УДК 615.31

ИССЛЕДОВАНИЕ ПРИМЕНИМОСТИ СПЕКТРОФОТОМЕТРИЧЕСКИХ МЕТОДИК ДЛЯ ОПРЕДЕЛЕНИЯ НИТРАЗЕПАМА В ФАРМАЦЕВТИЧЕСКОЙ СУБСТАНЦИИ И ГОТОВЫХ ЛЕКАРСТВЕННЫХ ФОРМАХ

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Для определения нитразепама использовали две спектрофотометрические методики, дающие воспроизводимые результаты. В качестве хромогенных агентов использовали фармацевтически чистые β-нафтол и *p*-диметил-амино-бензальдегид. В основе первой методики лежит реакция диазотирования востановленного нитразепама с β-нафтолом (метод А). В основе второй методики – реакция конденсации аминогруппы востановленного нитразепама и ароматического альдегида с образованием основания Шиффа (метод В). Продукты вышеописанных реакций имеют максимумы поглощения при 500 и 400 нм для методов А и В соответственно. В соответствии с законом Бера в концентрационных областях 0–40,0 и 0–5,0 мг/мл для методов А и В значения коэффициентов поглощения составляют $6{,}49 \cdot 10^3$ и $5{,}13 \cdot 10^4$ моль$^{-1}$·см$^{-1}$ соответственно. Статистическая проверка результатов, полученных по методу Стьюдента и F-тесту, показала совпадение с имеющимися в литературе данными. При использовании указанных методик определения нитразепама в готовых лекарственных средствах в таблетированной форме взаимодействия с содержащимися в них вспомогательными веществами не обнаружено. Разработанные методики – простые, высокоточные и недорогие - могут найти применение для контроля качества готовых лекарственных форм на основе субстанции нитрозепама.

Ключевые слова: *диазотирование, нитразепам, основания Шиффа, спектрофотометрия, фармацевтическая химия.*

Nitrazepam, (NTZ), a benzodiazepine class of drug, which is chemically known as 2-methoxyetyhyl1-methyl-ethyl 2,6-dimethyl-4-(-3-nitrophenyl)pyridine-3,5-dicarboxylate [1], is a hypnotic drug used in the treatment of insomnia which has sedative and motor impairing properties [2], as well as anxiolytic, amnestic, anticonvulsant and skeletal muscle relaxant properties. In humans, nitrazepam is metabolized mainly by the reduction of the nitro group in the hepatic microsomes, followed by acetylation. In brain, NTZ acts on benzodiazepine receptors, which are associated with GABA receptor [3].

Several methods have been reported for the detection and determination of nitrazepam in pharmaceuticals or in biological fluids include liquid chromatographic measurement in plasma [4–5], micellar electrokinetic capillary chromatography in urine [6], HPLC in plasma [7–8] and pulse polarography in serum [9]. GC [10], flow-injection analysis using voltammetric detector [11] and spectrophotometric methods [12–18] have been utilized to determine NTZ in pharmaceiuticals. Different manipulation steps are involved in some of these methods (except spectrpophotometry), which are not simple for the routine analysis of pharmaceutical formulations. The reported chromatographic techniques [4–8] require expensive experimental set-ups and not affordable in every laboratory for routine analysis. The polarographic and voltammetric technique requires sophisticated instrumentation, time consuming and involvement of scrupulous experimental condition. The reported spectrophotometric methods [12–18] are less sensitive and time consuming. Thus, there is a need to develop sensitive, accurate and cost-effective methods for its determination.

The aim of the present investigation is to develop a simple, highly sensitive, accurate and precise spectrophotometric methods for the analysis of nitrazepam in pure form and in pharmaceutical samples using β-naphthol (method A) and *p*-dimetyl amino benzaldehyde (PDAB) (method B) as chromogenic agents.

Experimental

Apparatus. A Systronics Model 166 digital spectrophotometer provided with 1-cm matched quartz cells were used for recording all absorbance measurements.

Reagents and Standards. All chemicals used were of Analytical grade. Double distilled water was used for dilution and preparation of all reagents. Sodium nitrite, hydrochloric acid and sodium hydroxide were obtained from E. Merck, sulfamic acid (Qualigens), β-naphthol (BDH chemicals, Poole, England) and *p*-dimetyl amino benzaldehyde (Nice laboratory reagent, India) Nitrazepam pure drug was obtained from Cipla Ltd., Mumbai (India). NTZ tablets were purchased from a local market.

Preparation of standard NTZ solution. Pharmaceutical grade NTZ was obtained from Cipla Ltd., Mumbai (India) as a gift sample. Accurately 10 mg of NTZ was weighed into a 100 ml beaker and dissolved 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 standard flask (100 μg/ml) (filter if necessary). Working solutions were prepared as required by dilution.

Results and Discussion

The method A involves the reaction of reduced NTZ with nitrous acid to form diazonium ion, and the formed diazonium ion then couples with β-naphthol in basic medium to produce orange colored azo-dye with a maximum absorption at 500 nm (Fig. 1). The proposed reaction pathway is shown in Scheme 1.

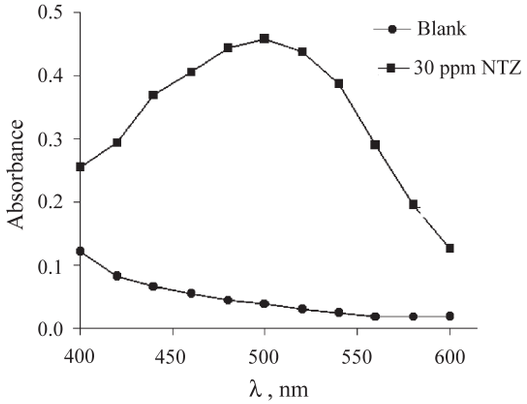


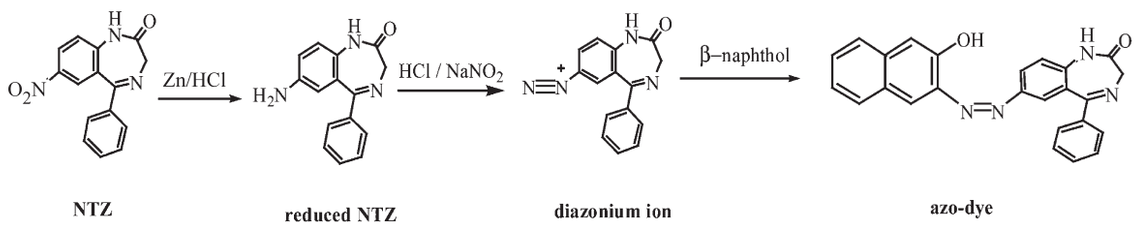
Fig. 1. Absorption spectra of NTZ using β-naphthol

The method B involves the condensation reaction of primary aromatic amino group of reduced NTZ with PDAB in methanol to produce yellow colored Schiff's base with a maximum absorption at 440 nm. The absorption spectrum of the formed complex is presented in Fig. 2. The $-NH_2$ group in NTZ donates a lone pair of electrons to the carbon present in the carbonyl group of PDAB which results in the formation of imine (Schiff's base) and then giving water and proton as by-products. The proposed reaction pathway is shown in Scheme 2.

Optimization of experimental variables. In order to achieve maximum sensitivity, various experimental pa-

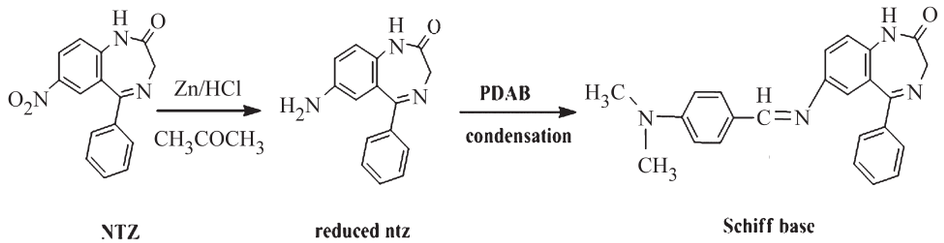
Scheme 1

Proposed reaction pathway for method A



Scheme 2

Proposed reaction pathway for method B



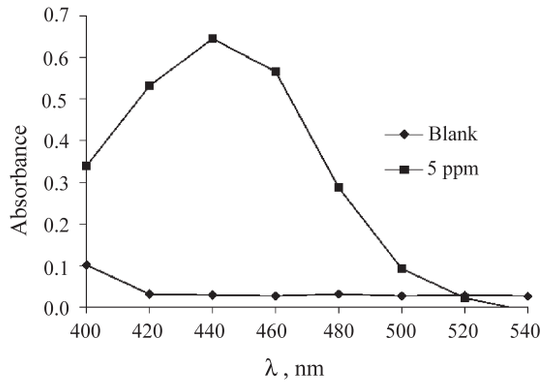


Fig. 2. Absorption spectra of NTZ using PDAB

rameters were optimized by varying one parameter at a time while keeping the others fixed and observing the effect produced on the absorbance of the colored species.

Method A. The developed method was optimized using different parameters such as sodium nitrite, hydrochloric acid and concentrations of β-naphthol for development of maximum color intensity. These experimental variables were studied with a fixed concentration (10 μg/ml) of nitrazepam.

The optimum concentration of β-naphthol (1% w/v) leading to maximum color stability was found to be 1.5 ml, and a volume of 1 ml each of the sodium nitrite (0.1%) and hydrochloric acid (1 M) were found to be suitable for stable diazonium ion, and the optimum concentration of sodium hydroxide leading to stabilize the colored azo-dye was found to be 2 ml of 4 M to in a 10 ml reaction mixture.

Method B. In order to study the effect of concentration of PDAB, a different volumes of the reagent (0–3.0 ml) was added to a fixed concentration (1 μg/ml) of nitrazepam. The optimum concentration of PDAB (2 %), leading to maximum color stability was found to be 1.0 ml in a 10 ml reaction mixture.

Stability study of the azo-dye and condensation product was carried out by measuring the absorbance values at time intervals of 10 min and was found to be stable for more than 1 and 2 h in the methods A and B, respectively.

Method validation. The proposed methods have been validated for linearity, sensitivity, precision, accuracy, selectivity and recovery.

Linearity and sensitivity. The calibration graphs were obtained by plotting the absorbance against the concentration of NTZ. Under the optimized experimental conditions, Beer's law was obeyed in the concentration range 0–40.0 and 0–5.0 μg/ml with molar absorption coefficients of 6.49×10^{3} and 5.13×10^{4} l mol^{-1} cm^{-1} for methods

Table 1

Optical characteristics and statistical data of the proposed spectrophotometric methods

Parameter	Method A	Method B
λ_{max} (nm)	500	440
Beer's law range (μg/ml)	0–40	0–5.0
Molar absorptivity (ε), l mol^{-1}cm^{-1})	6.49×10^{3}	5.13×10^{4}
Sandell sensitivity (μg cm^{-2})	0.0644	0.0082
Intercept (a)	–0.0095	–0.0045
Slope (b)	0.0161	0.1282
Correlation coefficient (r)	0.998	0.999
S_a	0.0222	0.0335
S_b	0.0006	0.0086
LOQ(μg/ml)	0.4633	0.0133
LOD(μg/ml)	0.1529	0.0044

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

A an B, respectively. The calibration graph is described by the equation: Y = a + bx, where y = absorbance, a = intercept, b = slope and x = concentration, obtained by the method of least squares. The correlation coefficient (r), intercept (a) and slope (b) for the calibration data and sensitivity parameters such as apparent molar absorptivity and Sandell sensitivity values, the limits of detection and quantitation calculated as per the current ICH guidelines [19] are compiled in Table 1.

Accuracy and Precision. To evaluate the accuracy and precision of the methods, pure drug solution at three different levels (within the working limits) was analyzed, each determination being repeated five times. The relative error (%) and relative standard deviation (%) were less than 2.0 and indicate the high accuracy and precision of the methods (Table 2).

Application to analysis of commercial samples. In order to check the validity of the proposed methods, NTZ was determined in some tablet formulations. The results in the Table 3, shows that the calculated paired t- and F-values are less than the theoretical ones confirming no significant difference between the performance of the proposed methods and the reference method [18] at 95% confidence level with respect to accuracy and precision .

Procedures for the determination of nitrazepam

Method A-Using β-naphthol. Different aliquots of standard drug solution ranging from 0–40.0 μg/ml (NTZ) were transferred into a series of separate 10 ml standard flasks. To each flask, 1 ml each of sodium nitrite (0.1% w/v) and 1 M hydrochloric acid were added. After 3 min, 0.5 ml of sulfamic acid (3% w/v) was added, and the contents of the flask were shaken for a minute. Then, volumes of 1.5 ml β-naphthol (1% w/v) and 2 ml 4 M sodium hydroxide were added. The contents were made up to the mark with distilled water and mixed well. The absorbance of the colored azo-dye was measured at 500 nm against the reagent blank prepared similarly omitting the drug content.

Method B-Using PDAB. Aliquots of standard drug solution ranging from 0–5.0 μg/ml were transferred into a series of separate 10 ml volumetric flasks. Then to each flask, 1 ml 2 % PDAB was added. The contents were

Table 2

Evaluation of accuracy and precision

Method	NTZ taken μg/ml	NTZ found* μg/ml	RE %	SD	SEM	RSD%	ROE**%
Method A	10	9.99	0.11	0.03	0.012	0.32	±0.32
	20	19.89	0.56	0.10	0.039	0.52	±0.52
	30	29.92	0.29	0.10	0.039	0.35	±0.35
Method B	1	0.98	2.18	0.01	0.003	0.72	±0.72
	2	1.99	0.64	0.01	0.004	0.48	±0.48
	4	4.01	–0.18	0.02	0.006	0.41	±0.41

RE. Relative error; SD. Standard deviation; SEM. Standard error of mean; RSD. Relative standard deviation; ROE. Range of error; * Mean value of five determinations; **At the 95% confidence level for 4 degrees of freedom.

Table 3

Results of the determination of NTZ in tablets and statistical comparison with the reference method

Tablet studied	Nominal amount, mg/tab	Found**(% of nominal amount ± SD)		
		Reference method [18]	Method A	Method B
Nitravet[a]	5.0	101.1±1.3	100.04±0.60 t=0.88, F = 4.68	100.16±0.54 t=0.81, F = 5.77

[a]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.

Table 4

Results of recovery experiments via the standard addition technique

Tablet brand name	Method A				Method B			
	NTZ tablet µg/ml	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[a] 5mg	10	5	14.98	99.75±0.60	1	0.5	1.49	99.43±0.81
	10	10	19.96	99.67±0.93	1	1	2.01	100.68±0.25
	10	15	25.11	100.7±0.30	1	1.5	2.51	100.38±0.57

made up to the mark with methanol and mixed well. The absorbance of the yellow colored product (Schiff's base) was measured at 440 nm after 15 min against the reagent blank prepared similarly without the drug content.

Procedure for tablets. Five tablets were weighed accurately and ground into fine powder. A quantity of the powder equivalent to 10 mg of NTZ was weighed accurately into a 100 ml standard flask and 10 ml acetone was added to it. Then, 5 ml 4 N hydrochloric acid and 1 g of zinc dust were also added into 100 ml standard flask and shaken thoroughly for about 30 min. Then, the volume was diluted to the mark with water and mixed well and filtered using a Whatman No.41 filter paper. The filtrate containing NTZ (100 µg/ml) was subjected to analysis by the procedures described above.

Recovery study. The accuracy and precision of the proposed methods were further ascertained by performing recovery studies. Pre-analyzed tablet powder was spiked with pure drug at three different concentrations and the total was found by the proposed methods. Each determination was repeated three times. The recovery of the pure drug added was quantitative and revealed that co-formulated substances such as talc, dextrose, alginate, acacia etc. did not interfere in the determination. The results of recovery study are given in Table 4.

In Summary, the developed spectrophotometric methods are simple, accurate, precise and highly sensitive and the methods employ inexpensive and easily available chemicals and instrument. These are considered to be normally available in common laboratories and can be applied at ambient temperature; color development is rapid and does not require strict pH control or any extraction procedures. The colored species are highly stable leading to high precision of the method. There is no interference from common additives and excipients added to the tablets. Thus, the proposed methods can be used for routine analysis in laboratories and for quality control purposes.

The authors are grateful to Cipla, Ltd., Mumbai (India) for the generous supply of pure drug sample. One of the authors HND is thankful to the University of Mysore, Mysore for providing necessary facilities.

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Поступила в редакцию 12.11.11

DEVELOPMENT AND VALIDATION OF SPECTROPHOTOMETRIC METHODS FOR NITRAZEPAM IN PURE AND THE TABLET DOSAGE FORM

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Two simple, sensitive, accurate and reproducible spectrophotometric methods have been described for the assay of nitrazepam (NTZ) in both pure and in pharmaceutical preparations using *β*-naphthol and *p*-dimethyl amino benzaldehyde (PDAB) as chromogenic agents. The first method is based on the diazo-coupling reaction of reduced nitrazepam with β-naphthol (Method A) and the second one is based on the condensation reaction of primary aromatic amine group of reduced nitrazepam with aromatic aldehyde PDAB to yield yellow colored Schiff's base (Method B). The formed products show maximium absorption at 500 and at 440 nm for methods A and B, respectively. Beer's law was obeyed in the concentration range of 0–40.0 and 0–5.0 μg/ml for methods A and B, respectively and the corresponding molar absorptivity values are 6.49×10^3 and 5.13×10^4 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 excipients normally added to the tablets. The proposed methods are simple, sensitive, rapid and economical, and could find application as in routine quality control analysis for nitrazepam.

Key words: *diazo-coupling, Schiff's base, spectrophotometry, nitrazepam, pharmaceuticals.*

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