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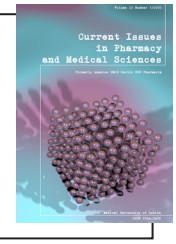

A batch and cloud point extraction kinetic spectrophotometric method for determining trace and ultra trace amounts of Benzodiazepine drugs (Clonazepam and Nitrazepam) in pure and pharmaceutical preparations

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ABSTRACT

Utilizing the batch Diazotization coupling reaction and the cloud point extraction kinetic spectrophotometric method, trace and Ultra trace amounts from reducing Nitrazepam and Clonazepam were evaluated by way of using 2,5-dimethoxyaniline as a new Chromogenic reagent to give colored products (red) in acidic medium which have a maximum absorption at 500 and 502 nm, respectively. In the work, the analytical data of batch and cloud point for Nitrazepam and Clonazepam depended on initial rate and fixation time. This involved concentrations of (0,3-9), (0.05-1.2), (0.5-10) and (0.025-1) μg mL^{-1}, as well as molar absorptivity of (3.8×10^4), (3.1×10^5) (3.39×10^4) and (3.47×10^5) L mol^{-1} cm^{-1}. According to our results, Sandall's sensitivity were (0.0074), (0.0009), (0.0092) and (0.0009) μg cm^{-2}, while detection limits were (0.055) and (0.069) μg mL^{-1}, (8.4) and (8.5) ng mL^{-1}. In addition, the measurements enrichment factors were (33.33) and (50), while preconcentration factors were (8) and (9.9), respectively. The reaction of Nitrazepam and Clonazepam with 2,5-dimethoxyaniline were a pseudo first order according to kinetic studies. The proposed methods are not affected by existence of excipients so the methods can be applied successfully for determination of Nitrazepam and Clonazepam in pharmaceutical preparations.

INTRODUCTION

Clonazepam (CZP) and Nitrazepam (NZP) are benzodiazepines. Benzodiazepine drugs are chemically different from each other in their activities yet share a similar structure. These drugs play prominent roles in the therapy of epilepsy, anxiety and insomnia, dyskinesia, and sedation prior to dental and medical procedures [1-3]. Both NZP and CZP are sturdy soporific drugs due to their high power Nitrobenzodiazepine structure. They are used to treat anxiety disorders, anti-panic and restless legs syndrome. Moreover, they can dampen the evolution and spread of epileptiform electrical activity in the central nervous system, hence, can be used in managing seizures [4-7]. NZP and CZP have been quantified via gas and Liquid chromatography [8-15], HPLC [16-23], Chemiluminescence and Electrochemiluminescence [24,25], Electrical methods [26-34], LC-MS/MS for estimation CZP in blood [35], flow-injection methods [36-41], as well as Colorimetric and Spectrophotometric techniques combined with Cloud point extraction methods for determining metals and drugs [42-60]. The chemical structures of both Clonazepam and Nitrazepam are shown in Scheme I [1-3].

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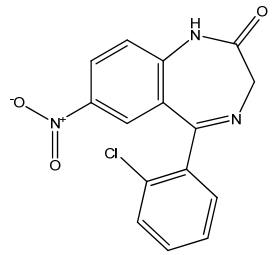
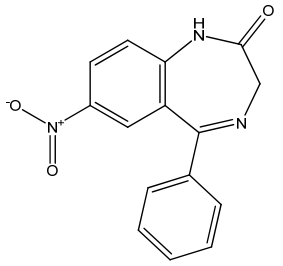
AIM

The present work involves using kinetic spectrophotometric method and Cloud point extraction (CPE) for estimating trace and Ultra trace amounts of Clonazepam and Nitrazepam drugs in pure and pharmaceutical states. The procedure depends upon a simple Diazotization coupling reaction between these drugs and 2,5-dimethoxyaniline in an acidic medium.



Clonazepam
5-(2-chlorophenyl)-7-nitro-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one
Chemical Formula: $C_{15}H_{10}ClN_3O_3$
Molecular Weight: 315.71
Physical properties: white to light yellow crystalline powder



Nitrazepam
7-nitro-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one
Chemical Formula: $C_{15}H_{11}N_3O_3$
Molecular Weight: 281.27
Physical properties: yellow, crystalline powder

Scheme I. Structural formula of Clonazepam and Nitrazepam

MATERIAL AND METHODS

Instruments

The scanning of all spectra and measurements of the absorbance at selected wavelengths were carried out by using a T80 UV-Visible Spectrometer PG Instrumental Ltd, UK, with quartz cell matched 1 cm, Melting points were estimated by means of a SMP30 Melting point Stuart apparatus, UK, while the pH was adjust using a 340i pH-meter WTW, Germany. An oven BS Size Two with a temperature range (0-300)°C made by Gallenkamp, England, was utilized for drying and a Heating-Cooling Water Bath from Haak Fe, Sartorius and a Balance Bp3015 (Germany) were used in sample preparation.

Chemicals and reagents

High purity Clonazepam ($C_{15}H_{10}ClN_3O_3$) and Nitrazepam ($C_{15}H_{11}N_3O_3$) were obtained from the State company for Drug Industries and Medical Appliance-(SDI), Samarra-Iraq. 2,5-dimethoxyaniline ($C_8H_{11}NO_2$), Sodium nitrite ($NaNO_2$), Absolute ethanol (C_2H_5OH), and Sulfamic acid (NH_2SO_3H) with Purity 99.00% were obtained from the BDH Company. Hydrochloric acid (HCl) with purity 37.00% was also obtained from BDH Company. Triton X-114 CAS#: 9002-93-1 with purity 100% was supplied by Arcos Organics, New Jersey, USA. Pharmaceutical preparations that were used in this study were Rivotril 2 mg/Clonazepam from Roche Farma S.A and Zipex 5 mg/Nitrazepam from Aburaihan phamaceutical Co, Tehran-Iran.

Solutions

Reduced Clonazepam and Nitrazepam Stock solutions (100 μg mL^{-1}) were prepared by taking (0.00500 g.) CZP and NZP and dissolving these in (25 mL) ethanol and (2 mL) of distilled water in separate beakers (125 mL). Subsequently, (2 mL) of Hydrochloric acid (~ 11.64 M) and (0.30 g) of zinc powder were added. These solutions were kept at room temperature for a quarter of an hour. Then the obtained solutions were filtered into a separated volumetric flask (50 mL), diluted to mark a volume with distilled water to obtain (100 μg mL^{-1}) solutions of reduced CZP and NZP [61]. Hydrochloric acid (1M) was prepared by dilution (4.3 mL) from concentrated Hydrochloric acid (~ 11.64 M) in (50 mL) distilled water. Sodium Nitrate (0.1 M) was prepared daily by dissolving (0.173 g) Sodium Nitrate in (25 mL) distilled water. Sulfamic acid (0.2) was prepared by dissolving (0.978 gm) in (50 mL) distilled water, while 2,5-Dimethoxyaniline (0.005 M) was prepared by dissolving (0.038 g.) 2,5-Dimethoxyaniline in (50 mL) absolute ethanol, and Triton X-114 (10%) was prepared by dilution (10 mL) from Triton X-114 in (100 mL) distilled water. Tablets sample (Rivotril 2 mg, Zipex 5 mg) solutions (100 μg mL^{-1}) were prepared after grinding by following the procedure of stock solution of reduced CZP and NZP, after weighing the suitable amount of each formulation (powder) equivalent to (5 mg), from CZP and NZP.

GENERAL PROCEDURE

Batch, Initial-rate method

The calibration curves of Nitrazepam and Clonazepam were constructed by using a series of (10 mL) volumetric flasks. In doing this, increasing volumes (0.03-0.9 mL) of reduced Nitrazepam (100 μg ml^{-1}) and (0.05^{-1} mL) of reduced Clonazepam (100 μg ml^{-1}) were added to volumetric flasks (10 mL), this is was followed by the addition of (0.1 mL) Hydrochloric acid (1 M), and (0.5 & 0.7 mL) Sodium Nitrate for Nitrazepam and Clonazepam solutions, respectively. The obtained solutions were mixed carefully and stood for (5 min) to complete the formation of a dizonium salt. Subsequently, (0.5 & 0.7 mL) Sulfamic acid (0.2 M) was added to the Nitrazepam and Clonazepam solutions, respectively, which then stood for (5 min) to removal excess amounts of Nitrous acid. Then (2 & 1.5 mL) 2,5-dimethoxyaniline (0.005 M) was added for Nitrazepam and Clonazepam solutions, respectively, and the volumes completed with distilled water. The absorbance for all solutions were measured at 500 nm for the Nitrazepam complex and 502 nm for the Clonazepam complex at (25°C) as a function with time against solution blank. The slope of the tangent absorbance-time curve gave the initial rate of reaction at different concentrations. The calibration curve was constructed by plotting the logarithm of the initial rate (log k) versus the logarithm of the molar concentration of Nitrazepam and Clonazepam (log C).

Batch, Fixed time method

The second procedure for treatment the calibration curves – the batch method – was performed by measuring the absorbance of color solutions. These contained varying amounts of Nitrazepam and Clonazepam at preselected fixed times (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 min.). Calibration curves were generated by plotting the absorbance against

the final concentrations of Nitrazepam and Clonazepam at fixed time intervals.

General procedure of Cloud Point extraction method (CPE)

A series of volumetric flasks (10 mL) with increasing volumes from 0.05 to 1.2 mL of solutions of reduce Nitrazepam (10 µg ml^{-1}) and (0.025^{-1} mL) from reduced Clonazepam (10 µg ml^{-1}) were first created. This solution was mixed carefully with (0.1 mL) Sodium Nitrate and stood for (5 min.) to insure complete formation of the dizonium salt. This then was followed by the addition of (0.1& 0.07 mL), respectively, Sulfamic acid (0.2 M) and stood for (5 min.) to remove excess amounts of Nitrous acid. Subsequently, (0.2 & 0.3 mL) 2,5-dimethoxyaniline (0.005 M) was added to the Nitrazepam and Clonazepam solutions, respectively, and then the volumes were completed with distilled water. The solutions were then transferred to centrifuge tubes following the addition (1 mL) of Triton X-114. Afterwards, the mixture was transferred into a hot water bath for about (10 min.) at (65°C) to form cloud solutions. The mixture was then separated into two phases by centrifuge (10 min.) at (3500 rpm). The aqueous phase was decanted and diluted with surfactant-rich phase by (0.5 mL) absolute ethanol. The absorbance of final solutions were measured at (500, 502 nm) for Nitrazepam and Clonazepam solutions, respectively, against a blank solution prepared in the same method as shown in (Fig. 1).

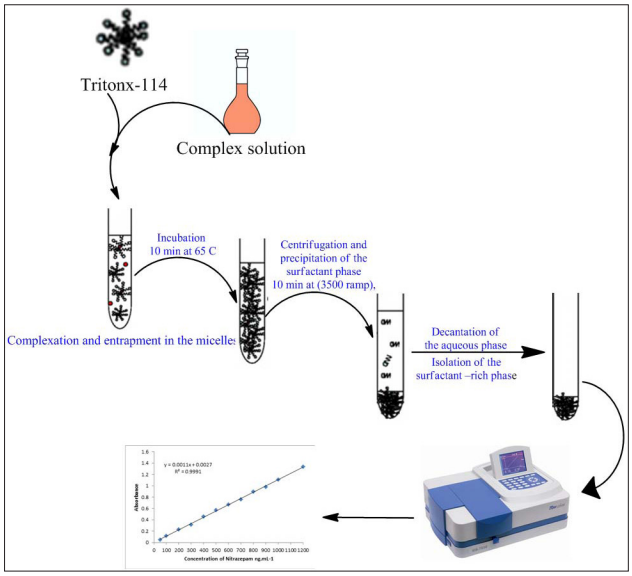


Figure 1. General steps for determination of Nitrazepam and Clonazepam by combining CEP with a UV-Visible technique

RESULT AND DISCUSSION

Identification of products

The qualitative study of color products for Nitrazepam and Clonazepam with 2,5-dimethoxyaniline in Batch and Cloud point extraction methods was carried out using a UV-Visible technique where the Azo-dyes complex (color product) was scanned at (700-300 nm) and showed a maximum absorption peak at (500-502 nm) versus blank solution. In contrast, the scanning of the blank solution versus water does not give any absorption at λ_{max} for colored products as shown in (Fig. 2). This property was adopted in quantitative estimation of trace and Ultra trace amounts from Nitrazepam and Clonazepam in pure and pharmaceutical preparations.

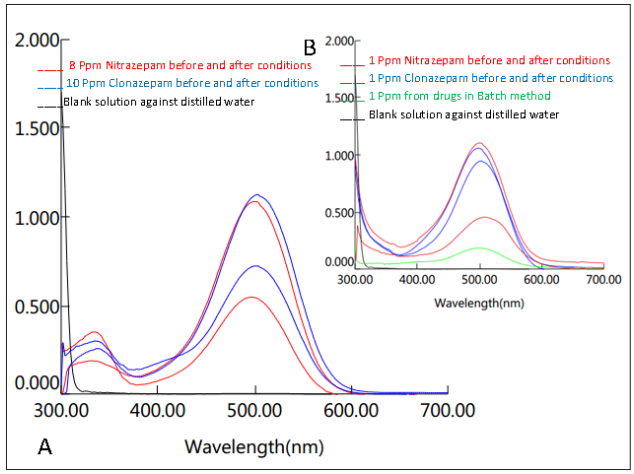


Figure 2. Spectra of azo-dye for Nitrazepam and Clonazepam with 2,5-dimethoxyaniline ***A.*** Batch method, ***B.*** Cloud point extraction method

OPTIMIZATION OF EXPERIMENTAL CONDITIONS

Optimization of Experimental Conditions for Batch Method

The effect of various experimental conditions on the absorption of colored solution was assessed by coupling dizonoum salt for reduced Nitrazepam and Clonazepam drugs with organic reagent 2,5-dimethoxyaniline in an acidic medium. Herein, the concentrations of 8 & 10 µg ml^{-1} from reduced Nitrazepam and reduced Clonazepam were used, respectively. To this, (0.1 mL) Hydrochloric acid (1 M) and (0.5 mL) Sodium nitrate (0.1 M) were incorporated and mixed carefully. The product then stood for (5 min) to complete the formation of a dizonium salt. Subsequently, (0.5 mL) Sulfamic acid (0.2 M) was added, and the product stood for (5 min) to removal excess amounts of Nitrous acid. Then (1 mL) 2,5-dimethoxyaniline (0.005 M) was added and the product was decanted into (10 mL) volumetric flasks. The absorbances of the solutions were measured at (λ_{max}=500, 502 nm) against blank solutions (5 min) after the beginning of the coupling reaction.

Effect of volume of coupling reagent (0.005 M)

The effect of various volumes of 2,5-dimethoxyaniline (0.005 M) solutions on colored products absorption for reduced Nitrazepam and reduced Clonazepam were studied, and the obtained results are shown in (Fig. 3). The increase in volume of 2,5-dimethoxyaniline led to an increase in the absorbance of colored products for Nitrazepam and Clonazepam, yet remain constant above (2 & 1.5 mL). Hence, these volumes were selected as the optimum volumes of coupling reagents with Nitrazepam and Clonazepam, respectively.

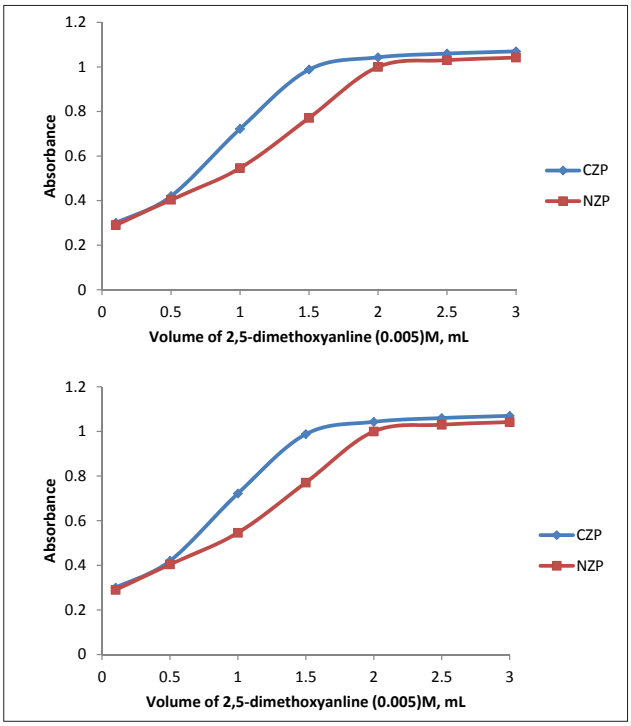


Figure 3. Effect of volume of the coupling Reagent (0.005 M)

Effect of volume of Hydrochloric acid (1 M)

The effect of addition of (0.05-2 mL) (1 M) Hydrochloric acid solution was studied, and the optimum volume to formulate a diazonium salt for reduced Nitrazepam and reduced Clonazepam was found (0.1 mL). Using higher volumes of hydrochloric acid led to a decline in the absorbance for color products because partial dissociation of color product occurred in high acidic media (Fig. 4).

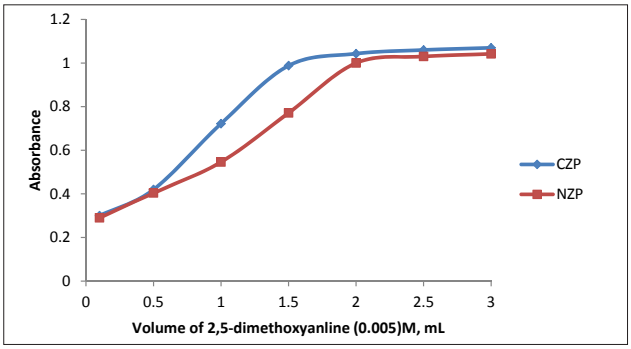


Figure 4. Effect of volume of (1 M) Hydrochloric acid, mL

Effect of volume of Sodium Nitrate (0.1 M)

The optimum volume to format a diazonium salt for reducing Nitrazepam and Clonazepam was found to be 0.5 and 0.7 mL, respectively. This experiment was performed to study the effect of the addition of 0.1-2.0 mL of 0.1 M of sodium nitrate solution on the absorption of color products. Accordingly, using higher volumes led to decrease absorption of color product due to notarization of the reagent (2,5-dimethoxyaniline) as shown in (Fig. 5).

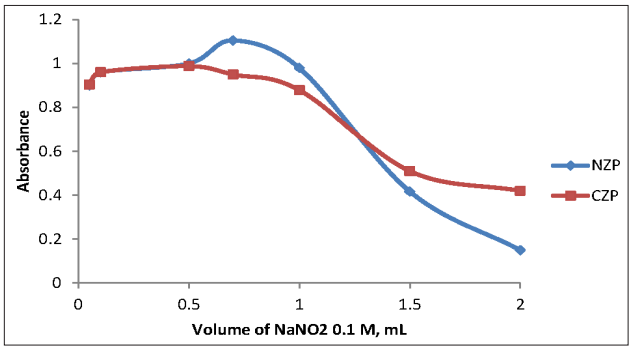


Figure 5. Effect of added volume of 0.1 M sodium nitrate on the formation of diazonium salt

Effect of volume of Sulfamic acid (0.2 M)

The effect of different volumes (0.1-2 mL) of (0.2 M) Sulfamic acid solution on the absorption of color products for Nitrazepam and Clonazepam was studied. The optimum volumes of Sulfamic acid needed for removing excess nitrous acid amounts were found to be (0.5 & 0.7 mL) for Nitrazepam and Clonazepam, respectively, as shown in (Fig. 6).

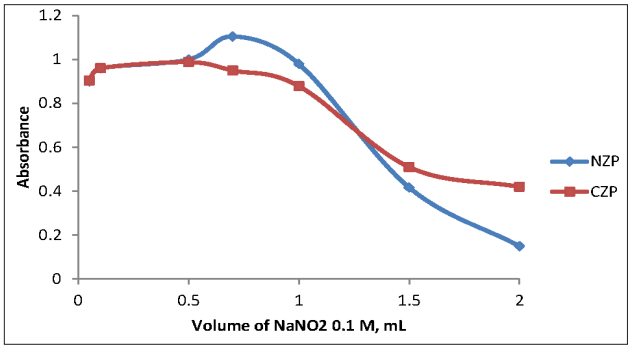


Figure 6. Effect of added volume of (0.2M) Sulfamic acid, mL

Effect of temperature

The effect of temperature on the formation of azo-dye for reduced Nitrazepam and reduced Clonazepam with 2,5-dimethoxyaniline was investigated. The colored products showed stable and maximum absorption spectrum at low and high temperatures. The obtained spectrum is shown in (Fig. 7).

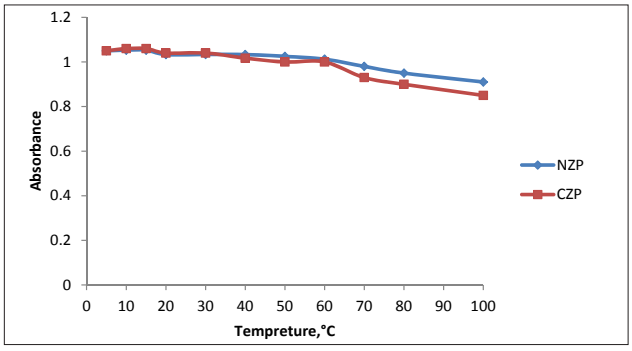


Figure 7. Effect of temperature on azo dye formation

Optimization of Experimental Conditions for the CPE method

The effect of various experimental conditions on absorption of azo-dye for reduced Nitrazepam and reduced Clonazepam with 2,5-dimethoxyaniline when applying the cloud point extraction method were studied. In each experiment, 1 µg ml^{-1} of the reduced drugs, (0.1 mL) (1M) Hydrochloric acid and (0.5 mL) (0.1 M) Sodium nitrate were combined and the result was then allowed to stand for (5 min) to complete the formation of a dizonium salt. Subsequently, (0.5 mL) Sulfamic acid (0.2 M) was added, and the product stood for (5 min) to removal excess amounts of Nitrous acid. After this, (1 & 0.2) mL Clonazepam) from 2,5-dimethoxyaniline (0.005 M) for both the Nitrazepam and Clonazepam solutions, respectively, was added, with the volume completed with distilled water. The solutions were then transferred to centrifuge tubes following the addition of (1 mL) Triton X-114. The tubes were then placed into a hot water bath for about (10 min) at (70°C) to form a cloud solution. The absorbance of final solutions were measured at (500, 502 nm) for Nitrazepam and Clonazepam solutions against a blank solution that was prepared in the same manner, after the surfactant-rich phase was separated by centrifuge (10 min) at (3500 ramp) and diluted with (0.5 mL) absolute ethanol.

Effect of volume of coupling reagent (0.005 M)

The coupling reagent concentration is an important factor affecting CPE and the formation of a surfactant rich phase. Varying the volumes of 2,5-dimethoxyaniline (0.005 M) on the absorbance of the surfactant rich phase for (1 µg mL^{-1}) led to changes in Nitrazepam and Clonazepam absorbance. These results are shown in (Fig. 8).

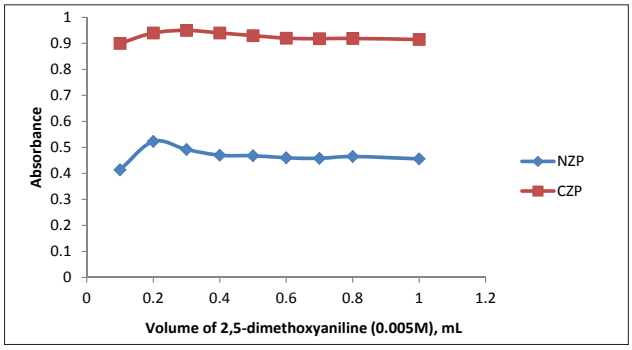


Figure 8. Effect of volume of the coupling reagent on diazonium salt formation

Effect of volume of Sodium Nitrate (0.1 M)

The optimum volume of Sodium Nitrate to formulate a diazonium salt for both reduced Nitrazepam and Clonazepam was found to be (0.1 mL). The effect of various volumes (0.05-1 mL) of (0.1 M) Sodium Nitrate solution on the absorbance of surfactant rich-phase is shown in (Fig. 9).

Effect of pH and volume of Hydrochloric acid (1 M)

The second factor that can effect the formation of a surfactant rich phase of azo-dyes is pH as the azo-dyes

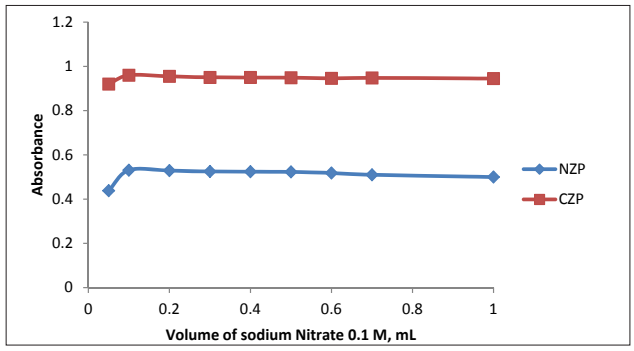


Figure 9. Effect of volume of (0.1 M) Sodium Nitrate, mL

for reduced Nitrazepam and reduced Clonazepam with 2,5-dimethoxyaniline are formulated in an acidic medium. The effect of reduced and increased acidity on azo-dyes were studied by using different volumes of Sodium Hydroxide and Hydrochloric acid. This part of the experiment showed that Azo-dyes solutions without hydrochloric acid showed best cloud point extraction are shown in (Fig. 10) and (Fig. 11). This means that the amounts of Hydrochloric acid coming from the reduction process for Nitro-Nitrazepam and Nitro-Clonazepam is sufficient to formulate the dizonium salt for these drugs.

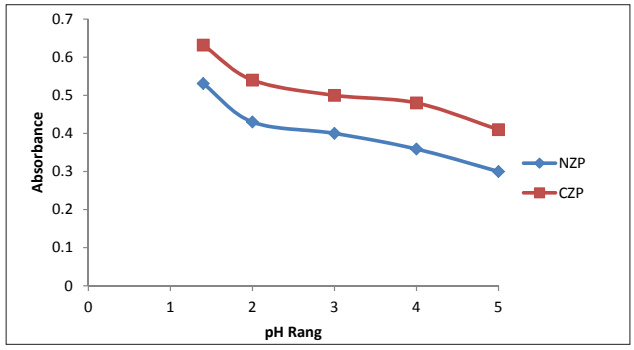


Figure 10. Effect of pH

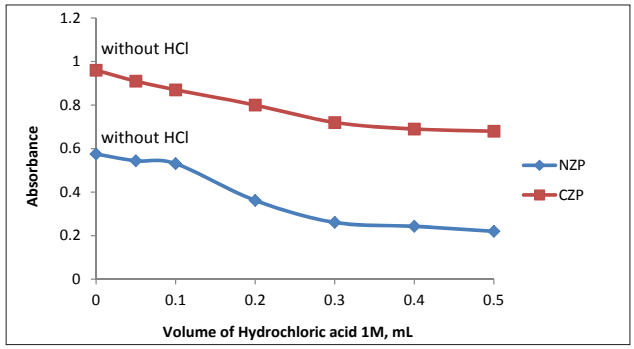


Figure 11. Effect of volume of (1 M) Hydrochloric acid, mL

Effect of volume of Sulfamic acid (0.2 M)

The effect of different volumes of 0.05-1 mL (0.2 M) Sulfamic acid solution on the formation of the surfactant rich-phase for reduced Nitrazepam and reduced Clonazepam was investigated. The optimum volumes of Sulfamic acid needed to remove excess nitrous acid amounts was found to be (0.1 & 0,07 mL) for Nitrazepam and Clonazepam, respectively, as shown in (Fig. 12)

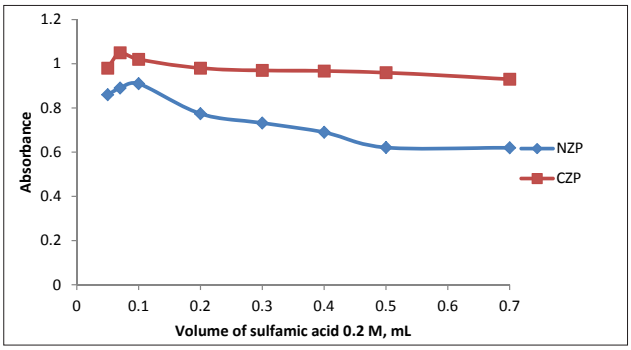


Figure 12. Effect of volume of (0.2M) Sulfamic acid, mL

Effect of volume of Triton X-114 10%

The optimum volume to formulate the surfactant rich phase for (1 µg mL^{-1}) needed for reduced Nitrazepam and reduced Clonazepam to improve the cloud point extraction efficiency was (1 mL). This study was carried out by investigating different volumes in the range of 0.2-1.4 mL (10%) Triton X-114 as shown in (Fig. 13)

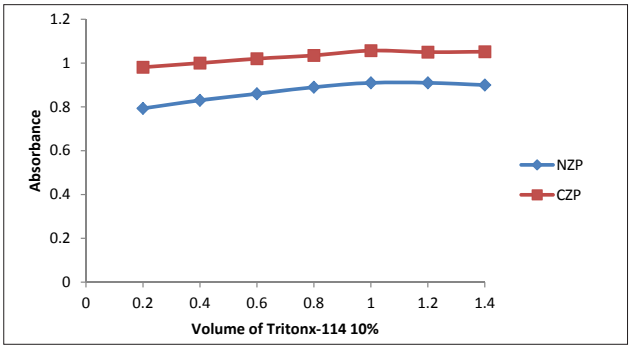


Figure 13. Effect of volume of Triton X-114 10%

Effect of temperature and incubation time on azo dye formation

We investigated the different temperatures in the range of 30-80°C needed at an incubation time of ten minutes. Moreover, the required time for incubation was examined. These results are shown in (Fig. 14, 15). The highest absorbance was obtained at 65°C for 10 min., this probably arises from color product dissociation that occurs at temperatures above this temperature.

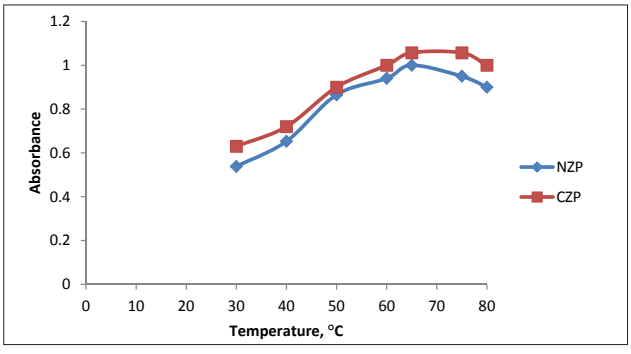


Figure 14. Effect of Temperature on azo dye formation

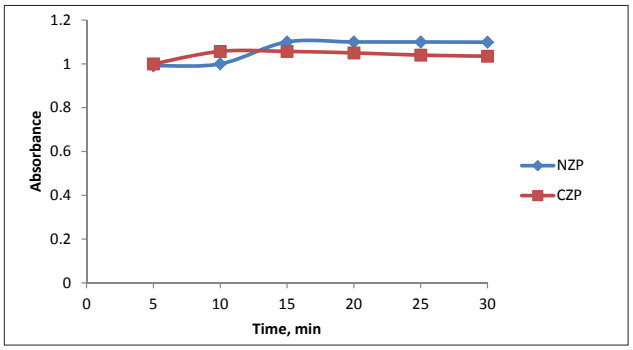


Figure 15. Effect of incubation time on azo dye formation

CALIBRATION CURVE

Calibration curve of initial-rate and fixed time method

After establishing all the optimum conditions for the reaction of reduced Nitrazepam and reduced Clonazepam with 2,5-dimethoxyaniline, a calibration curve was constructed using the initial-rate method. Herein, absorbance was measured at (500 and 502 nm) at (25°C) as a function of time against blank solution. The initial rate of reaction at different concentrations were obtained from the slope of the tangent to the absorbance-time curve (Fig. 16) and (Fig. 17). Suitable calibration curves were generated by plotting the logarithm of the initial rate (log k) versus the logarithm of the molar concentration of Nitrazepam and Clonazepam (log C) (Fig. 18) and (Fig. 19). The linear relationship over the concentration range was (0.3-9) µg mL^{-1} for Nitrazepam and (0.5-10) µg mL^{-1} for Clonazepam. The initial rate of each reactions would follow a pseudo first order rate constant and obeyed the following rate equations:

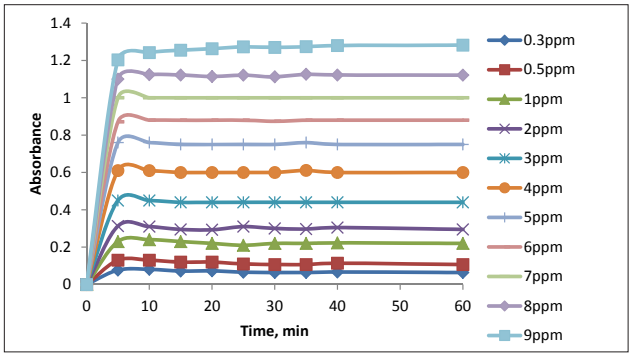


Figure 16. Absorbance versus time graphs for different concentrations of Nitrazepam

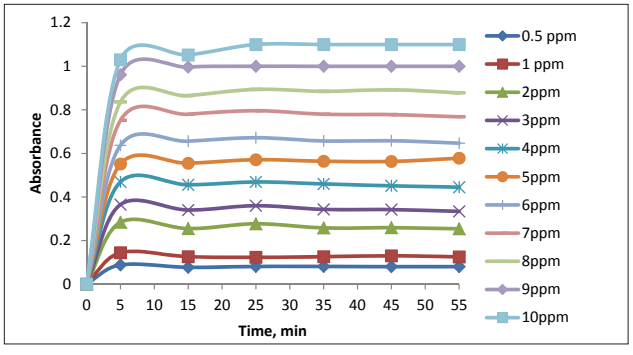


Figure 17. Absorbance versus time graphs for different concentrations of Clonazepam

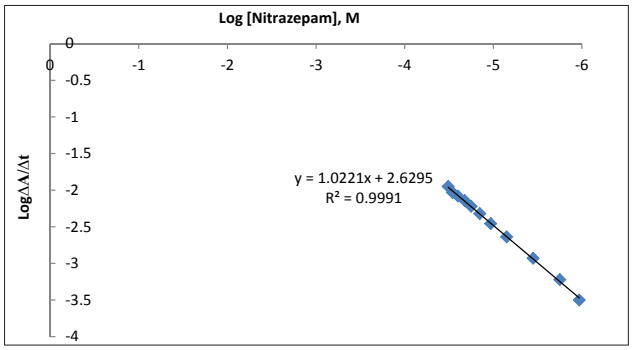


Figure 18. Calibration plots of logarithm rate of the reaction against logarithm molar concentration of Nitrazepam for the initial rate method

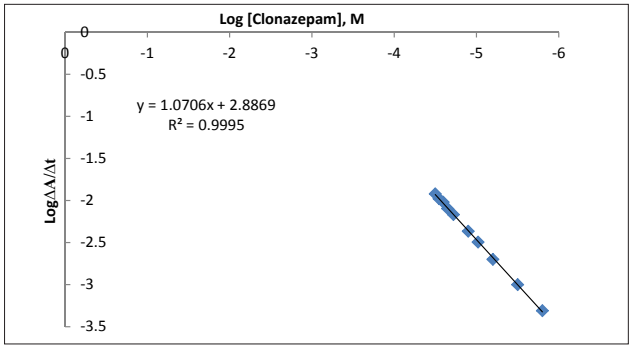


Figure 19. Calibration plots of logarithm rate of the reaction against logarithm molar concentration of Clonazepam for the initial rate method

$Rate = \Delta A/\Delta t = K'[Drug]^n$ $logK = log(\Delta A/\Delta t) = logK' + nlog[C]$

$K = \Delta A/\Delta t = K'[C]^n$ $logK = 2.6295 + 1.0221 log[Nitrazepam]$

A: Absorbane $logK = 2.8869 + 1.0706 log[Clonazepam]$

t: Measuring time log K': intercept, n: slope

K': Pseudo first order rate constant K' NZP =425.69 min^{-1}, K' CZP =770.72 min^{-1}

C: Concentration of the drug (mol L^{-1}) n NZP = 1.0221 ≈ 1, n CZP = 1.0706 ≈ 1

n: Order of the reaction

The straight line of slope (1.0221&1.0706 ≈ 1) confirmed that the reaction was first order. However, under the optimized reaction conditions, the concentration of 2,5-dimethoxyaniline was in excess when compared to that of the drugs, hence, in reality, the reactions followed a pseudo-first order reaction.

The second procedure for establishing the calibration curves was the batch method. The absorbance of color solution containing varying amounts of reduced Nitrazepam and Clonazepam was measured at preselected fixed times (5, 10, 15, 20, 25, 30, 35, 40, 45 and 60 min). Hence, herein, the curve was generated by plotting absorbance against the final concentrations of Nitrazepam and Clonazepam at fixed intervals. The statistical treatment of the calibration curves were summarized in Table 1 and Table 2. From these results, it can be seen that the slope increases with the development of reaction time. The most acceptable value of coloration coefficient, linear range and intercept were obtained at a fixed time (25 min) for Nitrazepam and (35 min) for Clonazepam (Fig. 20) and (Fig. 21), and the application of the fixed time method could be applied to determine reduced Nitrazepam and Clonazepam in pure form over the range of (0.3-9) μg $mL^{-1}$and (0.5-10) μg mL^{-1}, respectively. The analytical values of statistical treatments for the calibration curves are summarized in (Tab. 3) and (Tab. 4), according to the ICH guidelines for validation of analytical procedures [62].

Table 1. Regression equation for drugs at different fixed times for Nitrazepam

Nitrazepam Linear range (0.3-9) μg mL^{-1}		
Time (min)	Regression Equation	Correlation Coefficient
5	A=0.1298C+0.0729	0.9959
10	A=0.1331C+0.0656	0.9971
15	A=0.1347C+0.0541	0.9974
20	A=0.1355C+0.0491	0.9978
25	A=0.1368C+0.0448	0.9985
30	A=0.1361C+0.0457	0.9978
35	A=0.1368C+0.0478	0.9976
40	A=0.1365C+0.0480	0.9980
60	A=0.1373C+0.0427	0.9979

Table 2. Regression equation for drugs at different fixed times for Clonazepam

Clonazepam Linear range (0.5-10) μg mL^{-1}		
Time (min)	Regression Equation	Correlation Coefficient
5	A=0.0983C+0.0603	0.9976
15	A=0.1048C+0.0313	0.9984
25	A=0.1072C+0.0358	0.9988
35	A=0.1074C+0.0268	0.9995
45	A=0.1074C+0.0260	0.9995
55	A=0.1074C+0.0208	0.9992

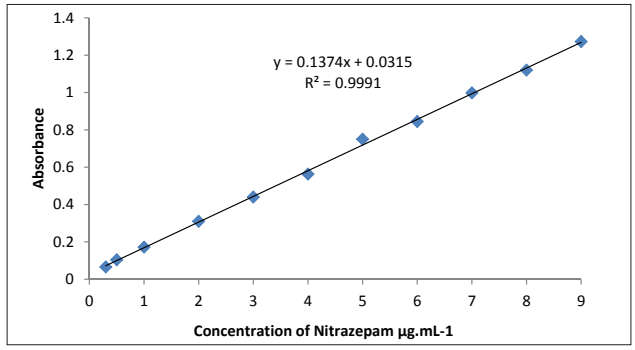


Figure 20. Calibration curve of Nitrazepam at fixed time 25 min.

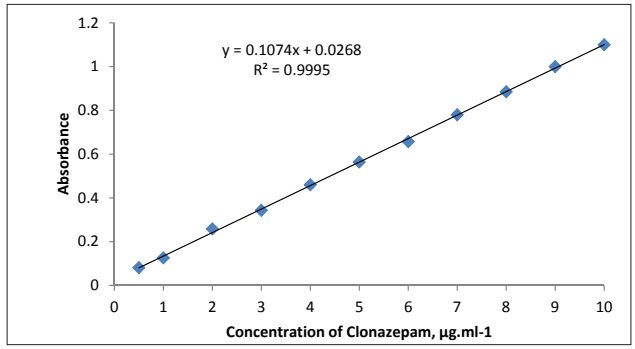


Figure 21. Calibration curve of Clonazepam at fixed time 35 min.

Table 3. Summary of analytical value for calibration curves of batch and CPE method for Nitrazepam

N	Parameter	Value	
		Batch method	CPE method
1.	Regression equation y = bx + a y: Absorbance, b:Slope, x:concentration, a:Intercept	y=0.1374x+0.0315	y=0.0011x+0.0027
2.	Slope $b = \Sigma_i [(x_i - \bar{x})(y_i - \bar{y})] / = \Sigma_i [(x_i - \bar{x})^2]$	0.1374	0.0011
3.	Intercept (a) a = y – bx	0.0315	0.0027
4.	Correlation coefficient	0.9991	0.9991
5.	Linear Range (µg mL^{-1})	0.5-9	50-1200 ng mL^{-1}
6.	Molar absorptivity (ε) (L $mol^{-1}.cm^{-1}$) ε = b × M × 103; M: Molecular weight	3.8×10^4	3.1×10^5
7.	Sandall's sensitivity (S) (µg cm^{-2}) S = M/ε	0.0074	0.0009
8.	Limit of Detection LOD (µg mL^{-1}) $LOD = (3SD_{Blank})/b$	0.055	8.4 ng mL^{-1}
9.	Limit of Quantitation LOQ (µg mL^{-1}) $LOQ = (10SD_{Blank})/b$	0.184	28.3 ng mL^{-1}
10.	Preconcentration factor	---	8
11.	Enrichment factor	---	33.33

Table 4. Summary of analytical value for calibration curves of batch and CPE method for Clonazepam

N	Parameter	Value	
		Batch method	CPE method
1.	Regression equation y = bx + a y: Absorbance, b:Slope, x:concentration, a:Intercept	y = 0.1074x+0.0268	y = 0.0011x+0.0032
2.	Slope $b = \Sigma_i [(x_i - \bar{x})(y_i - \bar{y})] / = \Sigma_i [(x_i - \bar{x})^2]$	0.1074	0.0011
3.	Intercept (a) a = y – bx	0.0268	0.0032
4.	Correlation coefficient	0.9995	0.9994
5.	Linear Range (µg mL^{-1})	0.5-10	25-1000 ng mL^{-1}
6.	Molar absorptivity (ε) (L mol^{-1} cm^{-1}) ε = b × M × 10^3; M: Molecular weight	3.4×10^4	3.47×10^5
7.	Sandall's sensitivity (S) (µg cm^{-2}) S =M/ε	0.0092	0.0009
8.	Limit of Detection LOD (µg mL^{-1}) $LOD = (3SD_{Blank})/b$	0.069	8.5 ng mL^{-1}
9.	Limit of Quantitation LOQ (µg mL^{-1}) $LOQ = (10SD_{Blank})/b$	0.235	28.3 ng mL^{-1}
10.	Preconcentration factor	---	9.9
11.	Enrichment factor	---	50

Calibration curve of CPE method

After establishing all the optimum conditions of the cloud point extraction method for the reaction of the reduced Nitrazepam and Clonazepam with 2,5-dimethoxyaniline, linear calibration curves were constructed as shown in Figure 22 and Figure 23. In this context, all the analytical values for estimation of these drugs by means of the CPE method are calculated and summarized in Table 3 and Table 4.

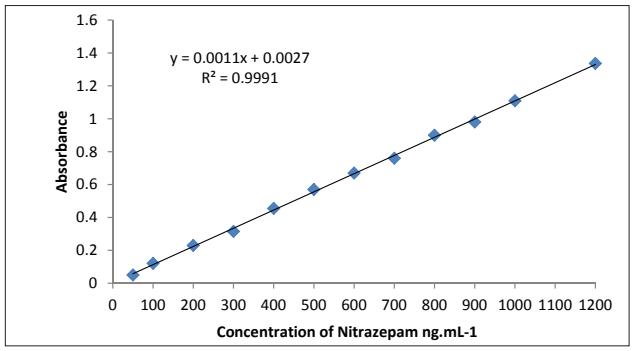


Figure 22. Calibration curve of Nitrazepam for CPE

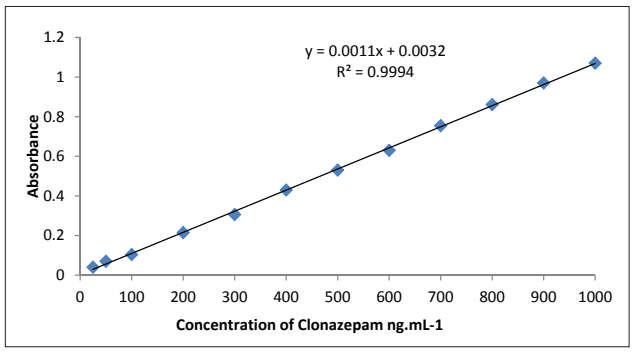


Figure 23. Calibration curve of Clonazepam for CPE

Accuracy and Precision

The calculation of [Relative Error (E%), Recovery (Rec%)] and [Relative standard deviation (RSD%)] affords accuracy and precision assessment for analytical methods respectively. The methods were checked by estimating Nitrazepam and Clonazepam at three standard different concentrations from the calibration curves of each variation of the batch method and from the CPE method, with five replications of every concentration under optimum conditions.

Table 5. Accuracy and precision of the proposed methods

	Concentration µgmL^{-1}				
	Taken	found	Error*%	Recovery*%	RSD*%
Clonazepam	Batch method				
	4	4.01	0.256	100.25	1.153
	6	5.91	-1.500	98.50	0.665
	8	8.04	0.500	100.5	0.162
	Cloud point Extraction method				
	0.3	0.299	0.326	99.67	0.458
	0.5	0.503	0.600	100.60	0.173
	0.7	0.697	-0.397	99.60	0.141
Nitrazepam	Batch method				
	4	3.94	-1.500	98.50	0.407
	6	5.93	-1.183	98.82	0.248
	8	7.90	-1.25	98.75	0.272
	Cloud point Extraction method				
	0.3	0.302	0.769	100.77	0.346
	0.6	0.599	-0.095	99.90	0.357
	1	0.998	-0.149	99.85	0.509

*Average of five values for each case

The results are shown in Table 5, and indicate that these methods have good accuracy and precision.

Stoichiometry of reaction and mechanism

The methods of Mole ratio and Continuous variation and Jobs method were used to detect the stoichiometry of colored products formation from the reaction of reagent 2,5-dimethoxyaniline with reduced Nitrazepam and Clonazepam drugs [63,64]. The obtained results are shown in (Fig. 24, 25). These results indicate that the ratio 2:1 is the ideal for both reduced Nitrazepam and Clonazepam to 2,5-dimethoxyaniline. To find the stability of colored products, the dissociation degree and stability constant were calculated by preparation of solutions containing stoichiometric amounts of reduced Nitrazepam and Clonazepam with 2,5-dimethoxyaniline, as well as other solutions containing the same amounts of reduced Nitrazepam (0.000355 M) and Clonazepam (0.000316 M) with an excess amount (triple) of 2,5-dimethoxyaniline. The average conditional stability constant of the colored products in water under optimum conditions was 1.44×109 L2 mol^{-2} for both reduced Nitrazepam and Clonazepam with 2,5-dimethoxyaniline. The color products have high stability because 2,5-dimethoxyaniline has an electron donating group active in ring creation, hence, high stability azo-coupling reactions between Benzodiazepine drugs and 2,5-dimethoxyaniline are easily generated [65]. The proposed mechanism of reaction is illustrated in Scheme II.

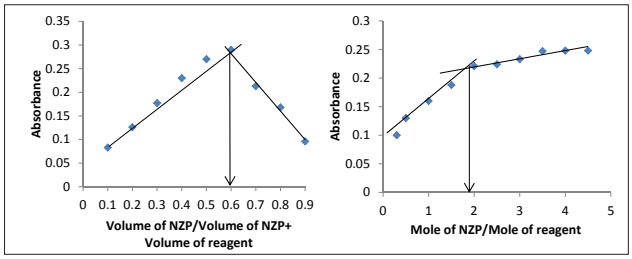


Figure 24. Mole Ratio and Continuous variation (0.000355 M) of Nitrazepam and 2,5-dimethoxyaniline

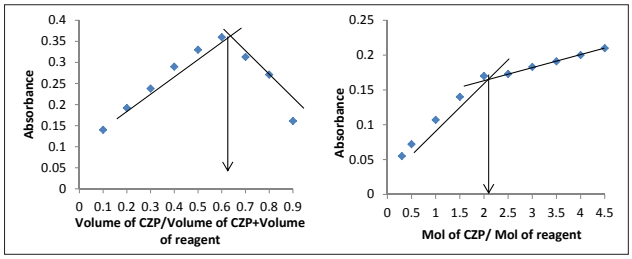
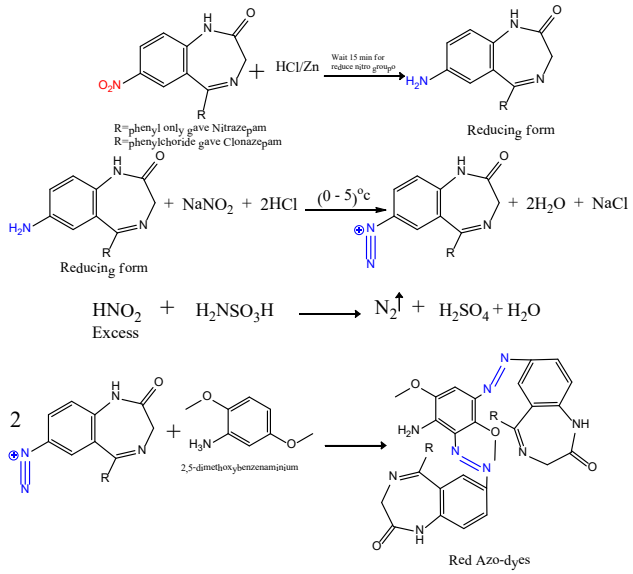


Figure 25. Mole ratio and continuous variation (0.000316 M) of Clonazepam and 2,5-dimethoxyaniline

INTERFERENCES

For evaluation of the selectivity of the proposed method to be applied on pharmaceutical preparations of the selected drugs, the influence of additives in the pharmaceutical preparations (Rivotril 2 mg) Clonazepam and (Zipex 5 mg) Nitrazepam were studied by adding separately excess amounts (10:1) of additives to (4 µg mL^{-1}) reduced Clonazepam solutions under optimum reaction conditions as



Scheme II. Scheme of the proposed reaction mechanism of coupling reduced Nitrazepam and Clonazepam with 2,5-dimethoxyaniline

indicated by the calibration curves. The results shown in (Tab. 6) reveal no influence of excipients on the proposed methods.

Table 6. Determination of (4 µg mL^{-1}) Clonazepam in the presence of excipients

Excipient	Clonazepam (4 µg mL^{-1})		
	Conce found µg mL^{-1}	Erorr%	Recovery*%
Pvp	3.95	-1.249	98.75
Lactose	3.99	-0.249	99.75
Starch	3.96	-1.000	99.00
Glucose	3.96	-1.000	99.00
Mg stearate	4.00	0.000	100.00

*Average of five values for each case

Pharmaceutical applications

The proposed methods of batch and cloud point extraction were applied successfully on pharmaceutical preparation solutions of reduced Nitrazepam and Clonazepam. The obtained results are shown in Table 7, and these indicate the applied recommended procedures to determine three concentration forms of Nitrazepam and Clonazepam in Zipex 5 mg and Rivotril 2 mg tablets, respectively. The proposed methods have good accuracy and precision for determining content levels of pharmaceutical tablets containing Nitrazepam and Clonazepam.

EVALUATING THE RESULTS OF THE PROPOSED METHODS

The standard methods for estimating Nitrazepam and Clonazepam in the British pharmacopoeia [66] were applied to pure drug and pharmaceutical preparations of the studied drugs, and the results obtained were compared with the proposed methods (Batch and cloud point extraction). The obtained results of F and t test values are summarized in

Table 8 and demonstrate no significant differences between the two methods.

Table 7. Application of proposed methods on pharmaceutical preparations of Nitrazepam and Clonazepam

	Concentration μg mL^{-1}				
	Taken	found	Error*%	Recovery*%	RSD*%
Rivotril 2 mg Clonazepam	Batch method				
	4	3.98	-0.500	99.500	0.763
	6	5.97	-0.456	99.540	0.787
	8	8.02	0.250	100.250	0.081
	Cloud point Extraction method				
	0.3	0.295	-1.660	98.33	0.109
	0.5	0.493	-1.400	98.60	0.036
	0.7	0.696	-0.571	99.42	0.857
Zipex 5 mg Nitrazepam	Batch method				
	4	3.92	-1.75	98.25	1.122
	6	5.97	-0.50	99.50	0.442
	8	7.95	-0.625	99.375	1.122
	Cloud point Extraction method				
	0.3	0.295	-1.660	98.33	0.700
	0.6	0.604	0.666	100.66	0.400
	1	0.997	-0.300	99.700	0.509

*Average of five values for each case

Table 8. Application of F, and t test for comparison between proposed and standard methods

	Pharmaceutical preparation	Proposed method		Standard method	
		Rec.*% (x_{i1})	$(x_{i1}-\bar{x}_{-1})^2$	Rec.*% (x_{-i2})	$(x_{i2}-\bar{x}_{-2})^2$
Batch method	Pure Nitrazepam	98.856	0.0088	99.96	0.0009
	Zipex 5	99.04	0.0081	99.90	0.0009
		$\bar{x}_{i1}$=98.95	Σ =0.0169	$\bar{x}_{i2}$=99.93	Σ=0.0018
	F-Value (experimental) = 9.380, Critical F-Value (19.000) t-Value (experimental) = -10.144, Critical t-Value (4.303)				
CPE method	Pure Nitrazepam	100.170	0.003	99.96	0.0009
	Zipex 5	100.060	0.003	99.90	0.0009
		$\bar{x}_{i1}$=100.115	Σ=0.006	$\bar{x}_{i1}$=99.93	Σ=0.0018
	F-Value (experimental) = 3.347, Critical F-Value (19.000)) t-Value (experimental) = 2.964, Critical t-Value (4.303))				
Batch method	Pure Clonazepam	99.75	0.000025	99.85	1.1342
	Rivotril	99.76	0.000025	101.98	1.1342
		$\bar{x}_{i1}$=99.755	Σ=0.00005	$\bar{x}_{i2}$=100.915	Σ=2.2684
	F-Value (experimental) = 0.000022, Critical F-Value (19.000) t-Value (experimental) = 1.06498, Critical t-Value (4.303)				
CPE method	Pure Clonazepam	99.956	0.343	99.85	1.1342
	Rivotril	98.783	0.343	101.98	1.1342
		$\bar{x}_{i1}$=99.369	Σ=0.686	$\bar{x}_{i1}$=100.915	Σ=2.2684
	F-Value (experimental) = 0.3025, Critical F-Value (19.000)) t-Value (experimental) = 1.2154, Critical t-Value (4.303))				

CONCLUSIONS

Simple, rapid, sensitive and selective kinetic Spectrophotometric methods were developed. These are not affected by excipients, so can be successfully applied for determination of trace and Ultra trace amounts of reduced Nitrazepama and Clonazepam in pure and pharmaceutical formulations based on coupling these drugs with 2,5-dimethoxyanline reagent and utilizing the diazonium coupling reaction and cloud point extraction method.

REFERENCES

1. Moffat A, Osselton M, Widdop B. *Clarkes Analysis of Drugs and poisons*. London: Pharmaceutical Press;2011.
2. Fleeger C. *USAN and the USP Dictionary of Drug Names*. USA: United States Pharmacopeial Convention;1992.
3. Parfitt K, Martindale W. *Martindale the Complete Drug Reference*. London: Pharmaceutical Press;1999.
4. Cowne P, Green A, Nutt D, Martin I. Ethyl beta-carboline carboxylate lowers seizure threshold and antagonizes flurazepam-induced sedation in rats. *Nature*.1981;290:54-5.
5. Browne T. Drug therapy reviews: clinical pharmacology of antiepileptic drugs. J Hosp Pharm. 1978;35:1048-56.
6. Wilson A. Schild H, Modell W. *Applied Pharmacologh*. London: Churchill Livingstone;1975.
7. Katzung B. *Basic and Clinical Pharmacology*. USA: The McGraw-Hill Companies; 2007:475.
8. Sane R, Ghorpade U, Nadkarni A, Dolas S. Gas chromatographic determination of nitrazepam and diazepam from pharmaceutical preparations. *Indian Drugs*. 1987;24:260-3.
9. Gunnar T, Ariniemi K, Lillsunde P. Determination of 14 benzodiazepines and hydroxy metabolites, zaleplon and zolpidem as tert-butyldimethylsilyl derivatives compared with other common silylating reagents in whole blood by gas chromatography-mass spectrometry. *J Chromatogr B*. 2005;818:175-89.
10. Salem AB, Barsoum BN, Izake EL. Spectrophotometric and fluorimetric determination of diazepam, bromazepam and clonazepam in pharmaceutical and urine samples. *Spectrochim Acta*. 2004;60:771-80.
11. Kakde R, Satone D, Gadapayale K, Kakde M. Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Escitalopram Oxalate and Clonazepam. *J Chromatogr Sci*. 2013;51:490-500.
12. Wilhelm M, Battista H, Obendorf D. Selective and sensitive assay for the determination of benzodiazepines by high-performance liquid chromatography with simultaneous ultraviolet and reductive electrochemical detection at the hanging mercury drop electrode. *J Chromatogr A*. 2000;897:215-25.
13. Cavedal L, Mendes F, Domingues C, Patni A, Monif T, Reyar S, et al. Clonazepam quantification in human plasma by high-performance liquid chromatography coupled with electrospray tandem mass spectrometry in a bioequivalence study. *J Mass Spectrom*. 2007;42:81-8.
14. Gandhi S, Dhavale N, Jadhav V, Sabnis S. Spectrophotometric and reversed-phase high-performance liquid chromatographic methods for simultaneous determination of escitalopram oxalate and clonazepam in combined tablet dosage form. *J AOAC Int*. 2008;91:33-8.
15. Pujadas M, Pichini S, Civit E, Santamarina E, Perez K, Torre R. A simple and reliable procedure for the determination of psychoactive drugs in oral fluid by gas chromatography-mass spectrometry. *J Pharm Biomed Anal*. 2007;44:594-601.
16. Mallikarjuna R, Agarwal N, Bichala P, Som S. Method development and validation for the simultaneous estimation of desvenlafaxine and clonazepam in bulk & tablet formulation by RP-HPLC methodIndian. *J Res Pharm Biotechnol*. 2013;1:525-32.
17. Bhagyasree T, Neelam I, Ajitha A. Assay method development and validation for simultaneous estimation of paroxetine and clonazepam by RP-HPLC. *J Pharm Res Anal*. 2014;4:421-7.
18. Rani N, Sahithi G, Divya K. New RP-HPLC method for simultaneous estimation of desvenlafazine and clonazepam tablets. *J Pharm Sci Drug Res*. 2015;7:182-7.
19. Ho P, Triggs E, Heazlewood V, Bourne D. Determination of nitrazepam and temazepam in plasma by high-performance liquid chromatography. *Ther Drug Monit*. 1983;5:303-8.

20. Kozu T. High-performance liquid chromatographic determination of nitrazepam and its metabolites in human urine. *J Chromatogr.* 1984;35:213-8.
21. Suzuki K, Johno I, Kitazawa S. High-performance liquid chromatographic determination of nitrazepam in plasma and its application to pharmacokinetic studies in the rat. *J Chromatogr.* 1988;69:435-40.
22. Pistos C, Stewart J. Direct injection HPLC method for the determination of selected benzodiazepines in plasma using a Hisep column. *J Pharm Biomed Anal.* 2003;33:1135.
23. Peiró M, Bose D, Domínguez A, Agustí M. Direct injection micellar liquid chromatographic determination of benzodiazepines in serum. *J Chromatogr B.* 2002;780:241-9.
24. Dai H, Lin Y, Wu X, Chen G Sens. A new electrochemiluminescent sensing interface for clonazepam based on titanate nanotubes self-assembled film. *Sensors Actuators B Chem.* 2010;145:320-6.
25. Chaichi M, Alijanpour S. A new chemiluminescence method for determination of clonazepam and diazepam based on 1-Ethyl-3-Methylimidazolium Ethylsulfate/copper as catalyst. *Spectrochim Acta A Mol Biomol Spectrosc.* 2013;24:36-41.
26. Jing D, Shi Y, Wang J. Qualitative and quantitative analysis of clonazepam and its metabolite 7-aminoclonazepam in blood by LC-tandem QTOF/MS and LC-MS/MS. *Forensic Sci.* 2014;4:45-52.
27. Jain R, Mishra R, Dwivedi A. Voltammetric behaviour of nitrazepam in solubilized system. *JSIR*. 2009;68:540-7.
28. Hanekamp H, Voogt W, Bos P, Frei R. A pulse polarographic detector for HPLC; Determination of Nitrazepam. *J Liquid Chromatogr.* 1980;3:1205-17.
29. Mishra A, Gode K. Polarographic assay of nitrazepam formulations. *Analyst.* 1985;110:1105-9.
30. Tomita M, Okuyama T. Application of capillary electrophoresis to the simultaneous screening and quantitation of benzodiazepines. *J Chromatogr B.* 1996;678:331-7.
31. Wozniakiewicz A, Wietecha-Posluszny R, Wozniakiewicz M, Bryczek E, Kosscielniak P. A quick method for determination of psychoactive agents in serum and hair by using capillary electrophoresis and mass spectrometry. *J Pharm Biomed Anal.* 2015;111:177-85.
32. Honeychurch K, Brooks J, Hart J. Development of a voltammetric assay, using screen-printed electrodes, for clonazepam and its application to beverage and serum samples. *Talanta*. 2016;147:510-5.
33. Habibi B, Jahanbakhshi M. Silver nanoparticles/multi walled carbon nanotubes nanocomposite modified electrode: Voltammetric determination of clonazepam. *Electrochim Acta.* 2014;118:10-7.
34. Halvorsen S, Jacobsen E. Electroreduction and polarographic determination of nitrazepam in serum. *Anal Chem Acta.* 1972;59:127.
35. Eldin A, Salem A, Barsoum B, Saad G, Izake E. Potentiometric determination of some 1,4-benzodiazepines in pharmaceutical preparations and biological samples. *J Electroanal Chem*. 2002;536:1-9.
36. Ruiz E, Blanco M, Abad E, Hernández L. Determination of nitrazepam and flunitrazepam by flow injection analysis using a voltammetric detector. *Analyst.* 1987;112:697-9.
37. Dolejs'ova J, Solich P, Polydorou C, Koupparis M, Efstathiou C. *J Pharm Biomed Anal.* 1999;20:357-62.
38. Al-Abachi M, Hammoudi M. Batch and flow injection spectrophotometric methods for the determination of clonazepam in pharmaceutical preparation via oxidative coupling with pyrocatecol. *Iraqi J Sci.* 2015;56:898-908.
39. Al-Abachi M, Hammoudi M. The use of spectrophotometric batch and flow injection estimation of clonazepam drug in pure and pharmaceutical preparations. *Iraqi J Sci.* 2015;56:2115-25.
40. Abdulsattar RS. Spectrophotometric determination of nitrazepam in pharmaceutical tablets using flow injection analysis. *JUAPS.* 2010;4:40-5.
41. Al-Abachi M, Hadi H. Flow injection-spectrophotometric determination of clonazepam based on its oxidative condensation with promethazine hydrochlorid. *Al-Mustansiriyah J Sci*. 2015;26:38-42.
42. El-Shabouri S. Spectrophotometric determination of nitrazepam in tablets. *Talanta*. 1986;33:743-4.
43. Walash M, Rizk M, El-Brashy A. Spectrophotometric determination of chlordiazepoxide and nitrazepam. *Talanta*. 1988;35:895-8.
44. Hassan S, Belal F, El-Din M, Sultan M. Spectrophotometric determination of some pharmaceutically important nitro compounds in their dosage forms. *Analyst.* 1988;113:1087-9.
45. Davidson A, Lia H. Spectrophotometric determination of nitrazepam in drug dosage forms. *J Pharm Pharmacol*. 2005;41:63-5.
46. Al-Ghabsha T, Azooz A, Namir A. Spectrophotometric method for determination of chloramphenicol in pharmacuetical preparations using o-chloranical reagent. *J Edu Sci.* 2008;21:147-63.
47. El-Brassy A, Aly F, Belal F. Determination of 1,4-benzodiazepines in drug dosage forms by difference spectrophotometry. *Mikrochim Acta*. 1993;110:55
48. Randez-Gil F, Daros J. Direct derivative spectrophotometric determination of nitrazepam and clonazepam in biological fluids. *J Pharm Biomed Anal.* 1991;9:539-45.
49. Thampi P, Premnath K. Simple spectrophotometric method for the determination of nitrazepam in pharmaceuticals. *Indian Drugs*. 1986;23:239-41.
50. Popovici I, Dorneanu V, Stan M, Cuciureanu R. Specrophotometric determination of nitrazepam. *Rev Chim* (Bucharest). 1984;35:266-7.
51. Revanasiddappa H, Deepakumari H, Mallegowda S, Vinay KB. Facile spectrophotometric determination of nimodipine and nitrezepam in pharmaceutical preparations. *Nalele UniversităŃii din Bucureşti.* 2011;20:189-96.
52. Sinan R, Al-Abachi M. Spectrophotometric Determination of Nitrazepam in Pharmaceutical Tablets by Oxidative Coupling Reaction with Pyrocatechol. *JUAPS*. 2009;3:6-12.
53. Abdullah H. Cloud-point extraction and spectrophotometric determination of clonazepam in pharmaceutical dosage forms. *Bull Chem Soc Ethiop.* 2017;31:373-82.
54. Al-Shaker Y. FIA-spectrophotometric determination of nitrazepam by oxidation with a solid-phase reactor and coupling with 2,2'-dihydroxybiphenyl reagent. *I Raf J Sci.* 2011;22:39-50.
55. Upadhyay K. Determination of Nitrazepam in its pure form, formulations and in biological samples. *Recent Res Sci Technol*. 2012;4:89-91.
56. Mahdi MI, Kadhim KH. Spectrophotometric Determination for Benzodiazepine Drugs (Clonazepam and Nitrazepam) in Pure and Pharmaceuticals Preparation. *Asian J Chem*. 2018;30:2686-92.
57. Ali S, Kadhim K. Spectrophotometric determination of Nitrazepam, using Thymol as a new chromogenic reagent. *Int J Chem Tech Res.* 2017;10:749-759.
58. Kadhim K, Al-Shirifi A, Abbas A. Spectrofluorimetry – Cloud point extraction for the determination of cadmium and lead using Furosemide as a complexing agent. *Kerbala J Pharm Sci.* 2015;9:39
59. Khammas ZA, Ghali AA, Kadhim KH. cadmium in honey samples using a new ligand. *Int J Chem Sci.* 2012;10:1185-204.
60. Kadhim K, ALsharifi A, Abbas A. Separation and preconcentration for determination of ultra trace of chromium (III) and zinc (II) using spectrofluorimetry techniques. *Asian J Chem*. 2014;26:139-42.
61. Hadi H. Spectrophotometric determination of clonazepam in pure and dosage forms using charge transfer reaction. *Iraq J Pharma Sci.* 2015;24:25-32.
62. International Conference on Harmonization, ICH Harmonised Tripartite Guidline-Text on Validation of Analytical Procedures. *Federal registar.* 1995;60:11260.
63. Levie RD. *Principles of Quantitative Chemical Analysis*. New York, London: McGraw-Hill;1997;24:718-725.
64. Okolo P, Ukebor E. Stoichiometry of Quinol/Ammonium-Nitrogen Complex Using Spectrophotometryjour. Chem Soc Pak. 2004;26:207-11.
65. Zollinger H. *Diazo chemistry I, aromatic and heteroaromatic compounds.* Weinheim: New York; 1994;11:305.
66. *British Pharmacopoeia*. The Stationery Office. London; 2009.