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Research Article

Spectrophotometric Estimation of Nitrazepam in Pure and in Pharmaceutical Preparations

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Three simple, sensitive, accurate, and rapid visible spectrophotometric methods (A, B, and C) have been developed for the estimation of nitrazepam in both pure and in pharmaceutical preparations. They are based on the diazotization of reduced nitrazepam with nitrous acid followed by coupling with acetyl acetone (method A), diphenylamine (method B), and citrazinic acid (method C) to form colored azo-dyes, exhibiting absorption maxima (λ_{max}) at 400, 550, and 460 nm, for methods A, B, and C, respectively. The produced colored azo-dyes are stable for more than 2 h. Beer's law was obeyed in the concentration range of 0.5–20, 0.3–14 and 0.5–12 μg/mL for methods A, B, and C, respectively and the corresponding molar absorptivity values are 1.01×10^4, 1.00×10^4, and 1.51×10^4 L mol^{-1}cm^{-1}. All variables have been optimized and the results were statistically compared with those of a literature method by employing the Student's t-test and F-test. No interference was observed from common adjuvants normally added to the tablets. The results obtained in the proposed methods are in good agreement with labeled amounts, when marketed pharmaceutical preparations are analyzed, which could be applied in the routine quality control analysis laboratory.

1. Introduction

Nitrazepam (NTZ), chemically known as 1,3-dihydro-1-nitro-2-oxo-5-phenyl-2H-1,4-benzodiazepine-2-one (Figure 1), is a hypnotic agent that belongs to the benzodiazepine class, and it has been used in the treatment of stress related disorders [1]. The official method for its determination is nonaqueous titrimetry reported in British Pharmacopoeia [2]. Various other methods based on HPLC [3–5], complexometry with cadmium-2-methyl-5-nitrobenzenesulphonate [6], polarography [7, 8], difference uv-spectrophotometry [9], densitometric TLC [10], gas-chromatography [11], micellar electrokinetic capillary chromatography [12], and derivative UV-spectrophotometry [13] have been reported for the assay of NTZ in pharmaceutical formulations. Different manipulation steps are involved in some of these methods, which are not simple for the routine analysis of pharmaceutical formulations.

Spectrophotometric methods are still considered to be a very convenient and cost-effective technique, and hence widely used for the determination of therapeutics in pure as well as in dosage forms. Very few spectrophotometric methods have been reported for estimation of NTZ [14–17]. These methods were suffering from one or other disadvantages such as lack of selectivity [14–16], the use of costly reagent [16], and lower sensitivity [15, 16]. Thus, there is a need to develop a simple, selective, cost-effective, and sensitive procedure for the determination of NTZ in pure and in pharmaceutical formulations.

The present paper describes the development and optimization of three visible spectrophotometric methods based on diazo-coupling reaction using acetyl acetone (AA), diphenyl amine (DPA), and citrazinic acid (CZA) as the coupling agents. The developed methods are simple, sensitive, and accurate for the determination of NTZ in pure and in pharmaceutical formulations.

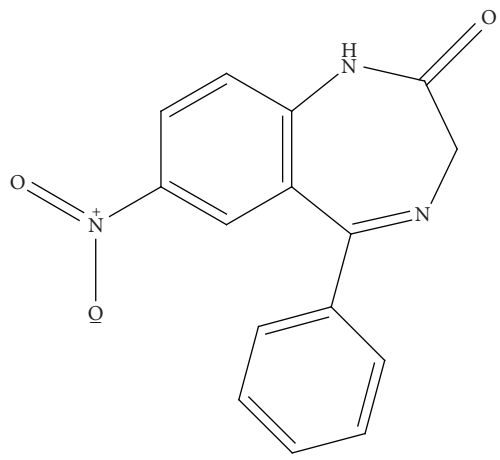


Figure 1: Structure of nitrazepam.

2. Experimental Section

2.1. Apparatus. All absorbance measurements were performed using a Systronics Model 166 digital spectrophotometer provided with 1 cm matched quartz cells.

2.2. Reagents and Standards. Analytical reagent grade chemicals were used, and double distilled water was used throughout the experiment to prepare all solutions.

(i) Standard NTZ solution. The pharmaceutical grade NTZ, certified to be 99.99% pure were received from Anglo French Drug & Industries Ltd., Bangalore India, as a gift sample and were used as received. Accurately 10 mg of NTZ was weighed into a 100 mL beaker and dissolved NTZ in 5 mL acetone. To this, 5 mL 4 N hydrochloric acid and 1 g of zinc dust were added and shaken thoroughly for about 15 min and then diluted up to the mark with water in a 100 mL calibrated flask (100 μg/mL), and filter through Whatman No.41 filter paper. Working solutions were prepared as required by dilution.

(ii) Acetyl acetone (AA) (5% w/v). Prepared by diluting 5.1 mL of AA (BDH chemicals, Poole, England) in 100 mL of methanol.

(iii) Diphenylamine (DPA) (0.5% w/v): Prepared by dissolving 0.5 g DPA (BDH chemicals, Poole, England) in 100 mL of methanol.

(iv) Citrazinic acid (CZA) (0.1% w/v). Prepared by dissolving 0.1 g of the citrazinic acid (Fluka, Switzerland) in 2 mL 4 M sodium hydroxide and diluting to 100 mL with distilled water.

(v) Others. Aqueous solutions of sodium nitrite (Merck) (0.1%), sulphamic acid (Qualigens) (3.0% w/v), sodium hydroxide (Merck) (4 M), and hydrochloric acid (Merck) (1 M and 6 M) were used.

2.3. Preparation of Calibration Graph

2.3.1. Method A. Aliquot of nitrazepam ranging from 0–20 μg mL^{-1} was transferred into a series of 10 mL volumetric flasks. To each flask, 1 mL sodium nitrite (0.1% w/v) and 1 mL of 1 M hydrochloric acid were added. After 3 min, 1 mL of sulfamic acid (3% w/v) was added to each flask. Then, 2 mL each of the acetyl acetone (5% w/v) and 4 M sodium hydroxide were added. The contents were made up to the mark with distilled water and mixed well. After 5 min, the absorbance of the yellow colored azo-dye was measured at 400 nm against the reagent blank.

2.3.2. Method B. Different aliquots of nitrazepam ranging from 0–14 μg/mL were transferred into a series of 10 mL volumetric flasks. To each flask, 1 mL each of the sodium nitrite (0.1% w/v) and 1 M hydrochloric acid were added. After 5 min, 1 mL of sulfamic acid (3% w/v) and then 2 mL diphenylamine (0.5% w/v) were added. The contents were made up to the mark with 6 M hydrochloric acid and mixed well. The absorbance of the violet colored azo-dye was measured at 550 nm against the reagent blank, after 5 min.

2.3.3. Method C. Into a series of volumetric flasks were added aliquots of NTZ ranging from 0–12 μg/mL, 1 mL each of the sodium nitrite (0.1% w/v) and 1 M hydrochloric acid. After 3 min, 1 mL of sulfamic acid (3% w/v) was added to each flask. After 1 min, 2 mL each of citrazinic acid (0.1% w/v) and 4 M sodium hydroxide were added. The volumes were made up to the mark with distilled water and mixed well. The absorbance of the orange colored azo-dye was measured at 460 nm against the reagent blank five minutes later.

The amount of nitrazepam present in the sample was computed from calibration curve Figures 2(a), 2(b), and 2(c).

2.4. Procedure for Pharmaceutical Preparation. Twenty tablets were weighed accurately and ground into a fine powder. Tablets powder equivalent to 10 mg of the NTZ was accurately weighed and transferred into a 100 mL beaker. To this 5 mL 4 N hydrochloric acid and 1 g of zinc dust were added and stirred thoroughly for about 30 min. The contents were diluted with 50 mL distilled water and filtered using Whatman No. 41 filter paper. The filtrate was received into a 100 mL calibrated flask and it was made up to the mark with water. Appropriate aliquots of the drug solution were taken and the proposed standard procedures were followed for analysis of the drug content.

3. Results and Discussion

The presence of the aromatic amino group in reduced nitrazepam undergo diazotization with nitrous acid and then coupling the resulting diazonium salt with acetyl acetone (method A), diphenylamine (method B), and citrazinic acid (method C) to form colored azo-dyes and exhibiting absorption maxima (λ_{max}) at 400, 550, and 460 nm, respectively for methods A, B, and C.

The reaction pathways of all the methods are shown in Scheme 1. In step (1), reduced NTZ is treated with nitrite solution in hydrochloric acid medium, undergoes diazotization to give diazonium ion. In step (2), the diazonium ion is coupled with the coupling agents acetyl acetone (method A),

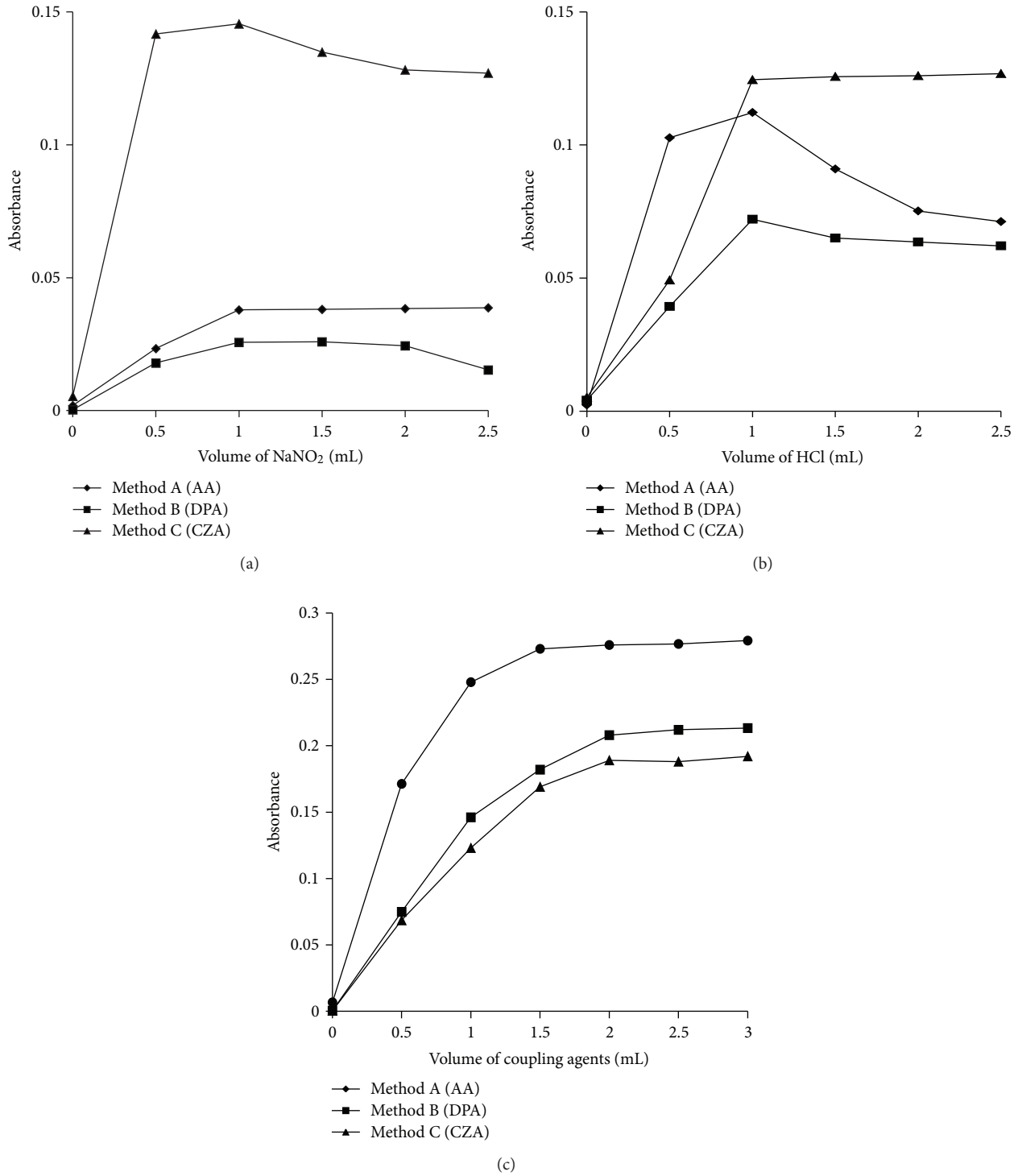
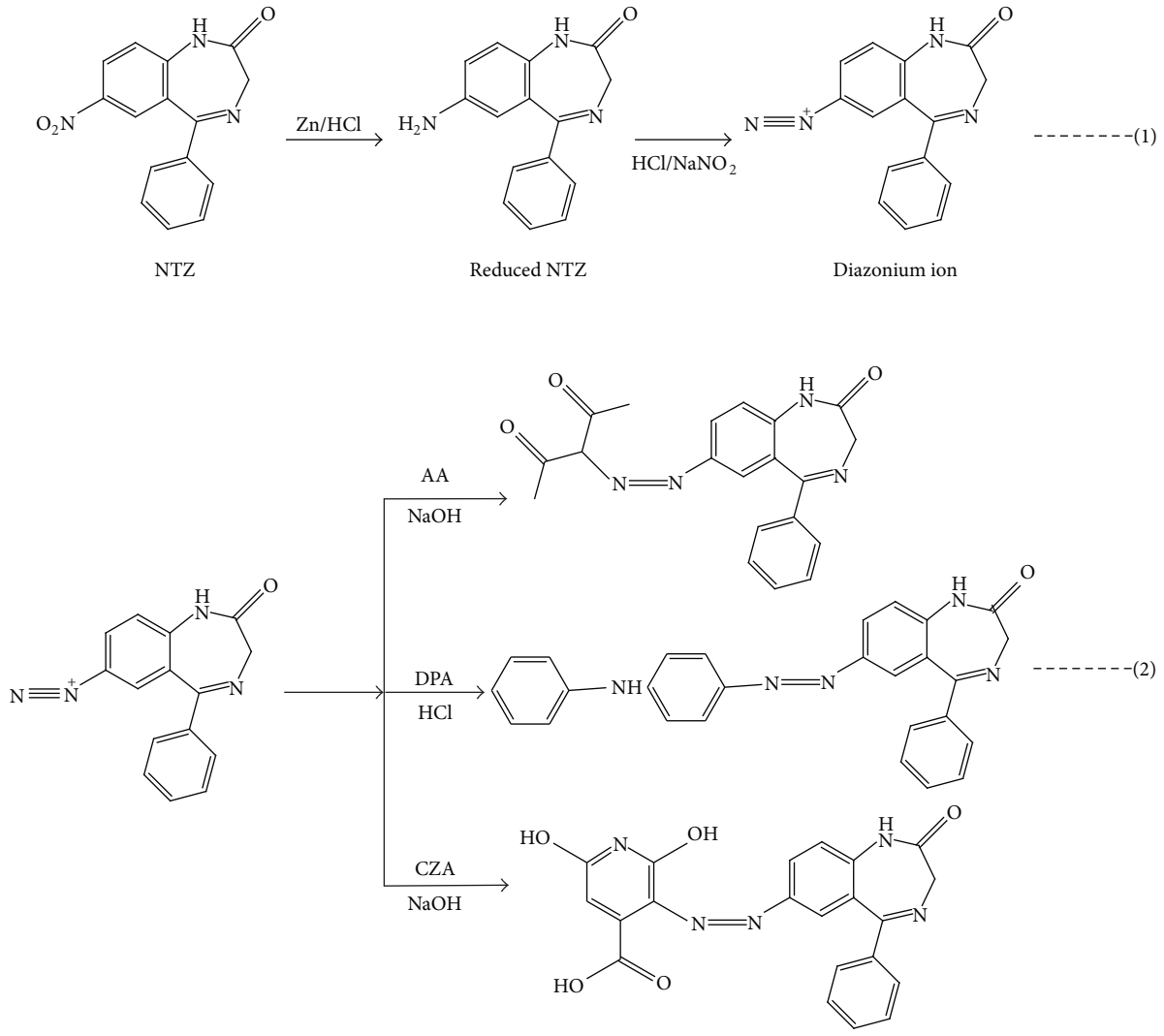


Figure 2: Effect of (a) sodium nitrite (b) hydrochloric acid and (c) coupling reagents.

diphenylamine (method B) and citrazinic acid (method C) to form colored azo-dyes in alkaline medium (methods A and C) and in acid medium (method B), to form colored azo-dyes. Based on the above observations, simple spectrophotometric methods to the determination of NTZ were developed and validated as per the current ICH guidelines.

3.1. Optimization of Experimental Parameters

3.1.1. Effects of Reagents, Acid, and Alkali. The methods developed were optimized using different parameters such as sodium nitrite, hydrochloric acid, and concentrations of acetyl acetone (AA) for method A, alcoholic diphenylamine



Scheme 1: Proposed reaction scheme for NTZ (1) diazotization (2) coupling reaction.

Table 1: Optical characteristics and precision.

Parameter	Method A	Method B	Method C
λ_{max} (nm)	400	550	460
Linear range (μg/mL)	0.5–20	0.3–14	0.5–12
Molar absorptivity (ε), (L mol^{-1} cm^{-1})	1.01×10^4	1.00×10^4	1.51×10^4
Sandell sensitivity (μg cm^{-2})	0.0281	0.0281	0.0186
Intercept (a)	0.0194	−0.0040	0.0042
Slope (b)	0.0327	0.0365	0.0530
Correlation coefficient (r)	0.9982	0.9999	0.999
S_a	0.0182	0.0037	0.0134
S_b	0.0012	0.0029	0.00127
LOQ (μg/mL)	0.1550	0.1213	0.0928
LOD (μg/mL)	0.0512	0.0400	0.0306

* $y = bc + a$ where c is the concentration of nitrazepam in μg/mL and y is the absorbance at the respective λ_{max}. S_a is the standard deviation of intercept, S_b is the standard deviation of slope.

Table 2: Evaluation of intraday and interday accuracy and precision results.

Method	NTZ taken, μg/mL	Intraday[a]			Interday[b]		
		NTZ found[c], μg/mL	Precision[d]	Accuracy[e]	NTZ found[c], μg/mL	Precision[d]	Accuracy[e]
A	5	4.96 ± 0.04	0.78	0.77	4.94 ± 0.02	0.39	1.19
	10	9.89 ± 0.04	0.39	1.11	10.08 ± 0.05	0.50	−0.78
	15	14.81 ± 0.09	0.66	1.28	15.10 ± 0.07	0.43	−0.68
B	4	4.02 ± 0.023	0.58	−0.46	4.18 ± 0.04	1.01	−4.45
	8	7.92 ± 0.042	0.53	0.22	7.95 ± 0.03	0.33	0.68
	12	12.00 ± 0.10	0.79	−0.04	11.83 ± 0.05	0.45	1.46
C	2	1.99 ± 0.02	0.96	0.37	2.054 ± 0.01	0.07	−2.72
	6	6.00 ± 0.05	0.79	−0.06	5.92 ± 0.03	0.47	1.37
	10	10.03 ± 0.05	0.47	−0.25	9.93 ± 0.03	0.27	0.69

[a]Mean value of five determinations, [b]mean value of five determinations, [c]mean value of three determinations, [d]relative standard deviation (%), [e]bias%: (found − taken/taken) × 100.

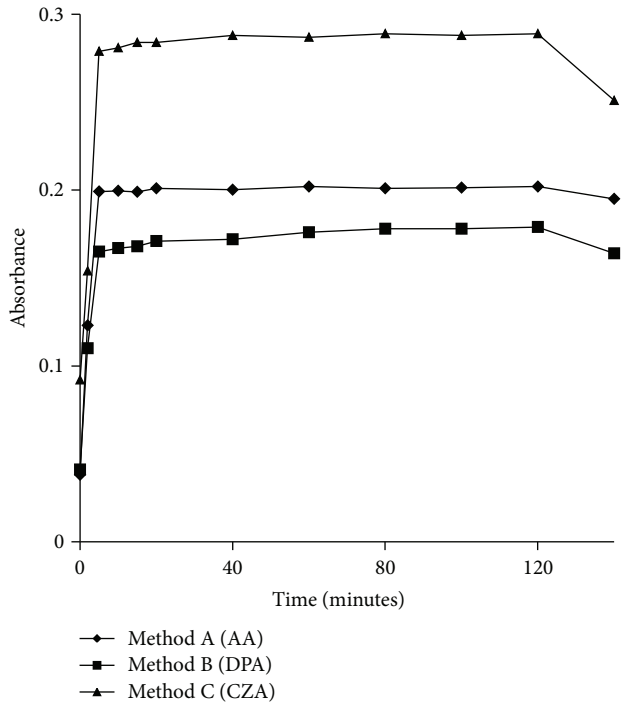


Figure 3: Effect of time and stability of the azo-dye.

(DPA) for method B, and aqueous citrazinic acid (CZA) for method C for development of maximum color intensity. These experimental variables were studied with a fixed concentration (5 μg/mL) of nitrazepam. Optimization is done by varying one parameter, keeping other constant.

The optimum concentrations of AA, DPA, and CZA leading to maximum color intensity were found to be 2 mL each of the above reagents per 10 mL of the reaction mixture in methods A, B, and C, respectively (Figure 2(c)). A volume of 1 mL each of the sodium nitrite (0.1%) (Figure 2(a)) and hydrochloric acid (1 M) (Figure 2(b)) were found to be suitable for getting the diazonium ion. In methods A and C, a volume of 2 mL 4 M sodium hydroxide was required to stabilize the colored azo-dye in a 10 mL reaction mixture. In method B, the violet colored azo-dye was stable in acid medium. Hydrochloric acid, sulphuric acid, and acetic acid were studied as a medium and as diluents. But, the azo-dye was stable in hydrochloric acid medium. Thus, hydrochloric acid (6 M) was used as a diluent in method B to stabilize the dye.

3.1.2. Effects of Reaction Time and Stability of the Azo-Dye. The colored azo-dyes developed rapidly after addition of the reagents and attained maximum intensity after about 5 min at room temperature in methods A–C. Stability study of the azo-dye was carried out by measuring the absorbance values at time intervals of 10 min and was found to be stable for more than 2 h in all the methods (Figure 3).

3.2. Method Validation

3.2.1. Linearity, Detection, and Quantitation Limit. Under the optimized experimental conditions for all the methods A–C, a linear correlation was found between the absorbance at respective wavelengths and concentration of NTZ in the ranges are given in Table 1. The regression analysis using method of least squares was made for the slope (b), intercept (a), and correlation (r) obtained from different concentrations and the results are summarized in Table 1. The optical characteristics such as absorption maxima, Beer's law limit, molar absorptivity and Sandell's sensitivity, the limits of detection and quantitation calculated as per the current ICH [18] guidelines are compiled in Table 1. The calibration curves of the methods A, B, and C are given in Figures 4(a), 4(b), and 4(c), respectively.

3.2.2. Accuracy and Precision. Intraday precision and accuracy of the proposed methods were evaluated by replicate analysis ($n = 5$) of calibration standards at three different concentration levels in the same day. Precision and accuracy of interday were measured by performing the calibration standards at cited three concentrations on five consecutive days. Both precision and accuracy were based on the calculated percent relative standard deviation (RSD, %) and percent relative error (RE, %) of found concentration

Table 3: Results of recovery by standard-addition method.

Tablet brand name	Method A				Method B			Method C			
	NTZ tablet μg/mL	Pure NTZ added, μg/mL	Total found μg/mL	Pure NTZ recovered* % ± SD	Pure NTZ added, μg/mL	Total found μg/mL	Pure NTZ recovered* % ± SD	NTZ tablet μg/mL	Pure NTZ added, μg/mL	Total found μg/mL	Pure NTZ recovered* % ± SD
NITRAVET 5 mg	5	2.5	7.52	100.81 ± 0.35	2.5	7.51	100.74 ± 0.33	4	2	6.01	100.31 ± 0.01
	5	5.0	9.98	99.56 ± 0.21	5	10.04	100.84 ± 0.19	4	4	8.03	100.64 ± 0.01
	5	7.5	12.53	100.52 ± 0.12	7.5	12.57	100.91 ± 0.15	4	6	9.97	99.55 ± 0.02

*Mean value of three determinations.

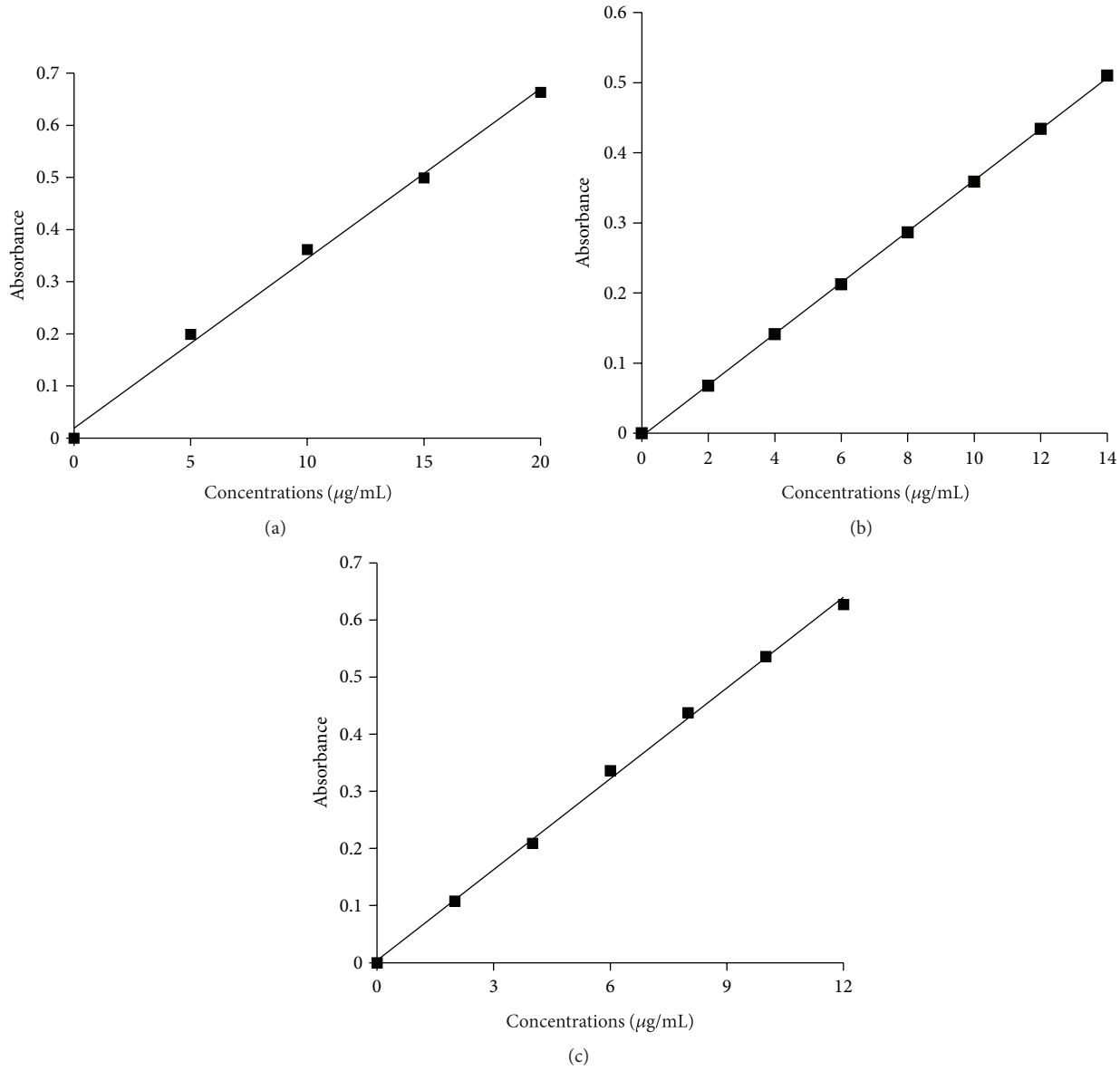


Figure 4: Calibration curves for (a) method A, (b) method B, and (c) method C.

Table 4: Results of determination of NTZ in tablets and statistical comparison with the reference method.

Tablet brand name	Nominal amount mg per tablet	Found** (% of nominal amount ± SD)			
		Reference method [17]	Method A	Method B	Method C
Nitravet*	5 mg	101.2 ± 1.3	99.96 ± 0.561 t = 1.23, F = 5.37	100.41 ± 0.539 t = 0.794, F = 5.81	100.22 ± 0.71 t = 0.935, F = 3.35

*Marketed by: *(Anglo French); **Mean value of five determinations.
Tabulated t and F values at 95% confidence level are 2.77 and 6.39, respectively.

compared to the theoretical one, respectively (Table 2). The result shows that these methods have reasonable precision Table 2. The proposed methods were successfully applied to the determination of nitrazepam in pharmaceutical dosage forms.

3.2.3. Recovery. The accuracy and precision of the methods developed were further ascertained by recovery experiments performed on synthetic mixtures of NTZ with several excipients such as talc (30 mg), dextrose (100 mg), sodium alginate (10 mg), starch (20 mg), acacia (15 mg), and magnesium

stearate (20 mg) by the proposed methods and recoveries obtained by each method were in the range 99.5 to 101.0% ($n = 5$).

In order to check the validity of the proposed methods, we applied the standard addition technique by adding nitrazepam to the previously analyzed tablets. The recovery of each drug was calculated by comparing the concentration obtained from the spiked mixtures with those of pure drugs. The results are summarized in Table 3.

3.2.4. Application to the Tablet Analysis. The proposed methods (A–C) were applied to the determination of NTZ in tablet solutions at three different concentrations. The results were compared with those of the reported method [17]. Statistical analysis of the results using the Student's t test and F test revealed no significant difference between the reported method at the 95% confidence level with respect to accuracy and precision (Table 4).

4. Conclusions

The developed visible spectrophotometric methods are simple, sensitive, accurate, precise, reproducible, and economical and can be successfully applied to the routine estimation of nitrazepam in bulk and in pharmaceutical dosage forms without the need of extraction or heating the reaction mixture. Diazotization was carried out at room temperature and cooling to 0–5°C was not necessary. The methods are unaffected by slight variations in the experimental conditions such as basicity, reagent concentrations, and temperature. The value of standard deviation was satisfactorily low and recovery was close to 100% which indicates the reproducibility and accuracy of the three methods.

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