used to keep the temperature of all solution at the working temperature (20±0.1°C). A stopwatch was used to record the reaction time.

*General procedure.* After initial kinetic spectrophotometric studies of the reaction system, the reagent concentrations (except the catalyst) were judiciously chosen for the analytical procedure. The catalyzed reaction was studied spectrophotometrically by monitoring the change in absorbance of the reaction mixture at 618 nm. To a series of 10 mL volumetric flasks, 0.8 mL of 2.0 mol/L sulfuric acid solution, 0.6 mL of $4.4\times10^{-4}$ mol/L Janus Green solution, and 1.0 mL of 10.0 μg/mL of lorazepam solution were added. The solution was mixed and diluted with water. Then 0.5 mL of $5.0\times10^{-2}$ mol/L bromate solution was added, and the volume was adjusted to the mark with water. The time measurement started just after adding the last drop of the oxidant solution. The solution was thoroughly mixed, and a portion of it was transferred to a glass cell. The absorbance of catalyzed reaction ($\Delta A_s$) was measured against water at 618 nm at 20°C and time interval 30–360 s. The measurement in the absence of lorazepam was repeated to obtain the values for the uncatalyzed reaction ($\Delta A_b$). Finally, the difference in the absorbance change was considered as the response ($\Delta A = \Delta A_s - \Delta A_b$). Under optimum experimental conditions, calibration curve was constructed by plotting the response against lorazepam concentration in lorazepam working standard solutions. The experimental data were also optimized using the RSM. According to ANOVA results, the proposed model can be used to navigate the design space.

*Analysis of the real sample. Pharmaceutical sample preparation.* Lorazepam tablet was used as pharmaceutical sample. Sample preparation was done as below: ten lorazepam tablets (in each dose of 1.0 mg/tablet and 5.0 mg/tablet) were powdered and mixed thoroughly. An amount corresponding to 5.0 mg of lorazepam was weighed and dissolved in water in the presence of a few drops of ethanol and diluted to 20 mL. After 10 min of sonication, the sample was filtered through Whatman filter paper (No. 1), transferred to a 25 mL volumetric flask. and diluted to the mark with water.

*Biological sample preparation.* Human serum and urine were used as biological samples for the determination of lorazepam. Real samples were prepared from a patient (female, 20 years old) with a time interval 4 h after oral administration of lorazepam tablet (5 mg/tablet). They were spiked with lorazepam, and the solid phase extraction technique with $C_{18}$ cartridge (Supelco Inc., 10 mL) was used for purification and pre-concentration of lorazepam from the samples. The extracted lorazepam was determined by the developed method.

*Experimental design and optimization.* The experimental results were subsequently analyzed by using the RSM for the experimental design and to find optimized conditions. In this study, the catalytic effect of lorazepam on the oxidation of Janus Green (JG) by bromate in acidic media was optimized using RSM by Design Expert 11.0.3.0. The I-optimal criterion can be used to select points for a mixture design in a constrained region. This criterion selects design points from a list of candidate points so that the variances of the model regression coefficients are minimized [33]. The set of candidate points to use should depend upon the order of the model the experimenter wishes to fit. The independent variables of sulfuric acid concentration, Janus green concentration, bromate concentration, temperature, and reaction time were coded in the I-optimal design against the catalytic effect of lorazepam response (dependent variable). The I-optimal designed experiments were carried out with five replications in order to evaluate the pure error and were carried out in randomized order as required in many design procedures. The performance of the process was evaluated by analyzing the response of absorb percent. In the optimization process, the responses can be simply related to the chosen factors by linear or quadratic models. A quadratic model, which also includes the linear model, is given as [34]:

$$\eta = \beta_0 = \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{i} \sum_{<j=2}^{k} \beta_{ij} x_i x_j + e_i \quad , \tag{1}$$

where $\eta$ is the response, $x_i$ and $x_j$ are variables, $\beta_0$ is the constant coefficient, $\beta_j$, $\beta_{jj}$, and $\beta_{ij}$ are the interaction coefficients of the linear, quadratic, and second-order terms, respectively, and $e_i$ is the error. In the study, absorbance percent data were processed by Eq. (1), including ANOVA to obtain the interaction between the process variables and the response. The quality of fit of the polynomial model was expressed by the coefficient of determination, namely $R^2$ and $R^2_{adj}$.

The experimental conditions run and absorb are shown in Table 1.

TABLE 1. The range of independent variables and the observed responses in the experimental design

| Factors | Name | Low level | High level |
|---|---|---|---|
| A | Janus green concentration, μmol/L | 22.0 | 35.2 |
| B | Bromate concentration, mmol/L | 2.0 | 3.2 |
| C | Sulfuric acid concentration, mol/L | 0.16 | 0.22 |
| D | Temperature, °C | 15 | 30 |
| E | Time, s | 30 | 540 |

| | Factors | | | | | Response |
|---|---|---|---|---|---|---|
| Run | A | B | C | D | E | Absorbance (%) |
| 1 | 33.1 | 2.0 | 0.22 | 17.4 | 45 | 0.02 |
| 2 | 26.8 | 2.4 | 0.22 | 27.4 | 540 | 0.32 |
| 3 | 35.2 | 3.2 | 0.17 | 30.0 | 81 | 0.19 |
| 4 | 22.0 | 2.7 | 0.19 | 22.5 | 310 | 0.43 |
| 5 | 29.8 | 2.6 | 0.16 | 21.1 | 30 | 0.09 |
| 6 | 35.2 | 3.2 | 0.16 | 26.3 | 540 | 0.28 |
| 7 | 29.6 | 3.2 | 0.22 | 21.4 | 293 | 0.31 |
| 8 | 35.2 | 2.7 | 0.22 | 15.0 | 540 | 0.34 |
| 9 | 22.0 | 3.0 | 0.22 | 15.0 | 30 | 0.25 |
| 10 | 22.0 | 2.0 | 0.17 | 15.0 | 30 | 0.10 |
| 11 | 35.2 | 3.2 | 0.19 | 15.0 | 76 | 0.07 |
| 12 | 25.3 | 3.2 | 0.16 | 15.0 | 540 | 0.27 |
| 13 | 22.0 | 3.2 | 0.22 | 30.0 | 30 | 0.13 |
| 14 | 22.0 | 2.0 | 0.22 | 30.0 | 30 | 0.05 |
| 15 | 28.6 | 2.7 | 0.19 | 30.0 | 310 | 0.71 |
| 16 | 22.0 | 2.7 | 0.19 | 22.5 | 310 | 0.67 |
| 17 | 35.2 | 2.1 | 0.16 | 30.0 | 481 | 0.11 |
| 18 | 22.0 | 2.0 | 0.16 | 30.0 | 540 | 0.26 |
| 19 | 22.0 | 2.0 | 0.22 | 15.0 | 433 | 0.31 |
| 20 | 22.0 | 3.2 | 0.16 | 30.0 | 30 | 0.02 |
| 21 | 29.9 | 2.0 | 0.19 | 21.0 | 540 | 0.28 |
| 22 | 29.6 | 3.2 | 0.22 | 21.4 | 293 | 0.6 |
| 23 | 35.2 | 2.1 | 0.16 | 15.0 | 310 | 0.32 |
| 24 | 35.2 | 2.0 | 0.17 | 30.0 | 30 | 0.07 |
| 25 | 29.9 | 2.0 | 0.19 | 21.0 | 540 | 0.36 |
| 26 | 22.0 | 2.7 | 0.19 | 22.5 | 310 | 0.43 |
| 27 | 35.2 | 2.0 | 0.22 | 30.0 | 438 | 0.39 |
| 28 | 24.5 | 3.2 | 0.17 | 15.0 | 134 | 0.19 |

**Results and discussion.** JG is a basic dye of a mono-azo group that can be oxidized by oxidizing agents such as bromate in acidic media at a slow reaction to produce a colorless oxidized form. It was used as an indicator for the catalytic determination of different species such as vanadate [35]. The absorption spectra of catalyzed and uncatalyzed reaction mixture at different time intervals are shown in Fig. 1 and the inset. As can be seen, the change in absorbance increases in the presence of lorazepam. Therefore, the sensitive proposed reaction system can be used for the determination of trace amounts of lorazepam. The proposed mechanism of the reaction for the oxidation of JG can be described by the following reactions:

$$JG_{(Red)} + BrO_3^- + H^+ \rightarrow JG_{(Ox)} + Br^- \quad (2)$$

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

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

In the presence of lorazepam, which has a catalytic effect, bromide generation was increased. This may be attributed to the following reaction:

$$\text{Lorazepam}_{(Red)} + BrO_3^- + H^+ \rightarrow \text{Lorazepam}_{(Ox)} + Br^-, \quad (5)$$

where Red is the reduced form and Ox is the oxidized form of reactant.

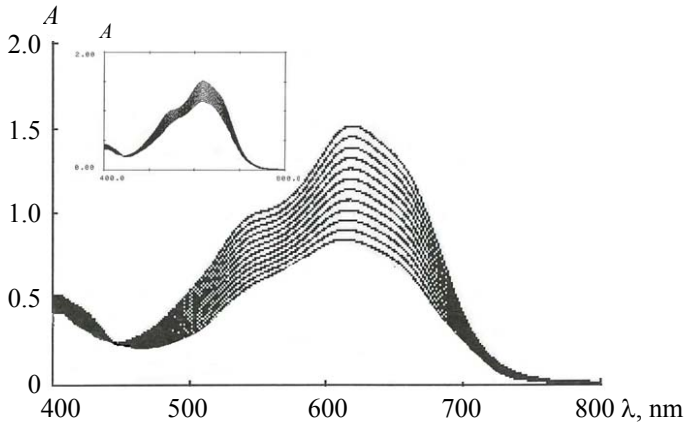



Fig. 1. The spectra of the sample (conditions: sulfuric acid 0.16 mol/L, Janus Green 28.6 μmol/L, lorazepam 10.0 μg, bromate 2.5 mmol/L, 20°C, and 6.0 min). Inset shows blank spectra that were recorded in the absense of lorazepam.

*Optimization of reaction variables. Optimization using kinetic spectrophotometric method.* In order to establish the experimental conditions under which the catalytic effect of lorazepam, and therefore the sensitivity in its determination, is maximum, the dependence of the reaction rate on reagent concentration, temperature, and time were studied. The change in absorbance after a fixed time as a measure of initial rate was used to plot the graph for each variable. Optimum conditions were taken from the graphs for the subsequent study of the variables. The reagent concentration optimization was carried out on the uncatalyzed and catalyzed reactions for a constant time of 360 s in the presence of 10.0 μg of lorazepam.

*Effect of sulfuric acid concentration.* The effect of sulfuric acid concentration on the uncatalyzed and catalyzed reactions was studied in the concentration range 0.16 to 0.22 mol/L. As shown in Fig. 2, the reaction rate increases with increasing concentration of sulfuric acid up to 0.19 mol/L. At higher concentrations, the reaction rate decreased. This decrease at higher acidic conditions may be attributed to the protonation of JG, which might stop oxidation or make oxidation difficult. Thus, 0.19 mol/L of sulfuric acid was used for further study.

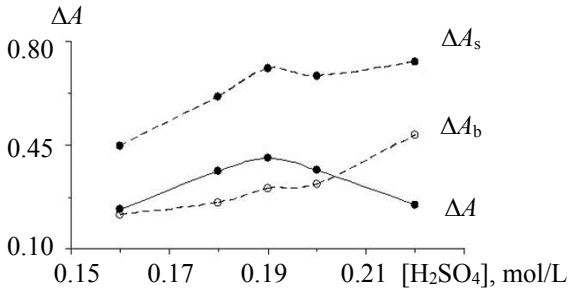



Fig. 2. Effect of sulfuric acid concentration on the rate of uncatalyzed ($\Delta A_b$) and catalyzed ($\Delta A_s$) reactions and response ($\Delta A$) (conditions: sulfuric acid 0.16–0.22 mol/L, Janus Green 26.4 μmol/L, lorazepam 10.0 μg, bromate 2.5 mmol/L, 20°C, and 6.0 min).

*Effect of Janus Green concentration.* The experimental results on the study of JG concentration effect in the range 22.0 to 35.2 μmol/L indicates that the difference in absorbance increases with concentration of JG up to 31.1 μmol/L (Fig. 3). Therefore, 31.1 μmol/L of JG was selected as the optimum value.

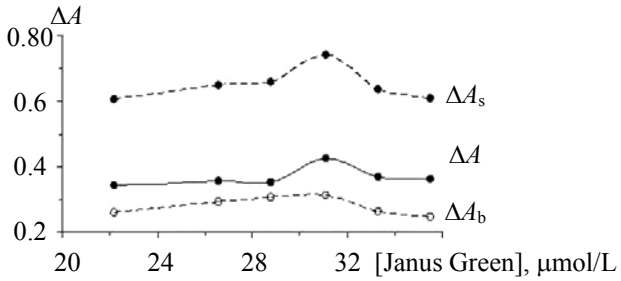



Fig. 3. Effect of Janus Green concentration on the rate of uncatalysed ($\Delta A_b$) and catalysed ($\Delta A_s$) reactions and response ($\Delta A$) (conditions: sulfuric acid 0.19 mol/L, Janus Green 22.0–35.2 μmol/L, lorazepam 10.0 μg, bromate 2.5 mmol/L, 20°C, and 6.0 min).

*Effect of bromate concentration.* The dependence of oxidation reaction rate on bromate concentration was studied in the concentration range of 2.0 to 3.2 mmol/L. As shown in Fig. 4, under optimum concentrations of $H_2SO_4$ and JG, the reaction rate increased up to 2.5 mmol/L of bromate. Therefore, the optimum value of 2.5 mmol/L of bromate was selected for the procedure.

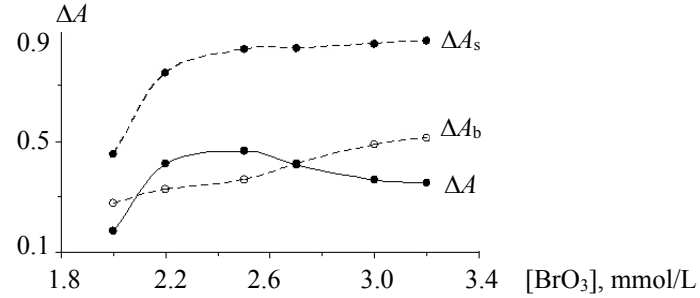



Fig. 4. Effect of bromate concentration on the rate of uncatalyzed ($\Delta A_b$) and catalyzed ($\Delta A_s$) reactions and response ($\Delta A$) (conditions: sulfuric acid 0.19 mol/L, Janus Green 31.1 μmol/L, lorazepam 10.0 μg, bromate 2.0–3.2 mmol/L, 20°C, and 6.0 min).

*Effect of temperature.* Under optimum reagent concentration, the temperature effect on the rate of reaction was studied in the range of 15–30°C. The maximum sensitivity was obtained at 25°C and selected as the optimum.

*Effect of reaction time.* The optimum time was found by measuring the change in the absorbance during 30–540 s. The reaction rate increased up to 330 s, and for longer times it decreased (Fig. 5). Therefore, 330 s was selected as optimum for further study.

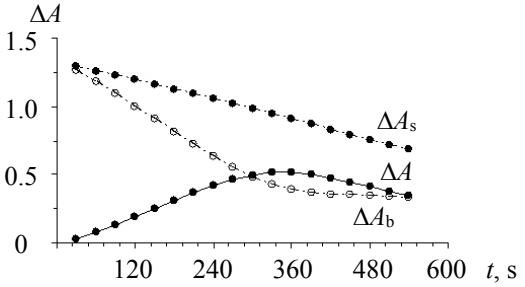



Fig. 5. Effect of time on the rate of uncatalyzed ($\Delta A_b$) and catalyzed ($\Delta A_s$) reactions and response ($\Delta A$) (conditions: sulfuric acid 0.19 mol/L, Janus Green 31.1 μmol/L, lorazepam 10.0 μg, bromate 2.5 mmol/L, 25°C, and 0.5–9.0 min).

*Optimization using response surface methodology.* In this study, the effect of operating variables of sulfuric acid concentration, Janus Green concentration, bromate concentration, temperature, and reaction time was investigated using response surface methodology according to the I-optimal design.

The batch runs were conducted in the I-optimal designed experiments to visualize the effects of independent factors on the response and the results along with the experimental conditions. Multiple regression analysis of experimental data was performed, and the model equation in terms of actual factors was obtained:

$$(\text{Absorb }\%)^{1/2} = -1.269 - 0.032A + 0.712B + 17.240C - 0.061D + 0.002E + 0.004AB +$$
$$+ 0.033AC + 0.0009AD - (8.510\times10^{-7})AE + 2.335BC + 0.004BD + 0.00003BE + 0.115CD - \quad (6)$$
$$0.0006CE - (6.348\times10^{-6})DE - 0.0001A^2 - 0.256B^2 - 66.765C^2 + 0.0001D^2 - (2.683\times10^{-6})E^2.$$

The ANOVA results of this quadratic model presented in Table 2 indicate that it can be used to navigate the design space. The model *F*-value of 3.13 in this table implies that the model is significant for the absorbance, and there is only a 3.38% chance that this large model *F*-value can occur due to noise. The adequate precision ratio of 8.395 indicates an adequate signal where it measures the signal-to-noise ratio; a ratio greater than 4 is desirable. *P*-values less than 0.0500 indicate that the model terms are significant, whereas values greater than 0.1000 are usually considered as nonsignificant. In designed experiments, $R^2$ is a measure of the amount of reduction in the variability of the response obtained by using the independent factor variables in the model. A high $R^2$ coefficient indicates a satisfactory adjustment of the proposed model to the experimental. However, a high value of $R^2$ does not necessarily imply that the regression model is a good one. Although, $R^2$ always increases on adding terms to the model, using an adjusted $R^2$ is preferred. There is a good chance that insignificant terms have been included in the model when predicted $R^2$ and adjusted $R^2$ differ dramatically. The proposed model fitted very well to the experimental data, and $R^2$ of 0.9319 is in reasonable agreement with the $R^2_{adj}$ of 0.7956.

TABLE 2. ANOVA results of the established model for responses.

| Source | Sum of squares | D*f* | Mean square | F-value | P-value |
|---|---|---|---|---|---|
| Model | 0.8779 | 20 | 0.0439 | 3.13 | 0.0338 Significant |
| A) Janus Green conc. | 0.0102 | 1 | 0.0102 | 0.7236 | 0.4149 |
| B) Bromate conc. | 0.0282 | 1 | 0.0282 | 2.01 | 0.1864 |
| C) Sulfuric acid conc. | 0.0257 | 1 | 0.0257 | 1.83 | 0.2059 |
| D) Temp. | 0.0018 | 1 | 0.0018 | 0.1292 | 0.7268 |
| E) Time | 0.3247 | 1 | 0.3247 | 23.14 | 0.0007 |
| AB | 0.0048 | 1 | 0.0048 | 0.3410 | 0.5722 |
| AC | 0.0006 | 1 | 0.0006 | 0.0405 | 0.8445 |
| AD | 0.0308 | 1 | 0.0308 | 2.19 | 0.1693 |
| AE | 0.000 | 1 | 0.0000 | 0.0019 | 0.9663 |
| BC | 0.0222 | 1 | 0.0222 | 1.58 | 0.2375 |
| BD | 0.0045 | 1 | 0.0045 | 0.3196 | 0.5843 |
| BE | 0.0003 | 1 | 0.0003 | 0.0244 | 0.8791 |
| CD | 0.0087 | 1 | 0.0087 | 0.6165 | 0.4506 |
| CE | 0.0004 | 1 | 0.0004 | 0.0257 | 0.8759 |
| DE | 0.0019 | 1 | 0.0019 | 0.1325 | 0.7234 |
| $A^2$ | 0.0002 | 1 | 0.0002 | 0.0175 | 0.8975 |
| $B^2$ | 0.0359 | 1 | 0.0359 | 2.56 | 0.1407 |
| $C^2$ | 0.0137 | 1 | 0.0137 | 0.9798 | 0.3456 |
| $D^2$ | 0.0002 | 1 | 0.0002 | 0.0166 | 0.9001 |
| $E^2$ | 0.1290 | 1 | 0.1290 | 9.19 | 0.0126 |
| Residual | 0.1403 | 10 | 0.0140 | | |
| Lack of fit | 0.0848 | 5 | 0.0170 | 1.53 | 0.3270 |
| Pure error | 0.0555 | 5 | 0.0111 | | |

The absorbance percent response surface graphs are shown in Figs. 6. The effect of Janus green concentration and bromate concentration on the absorbance percent at optimum 0.19 mol/L sulfuric acid, 22.5°C, and 285 s is shown in Fig. 6a as a semispherical response surface plot. The absorbance increases with the concentration of Janus green up to 28.6 μmol/L. The maximum absorbance was obtained at the optimum point of 28.6 μmol/L Janus green concentration.

Figure 6b shows the effect of Janus Green concentration and reaction time on the absorbance percent at optimum 2.5 mmol/L bromate, 0.19 mol/L sulfuric acid, and 22.5°C. The absorbance percent increased with increase in reaction time at the optimum value of 285, and above this value it decreased along with increasing reaction time.

In Fig. 6c, the effect of bromate concentration and sulfuric acid concentration on the absorbance is shown at optimum 28.6 μmol/L Janus Green, 22.5°C, and 285 s. The absorbance percent increased with increasing sulfuric acid concentration up to 0.19 mol/L. At higher sulfuric acid concentrations, the reaction rate decreased.

Figure 6d shows the effect of bromate concentration and reaction time on the absorbance percent at optimum 28.6 μmol/L Janus Green, 0.19 mol/L sulfuric acid, and 22.5°C. The maximum absorbance was obtained at the optimum point of 2.5 mmol/L Janus Green concentration.

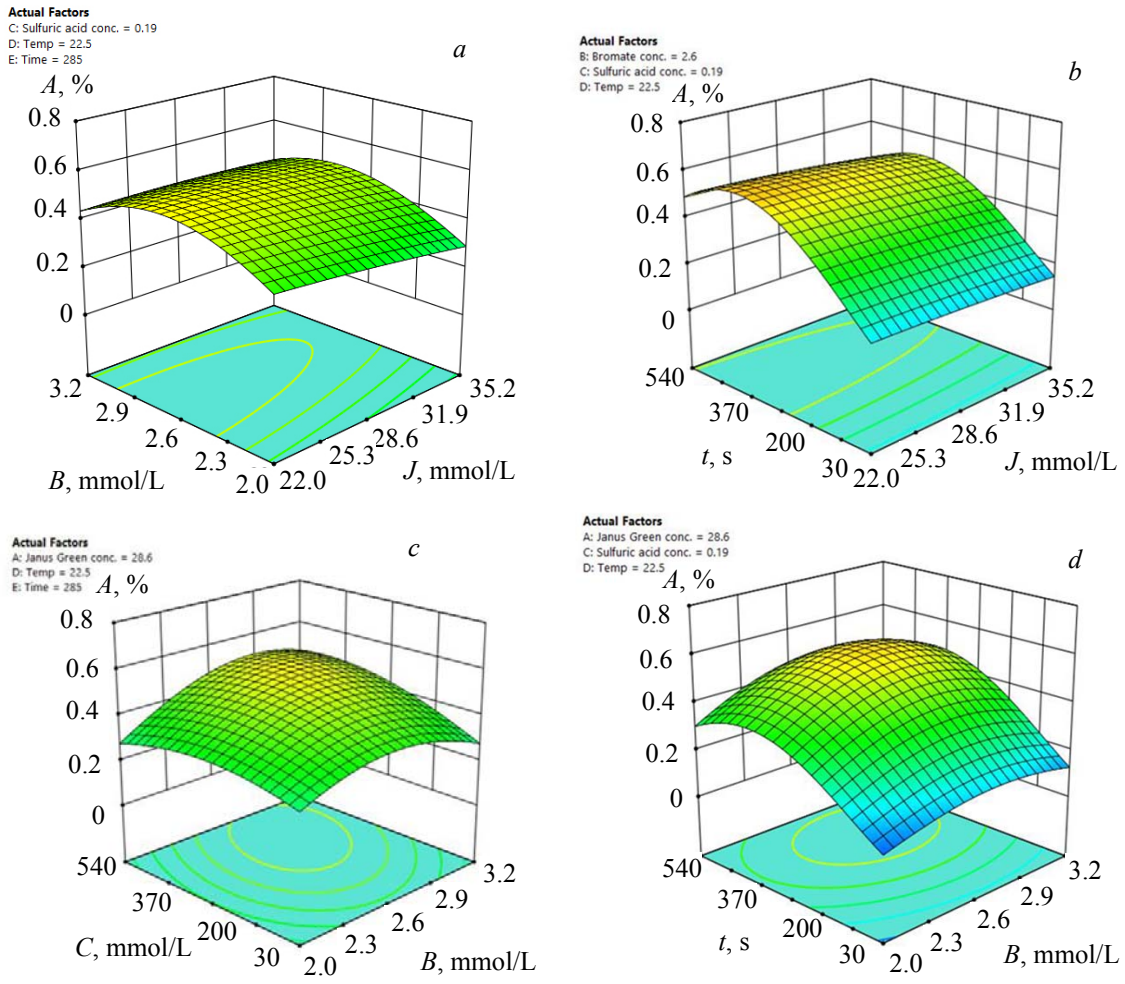



Fig. 6. a) Effect of Janus green concentration and bromate concentration (*B*) on catalyzed reaction (0.19 mol/L sulfuric acid, 22.5°C, and 285 s); b) effect of Janus green concentration (*J*) and reaction time on catalyzed reaction (2.5 mmol/L bromate, 0.19 mol/L sulfuric acid, and 22.5°C); c) effect of bromate concentration and sulfuric acid concentration (*C*) on catalyzed reaction (28.6 μmol/L Janus green, 22.5°C, 285 s); d) effect of bromate concentration and reaction time on catalyzed reaction (28.6 μmol/L Janus green, 0.19 mol/L sulfuric acid, and 22.5°C).

*Analytical parameters.* Under optimum experimental conditions, the calibration curve was obtained over the range 0.3–19.5 μg/mL of lorazepam, including two linear segments of 0.3–10.0 μg/mL and 10.0–19.5 μg/mL. An analysis of the data gave the following regression equation: $\Delta A$ = 0.0401[Lorazepam] + 0.0512 ($R^2$ = 0.9994) for the first and $\Delta A$ = 0.0082[Lorazepam] + 0.3696 ($R^2$ = 0.9998) for the second linear segment, where $\Delta A$ is the difference in absorbance between the blank and the sample, [Lorazepam] is the lorazepam concentration in μg/mL, and $R^2$ is the correlation coefficient. The detection limit ($3S_b/m$) was 0.08 μg/mL of lorazepam. The relative standard deviations ($n$ = 5) were 1.09 and 1.03% for 1.0 and 5.0 μg/mL and 0.97% for 15.0 μg/mL of lorazepam, respectively.

*Interference studies.* The interfering effect of foreign species in the determination of 5.0 μg/mL of lorazepam was investigated. The tolerance limit was defined as the concentration of the added species causing an error (analytical signal) greater than ±5% in 5.0 μg/mL of lorazepam. The results are given in Table 3. The obtained results show that nitrite and halide ions have serious interfering effects (less than 5.0 μg/mL), whereas they do not exist in the real sample matrix.

TABLE 3. Tolerance limit for foreign species on the determination of 5.0 μg/mL of lorazepam.

| Foreign species | Tolerance limit ($W_{Lorazepam}/W_{species}$) |
|---|---|
| $Na^+$, $K^+$, $NH_4^+$ | 1000> |
| $SO_4^{2-}$, $NO_3^-$ | >1000 |
| Sacarrose | 970 |
| Fructose, glucose | 940 |
| Ethanol | 910 |
| $HCO_3^-$, $CO_3^{2-}$, $NO_3^-$ | 900 |
| Urea | 775 |
| Uric acid | 750 |
| Lysine, glycine | 480 |
| Methionine | 375 |
| $I^-$, $Br^-$, $Cl^-$, $NO_2^-$ | <1 |

*Real sample analysis.* The accuracy and applicability of the proposed method has been confirmed by the determination of lorazepam in pharmaceutical and biological samples. Pharmaceutical sample preparation was performed using the mentioned procedure. An appropriate amount of the samples was analyzed by the recommended procedure and HPLC as an alternative method. The results of four replicate determinations are given in Table 4. The obtained results indicated that lorazepam content determination by the two procedure is in good agreement. The precision (RSD%) varies in the range 0.99–1.17 and 0.97–1.09% for lorazepam tablet (in dosages of 1 and 5 mg/tablet) using the recommended procedure and HPLC method, respectively. Moreover, the procedure was used for the determination of lorazepam in human serum and urine samples collected from a patient who has taken 5 mg/tablet of lorazepam tablet after 4 h of oral administration. After sample preparation, the samples were spiked with different amounts of lorazepam, including two linear segments of the calibration curve, and analyzed using the recommended procedure and the HPLC method. The obtained results of three replicate determination are given in Table 5. The values of RSD% of the spiked serum and urine samples using the recommended procedure vary over the range 1.05–1.19 and 0.92–1.19%, respectively. The statistical $t$-test did not show any significant difference between the data obtained from the two methods (at 95% confidence level). Also, the precision of the proposed method and the HPLC method was evaluated using the $F$-test. The precision of the two methods is the same, as confirmed by the obtained results. Therefore, the developed method is free from interference from the matrix effect and is suitable for analysis of lorazepam in different samples.

TABLE 4. Determination of Lorazepam in Lorazepam Tablet in Dosage 1 and 5 mg/tablet Using the Developed Procedure and HPLC

| Sam-ple | Proposed method Found [a] (mg/tablet) | RSD, % | HPLC method Found [a] (mg/tablet) | RSD (%) | Statistical test *t* test [b] | Statistical test *F* test [c] | Pharmaceutical Co./ Batch No. |
|---|---|---|---|---|---|---|---|
| *Lorazepam tablet (1 mg)* | | | | | | | |
| 1 | 0.011 ± 1.01 | 1.09 | 0.011 ± 1.01 | 1.09 | 1.43 | 1.36 | Abidi-Iran/3208 G |
| 2 | 0.013 0.99 | 1.08 | 0.013 ± 1.00 | 0.98 | 1.54 | 1.00 | |
| 1 | 0.011 ± 0.99 | 1.11 | 0.010 ± 0.98 | 1.14 | 1.67 | 1.44 | Abidi-Iran/3211 G |
| 2 | 0.014 ±1.02 | 1.17 | 0.011 ±1.01 | 1.10 | 2.86 | 1.62 | |
| *Lorazepam tablet (5 mg)* | | | | | | | |
| 1 | 0.052 ± 4.97 | 1.05 | 0.050 ± 4.99 | 1.00 | 1.15 | 1.08 | Abidi-Iran/0118 E |
| 2 | 0.051 ± 5.02 | 1.02 | 0.051 ± 5.00 | 1.02 | 0.78 | 1.15 | |
| 1 | 0.052 ± 5.03 | 1.03 | 0.054 ± 5.06 | 1.07 | 1.15 | 1.01 | Abidi-Iran/0121 E |
| 2 | 0.050 ±5.05 | 0.99 | 0.049 ± 5.04 | 0.97 | 1.26 | 1.04 | |

[a] Mean ± standard deviation ($n$=4)
[b] Tabulated $t$-value for three degrees of freedom at $P$(0.95) is 3.18.
[c] Tabulated $F$-value for three degrees of freedom at $P$(0.95) is 9.28

TABLE 5. Determination of Lorazepam in Human Serum and Urine of a Patient Using the Developed Procedure and HPLC

| Sample | Added | Proposed method Found (μg/mL) | Proposed method RSD, % | HPLC method Found [a] (μg/mL) | HPLC method RSD, % | Statistical test *F* test [b] |
|---|---|---|---|---|---|---|
| Human serum 1 | – | <D.L | – | <D.L | – | – |
| | 5.0 | 0.06 ± 5.19 | 1.15 | 0.06 ± 5.20 | 1.15 | 1.00 |
| | 15.0 | 0.16 ± 15.21 | 1.05 | 0.14 ± 15.18 | 0.92 | 1.31 |
| Human urine 1 | – | <D.L | – | <D.L | – | |
| | 5.0 | 0.06 ± 5.04 | 1.19 | 0.05 ± 5.02 | 1.00 | 1.44 |
| | 15.0 | 0.15 ± 15.07 | 0.99 | 0.15 ± 15.10 | 1.00 | 1.02 |

[a] Mean ± standard deviation ($n$ = 3)
[b] Tabulated $F$-value for two degrees of freedom at $P$(0.95) is 9.28.

**Conclusions.** This study reports a sensitive and relatively selective spectrophotometric method for the determination of lorazepam. The developed method possesses distinct advantages over other chromatographic methods in cost, simplicity, ease of operation, and applicability to real sample analysis. The experimental data were also optimized using the RSM, and the ANOVA results were evaluated. Moreover, the reliability of this method permits the analysis of pharmaceutical and biological samples with satisfactory results.

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