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Spectrophotometric determination of Nitrazepam, using Thymol as a new chromogenic reagent

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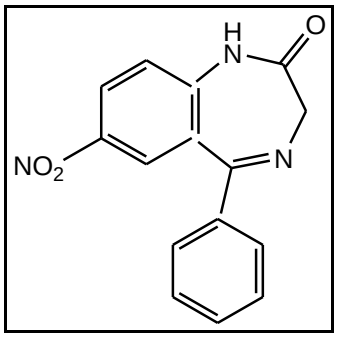
Abstract : A simple and sensitive spectrophotometric method for the determination of Nitrazepam in pure form and in pharmaceutical dosage was developed. The method is based on reduction of the Nitrazepam with zinc powder and hydrochloric acid, followed by reaction with thymol as a new organic reagent and ammonium hydroxide in the Diazo-coupling reaction, to form a stable chromophore which absorbs at (477nm). The method showed a good linearity in the range (0.1– 12 µg. mL^{-1}) with molar absorptivity of ($2.264*10^4$ $L.Mol^{-1}cm^{-1}$) This method is a free from the interference of common excipients that found in pharmaceutical dosage. It was also applied for the determination of chlorpromazine hydrochloride (NZP) in some of pharmaceutical dosage sample containing of this drug.
Keywords : Nitrazepam, spectrophotometric, diazo-coupling, Thymol.

Introduction:

Nitrazepam is a powerful hypotonic drug, anticonvulsant and sedative in the group of drugs. It belongs to the benzodiazepine group, they are a class of antidepressants, anti-panic agents, and muscle relaxants [1].The scientific name of Nitrazepam is: (7-nitro-5-phenyl-1,3 dihydro-2H- 1,4benzodiazepin-2-one), The chemical structure is:



The chemical formula is $C_{15}H_{11}N_3O_3$, molecular weight is ($281.3 g.mol^{-1}$). It is a yellow, crystalline powder, soluble in alcohol, acetone, chloroform, and hexan[2] , there are several analytical methods that are used in the estimation of the (NZP)[3], spectrophotometric methods are most important one, such as: Spectrophotometry-with FIA[4], Oxidative Coupling[5], kinetic methods[6], Charge transfer complex[7]. The other methods have been used to determination of (NZP) are Electrical methods[8], and Chromatography[9-18]. In this paper describes a simple, rapid and sensitive method for the determined of small amounts of Nitrazepam (NZP) depending on the diazotization-coupling reaction between reduced (NZP) and thymol reagent in the alkaline medium of (NH_4OH). And study the optimum condition for this reaction. Then applying this method on pharmaceutical Preparations which is contained (CPZ) compound with high accuracy and precision.

Experimental

All spectral and absorbance measured by T80 UV-Visible Spectrophotometer PG Instrumental Ltd, UK. With quartz cells, which have (1 cm) optical path.

Oven BS Size Two, Gallenkamp, England. With (0-300 ^{0}C) thermal range.
Heating, Cooling Water Bath – Haak Fe, Sartorius, Balance Bp3015 –Germany

Material and Method

All materials used have a high degree of purity, they were prepared by the following:

1- Nitrazepam standard material from the state company for drug industries and medical appliances Samara-Iraq (SDI). The standard stock solution of reduced (NZP) at (100 µg. ml^{-1}) was prepared by dissolving (0.005 gm) of NZP in ethanol, then transferred into (25 ml) volumetric flask, and diluted to mark with the same solvent. The solution was transferred into a beaker of (125 ml)a (2 ml) of distilled water, (2 ml) of hydrochloric acid (~11.64 M), and (0.3 gm) of zinc powder were added. The content was allowed to stand for (15 min) at room temperature, then the solution was filtered into (50 ml) volumetric flask, washed the residues with distilled water, and diluted to mark the volume with distilled water to obtain (100 µg ml^{-1})of Nitrazepam reduction solution. The other diluted solutions of NZP were prepared daily dilution using distilled water.
2- **Thymol Solution (0.2) M:** It was provided from (SDI) company. This solution was prepared by dissolving (1.502 gm) of thymol by ethanol and diluting into (50ml) with the same solvent in a volumetric flask as a stock solution.
3- **Ammonium hydroxide Solution (4 M):** It was prepared from (BDH) company, used for preparation (4M) solution.
4- **Sodium Nitrate Solution (0.1 M):** It was prepared from (BDH) company at percentage (%99), it was used for preparation (0.1M) solution
5- **Sulfuric acid H_2SO_4 (1M):** It was prepared from (GCC) at percentage (%98) company and used for preparation (1M) solution.

Recommended Procedure

A group of (10 ml) volumetric flasks was taking, and added to them an increasing volume (0.01-1.2 ml) of (100 µg/ml) of the reduced NZP solution, and constant volume (1 ml) of (0.1 M) of sodium nitrate, then added (0.1 ml) of (1 M) HCl, After that the solution was to leave for a few times to complete diazotization reaction. Then (1 ml) of (0.05 M) of thymol, and (1.2 ml) of (4 M) of ammonium hydroxide were added. Volume was completed to the mark with distilled water, mixed well and measured the absorbance of all solutions against reagent blank solution, at room temperature after passing (5) minutes for the dilution process at the wavelength (477nm). And calibration curve was constructed.

Procedure for Assay of Nitrazepam in Pharmaceutical Preparations. As the preparation Zipex containing (NZP) as an active material was taken and it included the following, Table (1):

Table (1): Pharmaceutical Preparations of NZP

Pharmaceutical	Contains	Company
Zipex Tablets (30-Tablets)	Per tablet: 5 mg. Nitrazepam	Aburaihan Pharmaceutical Co. Tehran-IRAN

Procedure for Tablets :

The amount of tablets was accurately weighted and finely powdered, an amount of the powder equivalent to (5 mg) of NZP was dissolved in (30 ml) of ethanol. The solution was filtered into (25 ml) volumetric flask, the residue was washed with ethanol and diluted to volume with the same solvent to obtain (100 µg ml^{-1}) of NZP. This solution was transferred into (125) ml beaker and was reduced as procedure of pure NZP, and this solution was used to apply the proposed method by dilution with distilled water.

Different conditions were studied which are affecting the absorbance of the product formed such as:

1- Concentration of the reagent (Thymol):

It has been added (1 ml) of different concentrations ranging (0.001-0.2 M) of the reagent (thymol), the results shown in Fig (1). It was found that the best concentration of thymol was (0.05 M), and the absorbance was decreased after this concentration, so this concentration was used in the subsequent experiments.

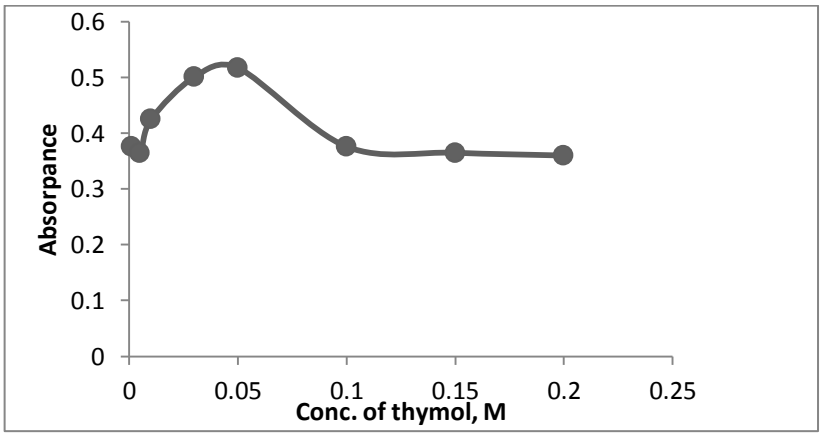


Figure (1): Effect of the reagent concentration

2- volume of the reagent (0.05 M):

The effect of the reagent volume on the absorption signal of the colored product was studied. It has been taking various volumes ranging from (0.1 -1.6 ml) of (0.05 M) of the reagent, it found that the best volume is (1 ml), where after this volume the absorbance remain almost fixed, so this volume was used in subsequent experiments. Fig (2) exhibit this.

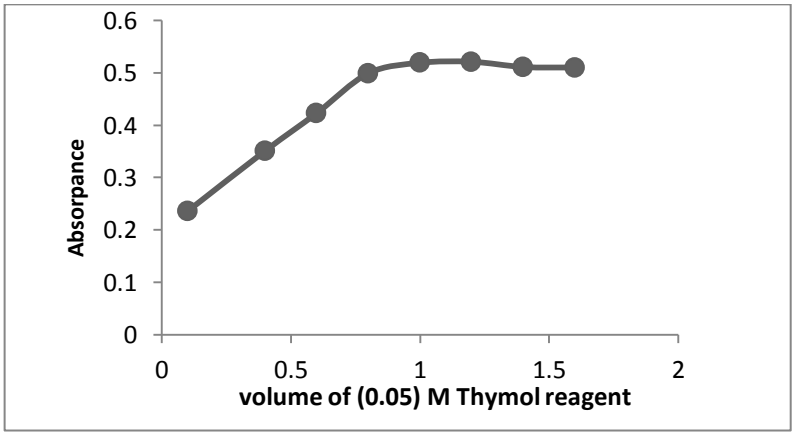


Figure (2): Effect of volume of (0.05) M of Thymol

3-Type of Acid:

Different types of strong and weak acids have been chosen, to study the effect of force and type on the formation and stability of diazonium salt of reduced NZP, by adding (1 ml) of (1 M) of each acids, and measuring the absorbance at the wavelength (477 nm). The results in Table (2) show a significant change in the intensity of absorption of colored product, when replacing acid by another. Therefore, (1 M) of HCl was chosen for diazotization reaction of the reduced NZP, to give a better absorbence of colored product.

Table (2): Effect of type of acid

Acid (1) M	Absorbance
Hydrochloric acid	0.519
Sulfuric acid	0.172
Nitric acid	0.350
Acetic acid	0.063

4- The volume of acid (HCl):

The effect of the volume of (1 M) of HCl on the absorption signal of colored product has been studied, by taking volumes ranging from (0.05 -1.2 ml) of this acid. From the results obtained, the best volume of the acid is (0.1 ml), it was selected in the following experiments. Fig (3) exhibit this.

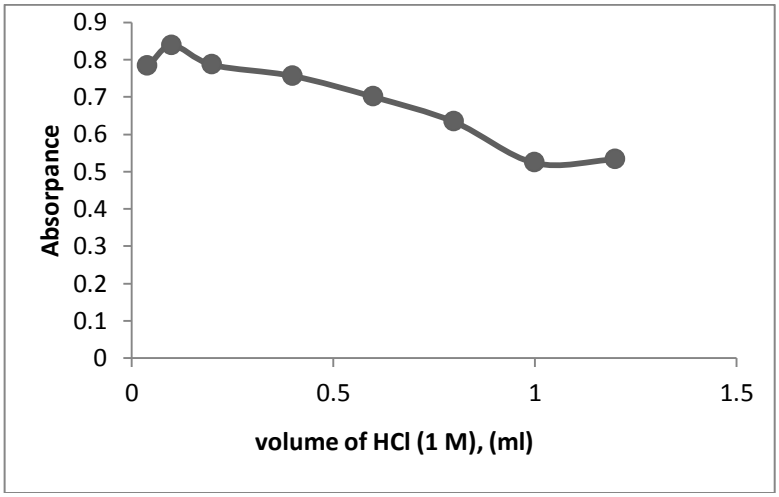


Figure (3): Effect the volume of HCl

5- Effect of $NaNO_2$ Volume:

The volume effect of (0.1 M) $NaNO_2$ on the intensity of the absorption of colored product has been studied, by taking volumes ranging from (0.1 -1.6 ml). From the results obtained, the best volume of the sodium nitrate is (1 ml). As shown in the Fig (4). The use of excessive volume of sodium nitrite in the reaction mixture leads to increased the concentration of nitrous acid, and this is undesirable, because it may not lead to the preferred side reactions, such as Nitration reaction of the reagent, especially it has a electrons with donating groups. So it was installed this volume in subsequent experiments.

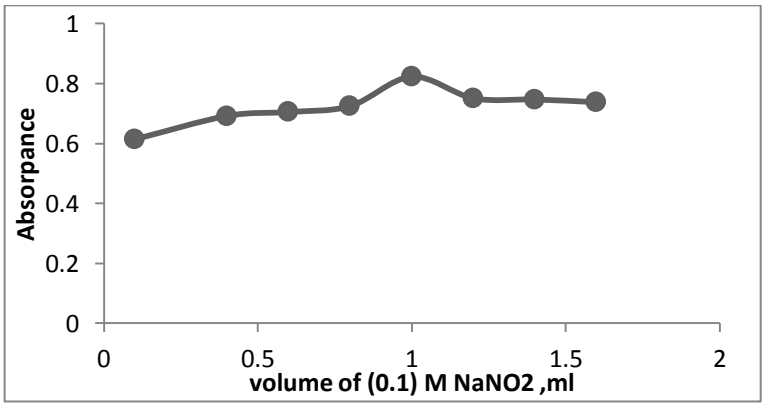


Figure (4): Effect of $NaNO_2$ volume

6- Effect type of Base:

Different types of strong and weak bases have been chosen, to study the effect of these bases on the formation and stability of coloured product, by adding (1 ml) of (4 M) of each base, measuring the absorbance at the wavelength (477 nm). The results in Table (3) show a significant change in the intensity of absorption of colored product, when replacing one base by another. Therefor, (4 M) of NH_4OH was chosen for diazotization reaction of the drug, to give a better absorbency of colored product.

Table (3): Effect of type of alkaline medium

Type of Base	Absorpance
Without adding any base	**0.09**
Sodium hydroxide	-
Potassium hydroxide	-
Ammonium hydroxide	0.832
Sodium acetate	0.352
Sodium carbonate	0.029
Potassium carbonate	0.150

7- The Volume of (4 M) NH_4OH:

The volume effect of (4 M) of NH_4OH on the intensity of the absorption of colored product has been studied, by taking volumes ranging from (0.4 -1.6 ml) From the results obtained, the best volume of the base is (1.2 ml), it was selected in the following experiments. As shown in Fig (5).

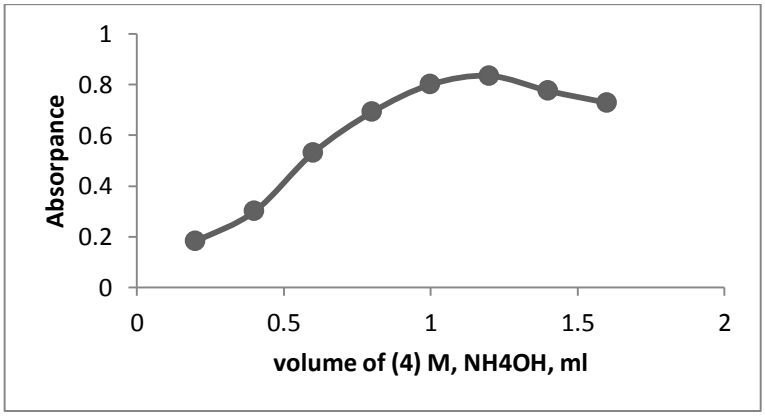


Figure (6): Volume effect of base NH_4OH

8- Sequence of addition:

Number of sequences were tested, shown in the table (4) for the purpose of estimating the best sequence of addition in the form of colored product. It was found that the best sequence of addition that gives the highest absorption (D+R +B), where: (D = Diazotized Drug, R = Reagent, and B= Base) which selected in following experiments.

Table (4): Effect of sequence of addition

Added sequence	Absorbance
D+R+B	0.847
D+B+R	0.798
R+B+D	0.539
R+D+B	0.502

9- Effect of Reaction Time:

The color intensity appears its maximum after the diazotized reduced drug (NZP) had been reacted immediately with the reagent, in the alkaline medium of (4 M) of ammonium hydroxide, became stable after (5) minutes. Therefore (5) minutes was selected as an optimum time in the general procedure. The color product obtained was stable for at least (60) minutes. As in Fig (6)**.**

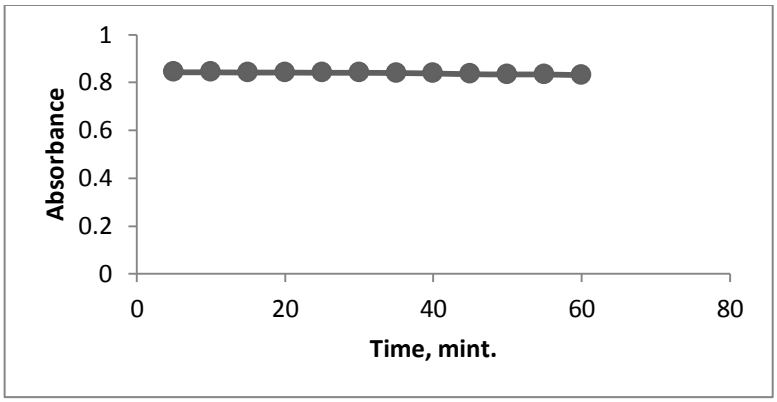


Figure (6): Effect of reaction time

10- Effect of Temperature:

The resulting product of the proposed method was studied at different temperatures. The results referred that the absorbance values remain nearly constant in the temperature range (5-30ºC), whereas, at higher temperatures the absorbance value decreases, referring to the partial dissociation of the colored product when heated for a long time. The color product was stable at room temperature (25ºC) which was given the highest absorbance. The room temperature was selected in this method of determination. This explains in Fig (7).

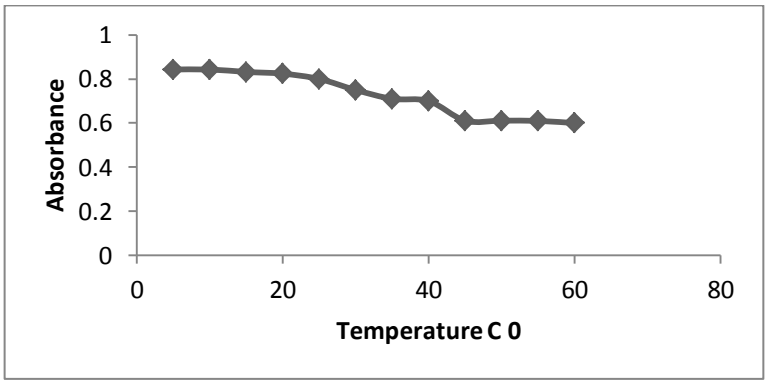


Figure (7): Effect of temperature

Absorption Spectra

The spectral scan has been done to get the highest wavelength absorption of the resulting compound getting after fixing the optimum conditions for the reaction against blank solution which contain all the additives except for the drug compound, Fig (8) shows the spectrum of complex product from the reaction between (NZP) and Thymol against blank solution.

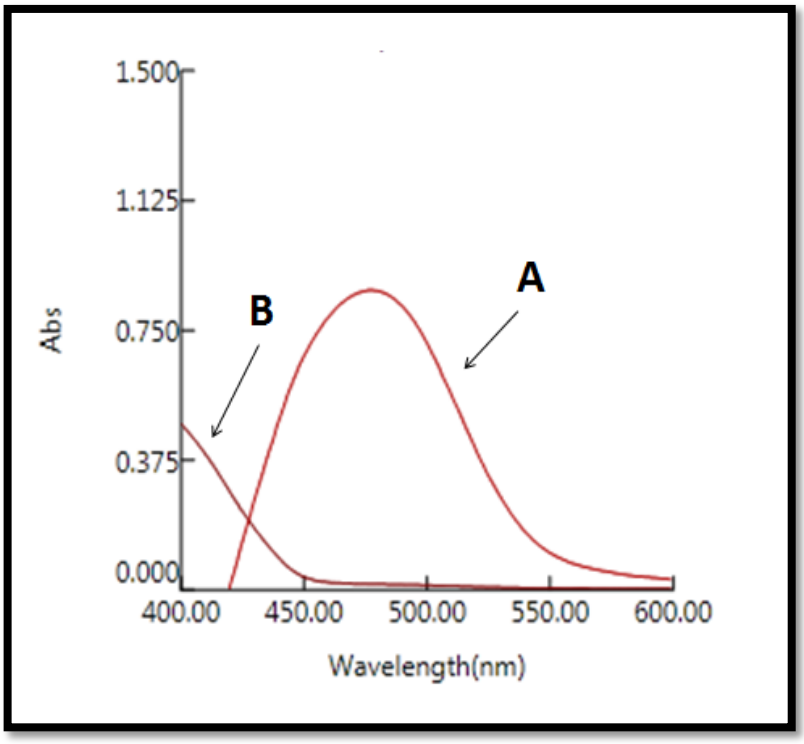


Figure (8): Absorption spectra of the: (A): product obtained from reaction between 10 µg/ml diazotization reduced NZP with thymol, versus reagent blank. (B): reagent blank versus distilled water

Calibration curve

According the conditions described in the procedure, a linear calibration curve for Nitrazepam is obtained Fig 9, which shows that Beer's law is obeyed over the concentration range of (0.1–12 µg/ml) with a correlation coefficient of (0.9974) and an intercept was (0.0454), the slope of the curve was (0.0805). The conditional molar absorptivity of the blue product formed was found to be (2.264*104 L.mol-1. cm-1). The Sandell's sensitivity was (0.0124µg. cm –2). LOD was (0.020µg/ml-1). LOQ was (0.072µg/ml-1).

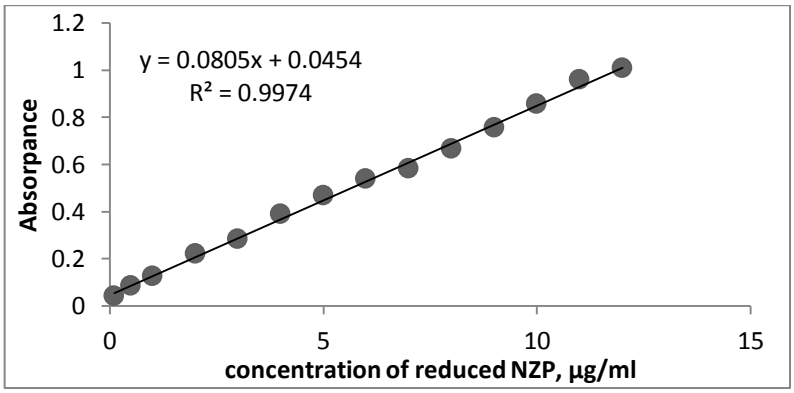


Figure (9): Calibration curve of reduced NZP with thymol reagent

Accuracy and precision

The accuracy and precision of the method were checked by determination the Nitrazepam at three different concentrations from calibration curve (five solution for each concentration) under optimum condition. And calculation relative error (E%), recovery (Rec %), and relative standard deviation (RSD %). The results represented in Table (5) indicate that the method is satisfactory, and have high accuracy and precision.

Table (5): Accuracy and Precision for proposed method

Concentration of reduced NZP µ/ml		%E*	%Rec*	%RSD*
taken	found			
3	2.980	-0.353	99.67	0.886
5	4.933	-1.232	98.67	1.088
7	7.010	+0.240	100.24	0.223

***Each value, it's the average of five readings**

Stoichiometry of reaction

The stoichiometry of the reaction between Nitrazepam and the reagent (thymol) was investigated by applying Job's method and mole ratio method; the results obtained that (2:1) drug to reagent complex was formed as (477nm) shown (Fig 10). The product formed was soluble in water. To know the stability of the product, degree of dissociation and stability constant were calculated by comparing the absorbance of a solution containing stoicheiometries amount of Nitrazepam and the reagent at a concentration (3*10-4 M), with other solutions containing the same amount of the drug and five times the amount of reagent. The average conditional stability constant of the color product in the water under the described experimental conditions was (2.27*1011 L2 Mol-2).

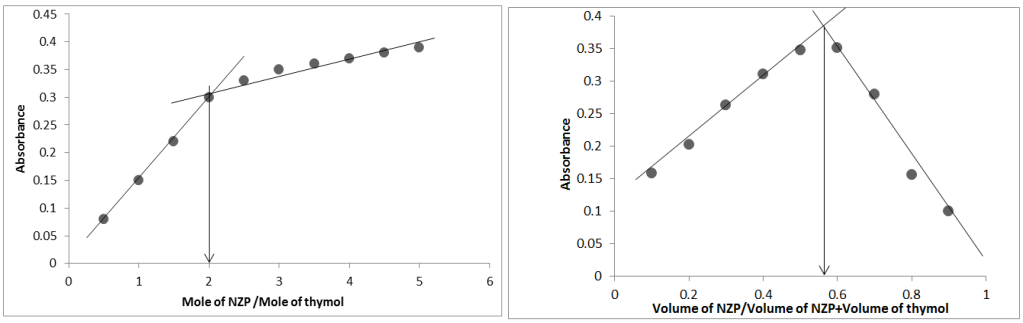


Figure (10): mole ratio and job methods, for diazo-coupling reaction between (NZP) with thymol in the presence of NH_4OH

The formation of the color product between Nitrazepam and Thymol in the presence of NH_4OH was suggested at the scheme of reaction probably occurs as the following equation Fig (11):

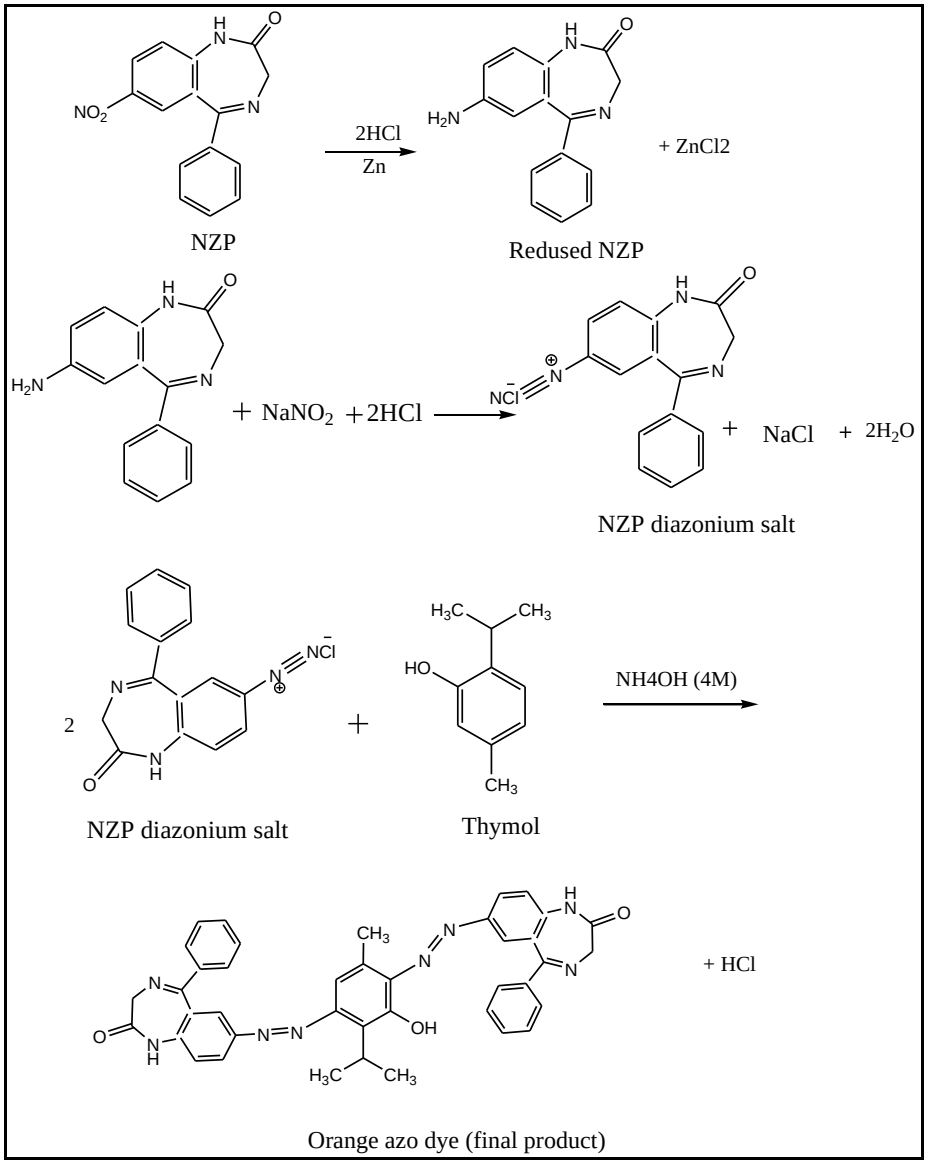


Figure (11): proposed reaction between NZP and thymol reagent

Interferences

The effect of additives which are found in the pharmaceutical contain (NZP) were studied, to ensurefrom the selectivity of the proposed method for the purpose of application of the pharmaceutical contain (NZP). For this study, the solution was contained (4µg / 10 ml) of (NZP) and each one of the excipients was taken separately in concentrations, ten-times greater than of (NZP) were analyzed under the same procedure in the Calibration curve. The level of interference was considered to be acceptable if the error was not higher than (± 2%) relative to the expected No interferences were observed in the determination of (NZP) in the presence of the excipients studied, show in table (6) (Average of five determinations).

Table (6): Effect of excipients in determination of NZP (4µg /10 ml)

Excipient	Conc of NZP µg/ml (found)	%Error*	%Rec*
Lactose	3.96	-1.00	99.00
PVP	3.97	-0.75	99.25
starch	3.94	-1.50	98.50
Mg stearate	3.98	-0.50	99.50

***Each value, it's the average of five readings**

Application of the method

The applicability of the method for the assay of pharmaceutical formulation, it has been studied. The result of experiment for available formulations of Nitrazepam drug is summarized in Table (7):

Table (7): Application on pharmaceutical formulations of NZP

Pharmaceutical preparation	Proposed method		*Error%	Rec%*	*RSD%
	taken	Found			
Zipex (Tablets)	3	2.97	-1.00	99.00	0.740
	5	5.01	+0.20	100.2	1.308
	7	6.97	0.42-	99.57	0.254

***Each value, it's the average of five readings**

For assessing proposed method and its success in the spectral estimation of (NZP) in some pharmaceuticals, it has applied the standard method to estimate (NZP) were taken from British pharmacopoeia (2009). Then comparison with The proposed analytical method. The results are summarized in the table (8).

Table (8): Application of F, and t test for comparison between proposed and standard methods

Pharmaceutical preparation	Proposed method		Standard method	
	(x_{i1}) %Rec	$(x_{i1} - \bar{x}_1)^2$	%Rec (x_{i2})	$(x_{i2} - \bar{x}_2)^2$
Pure NZP	99.52	0.01	99.96	0.0009
Zipex	99.72	0.01	99.90	0.0009
	$\bar{x}_1$ = 99.62	= 0.02Σ	$\bar{x}_2$= 99.93	= 0.0018Σ
T Value (exp)= 10.33, Critical Value = 12.706 F Value (exp)= 1.111, Critical Value = 19.000				

The results showed no significant differences between the two methods, this means the proposed method in this research with the possibility and validity of Good apply to all pharmaceuticals used.

Conclusion

A simple, rapid, precise and sensitive spectrophotometric method has been developed for the determination of trace amounts of Nitrazepam in aqueous solution based on diazotization coupling reaction with the organic reagent Thymol and $NaNO_2$ and HCl in the presence of ammonium hydroxide. The proposed method does not require temperature control or the solvent extraction step, the method was applied, successfully for the determined of small amounts commercial (NZP) drug.

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