Spectrophotometric Determination for Benzodiazepine Drugs (Clonazepam and Nitrazepam) in Pure and Pharmaceuticals Preparation

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Depending on the oxidative coupling reaction as a rapid and selective spectrophotometric method, reduced clonazepam and nitrazepam drugs were evaluated by using potassium bromate as oxidation agent for these drugs and phenylephrine hydrochloride as a new chromogenic reagent to give red coloured products for clonazepam and nitrazepam at a maximum absorption of 491 and 488 nm, respectively. The linear ranges was $2\text{--}60\text{ }\mu\text{g mL}^{-1}$ and $5\text{--}90\text{ }\mu\text{g mL}^{-1}$ while the molar absorptivity are $3.157 \times 10^3\text{ L mol}^{-1}\text{ cm}^{-1}$ and $3.825 \times 10^3\text{ L mol}^{-1}\text{ cm}^{-1}$ and their detection limits are $0.141\text{ }\mu\text{g mL}^{-1}$ and $0.104\text{ }\mu\text{g mL}^{-1}$, respectively for clonazepam and nitrazepam. The proposed methods did not require any temperature control and also not affected by foreign excipients and henceforth successfully applied to determine clonazepam and nitrazepam in pharmaceutical preparations.

Keywords: Spectrophotometric determination, Benzodiazepine drugs, Clonazepam, Nitrazepam, Oxidative coupling reaction.

INTRODUCTION

Clonazepam and nitrazepam are the member of benzodiazepine drug series where it play a prominent roles in epilepsy therapy [1-3]. Benzodiazepine drugs chemically much similar with a suitable structural but differ from each other in their activities. Drugs in general have many applications such as furosemide to determine the heavy metals, while others drugs clonazepam has high potency of nitro-benzodiazepine which normally requires in epilepsy therapy, anxiolytic, removal of insomnia, tranquilizer, relaxation of muscle spasms and use as sedation prior to medical procedures. While nitrazepam is a sturdy soporific drug, anticonvulsant, antidepressant, anti-panic, sedative and muscle relaxants also it can dampen the evolution and spread of epileptiform electrical activity in the central nervous system [4-8].

Several methods have been reported in the literature for the estimation of drugs of clonazepam and nitrazepam in either pharmaceutical forms or biological fluids, including HPLC [9-16], liquid and gas chromatography [17-24], clonazepam in blood using LC-MS/MS [25], chemiluminescence and electrochemiluminescence [26,27], electrical methods [28-36], flow-injection [37-42], colorimetric and various spectrophotometric

methods [43-59]. The present paper involves the development of a rapid and selective spectrophotometric methods for determination of clonazepam and nitrazepam drugs in pure pharmaceutical formulations based on the oxidative coupling reaction between these drugs with a phenylephrine-HCl as a new chromogenic reagent in the presence of potassium bromate as oxidizing agent.

EXPERIMENTAL

The scanning of all spectra and measurements of the absorbance at selected wavelengths achieved by T80 UV-visible Spectrometer PG Instrumental Ltd., U.K., with quartz cell matched 1 cm, Oven BS Size Two, Gallenkamp, England, for drying with a temperature range $0\text{--}300\text{ }^{\circ}\text{C}$, heating-cooling Water Bath-Haak Fe, Sartorius and Balance Bp3015-Germany were used.

Highest purity of clonazepam (m.f. $\text{C}_{15}\text{H}_{10}\text{N}_3\text{O}_3\text{Cl}$), nitrazepam (m.f. $\text{C}_{15}\text{H}_{11}\text{N}_3\text{O}_3$) and phenylephrine hydrochloride (m.f. $\text{C}_9\text{H}_{14}\text{NO}_2\text{Cl}$) were obtained from State Company for Drug Industries and Medical Appliance (SDI), Samarra, Iraq. Potassium bromate was obtained from the BDH company having purity 99.00 %. Pharmaceutical preparations that were used in this

study were rivotril (2 mg/clonazepam), zipex (5 mg/nitrazepam) from Roche farma S.A. and Aburaihan Phamaceutical Co., Tehran, Iran.

Preparation of reduced clonazepam and nitrazepam stock solutions ($200 \mu\text{g mL}^{-1}$): These solutions were prepared by taking 0.0100 g clonazepam and nitrazepam separately in 125 mL separated beakers and dissolved in 25 mL of ethanol. Distilled water (4 mL), hydrochloric acid (4 mL, $\sim 11.64 \text{ M}$) and zinc powder (0.60 g) were mixed and allowed to stand for a 0.25 h at room temperature, then filtered into 50 mL of separate volumetric flask, rinsed the remains and diluted to mark a volume with distilled water to get $200 \mu\text{g mL}^{-1}$ solutions of reduced clonazepam and nitrazepam [60].

Phenylephrin-HCl solution (0.05 M) was prepared by dissolving 0.509 g phenylephrine-HCl in 50 mL distilled water, while potassium bromate solution (0.1M) was prepared by dissolving 0.835 g potassium bromate in 50 mL distilled water.

Tablets sample (Rivotril 2 mg, Zipex 5mg) solutions ($100 \mu\text{g mL}^{-1}$) prepared after grinding by following the procedure of stock solution of reduced clonazepam and nitrazepam, where weighed the suitable amount of each formulation (powder) equivalent to 5 mg of clonazepam and nitrazepam.

General procedure of determination of clonazepam and nitrazepam: A series of 10 mL volumetric flask, 1 mL of phenylephrine-HCl (0.05 M) mixed with 0.5 mL KBrO_3 (0.1M) followed by the addition by increasing volume 0.1-3 mL of reduced solution of clonazepam ($200 \mu\text{g mL}^{-1}$). Increasing volume of 0.25-4.5 mL of reduced solution of nitrazepam ($200 \mu\text{g mL}^{-1}$) mixed with 2 mL of phenylephrine-HCl (0.05 M) and 1 mL of KBrO_3 (0.1 M). All the solutions were diluted to the mark using distilled water and after 5 min at 20°C , the absorbance for each solution was measured at 491 nm for clonazepam and 488 nm for nitrazepam against the blank solution.

RESULTS AND DISCUSSION

Absorption spectra: Potassium bromate is used as a oxidizing agent in order to make oxidation and coupling the mixture from reduced clonazepam, nitrazepam with phenylephrine-HCl to produce coloured products (red) which gave maximum absorption at 491 nm (for clonazepam) and 488 nm (for nitrazepam) against blank solution. The scan of the blank solution against distilled water doesn't give any absorption at λ_{max} for each a coloured product (Fig. 1).

Effect of concentration of coupling reagent: The effect various concentrations of phenylephrine-HCl on coloured products absorption of clonazepam and nitrazepam are shown in Fig. 2, where it is found that an increase in the concentration of phenylephrine-HCl lead to increase the absorbance of a coloured products and remained constant above (0.05 M), so selected this concentration as optimum concentration for both drugs.

Effect of volume of coupling reagent: The effect of vary volume (0.1-4.0 mL) of (0.05 M) phenylephrine-HCl solution on a coloured absorption products were studied. The optimum volume of clonazepam and nitrazepam drugs were found to be 1 and 2 mL, respectively, where using higher volume gave a constant response for nitrazepam and decreases in absorption for clonazepam (Fig. 3).

Fig. 1. Spectra of oxidation $30 \mu\text{g mL}^{-1}$ from clonazepam and nitrazepam with phenylephrine-HCl

Fig. 2. Effect concentration of coupling reagent on benzodiazepine drugs

Fig. 3. Effect volume of coupling reagent on benzodiazepine drugs

Effect of volume of oxidation agent: The effect of varying volume from 0.1 to 4 mL of KBrO_3 (0.1 M) on a coloured absorption products were studied. The optimum volume of this oxidant for clonazepam and nitrazepam drug was found to be 0.5 and 1 mL, respectively (Fig. 4).

Effect of addition order: Three additions were examined as shown in Table-1. The optimum addition order no. 1, 2 for nitrazepam and clonazepam solutions, because its gave maximum

Fig. 4. Effect volume of (0.10 M) KBrO₃ on benzodiazepine drugs

Fig. 6. Effect of reaction time on benzodiazepine drugs

TABLE-1 EFFECT OF ADDITION ORDER		
Addition order	Absorption	
	Clonazepam (CZP)	Nitrazepam (NZP)
D + R + O	0.312	0.396
R + O + D	0.321	0.384
D + O + R	—	—

D = drug, R = Reagent, O = Oxidizing agent

absorption. No. 3 did not show any absorption at ($\lambda_{\max} = 491$ and 488 nm), because the reaction occurs between drugs and KBrO₃.

Effect of temperature: The obtained coloured products are stable and give maximum absorption at low and room temperature (10-30 °C). However at high temperature, low absorption occurred because partial dissociation of a coloured products (Fig. 5).

Fig. 5. Effect of temperature on benzodiazepine drugs

Effect of reaction time: After the optimization of all the best conditions, time required for complete reaction and development absorption for a coloured products was studied. A coloured products formation for clonazepam and nitrazepam are obtained after 5 min, which remain stable at least for 1 h (Fig. 6).

Calibration curve: After the optimum conditions of coloured products for reaction clonazepam and nitrazepam with phenylephrine-HCl in the presence of KBrO₃, a linear calibration curve were constructed as shown in Figs. 7 and 8. All the analytical values for the methods used to estimate clonazepam and nitrazepam drugs are summarized in Table-2.

Fig. 7. Calibration curve of clonazepam (CZP)

Fig. 8. Calibration curve of nitrazepam (NZP)

TABLE-2 SUMMARY OF ANALYTICAL VALUE FOR CALIBRATION CURVES		
Parameter	Value	
	Clonazepam	Nitrazepam
Regression equation: $y = bx + a$	$y = 0.01x + 0.0072$	$y = 0.0136x - 0.0257$
Slope	0.01	0.0136
Intercept (a)	0.0072	0.0257
Correlation coefficient	0.998	0.9998
Linear range ($\mu\text{g mL}^{-1}$)	2-60	5-90
Molar absorptivity (ϵ) ($\text{L mol}^{-1} \text{cm}^{-1}$)	3.157×10^3	3.825×10^3
Sandall's sensitivity (S) ($\mu\text{g cm}^{-2}$)	0.1000	0.0995
Limit of detection ($\mu\text{g mL}^{-1}$)	0.141	0.104
Limit of quantitation ($\mu\text{g mL}^{-1}$)	0.472	0.347

Accuracy and precision: The calculation of relative error (E %), recovery (Recov %) and [relative standard deviation (RSD %)] gives accuracy and precision for analytical methods respectively. The methods were checked by the estimation of clonazepam and nitrazepam at three standard different concentrations from calibration curve with five replications of each concentration under optimum condition. The methods have a good accuracy and precision (Table-3).

Stoichiometry of reaction and mechanism: The methods of mole ratio and continuous variation Job method were applied to find the stoichiometry of coloured products formation for

reaction of phenylephrine-HCl (Phy) with clonazepam and nitrazepam drugs. Figs. 9 and 10 show that 1:1 drug to reagent was formed at 491 nm for clonazepam and 488 nm for nitrazepam. For known stability of coloured products, the dissociation degree and stability constant were calculated by prepared solutions contained the stoichiometries amount of reagent and drugs, and other solutions containing the same amount of drugs, and an excess amount (triple) of a reagent (0.0017 M). The average conditional stability constant of coloured products in water at optimum conditions was $9.54 \times 10^6 \text{ L}^2 \text{ mol}^{-2}$ for clonazepam-Phy and $5.67 \times 10^6 \text{ L}^2 \text{ mol}^{-2}$ for nitrazepam-Phy. The proposed

TABLE-3
ACCURACY AND PRECISION OF THE PROPOSED METHODS

Clonazepam					Nitrazepam				
Concentration ($\mu\text{g mL}^{-1}$)		Error* (%)	Recovery* (%)	RSD* (%)	Concentration ($\mu\text{g mL}^{-1}$)		Error* (%)	Recovery* (%)	RSD* (%)
Taken	Found				Taken	Found			
10	10.140	1.441	101.440	1.346	10	9.981	-0.190	99.810	0.776
20	20.070	0.300	100.300	0.582	20	19.983	-0.085	99.915	0.350
30	30.050	0.186	100.180	0.278	30	30.120	0.400	100.400	0.136

*Average of five determinations

Fig. 9. Mole ratio (0.0017 M) of on benzodiazepine drugs and reagent

Fig. 10. Continuous variation, Jobs method (0.0017 M) of drugs and reagent

mechanism of reaction between phenylephrine-HCl and the benzodiazepine drugs is illustrated in **Scheme-I**. The reduced drugs clonazepam and nitrazepam have strong electron donating ability coupling with phenylephrine-HCl after the oxidation by KBrO_3 to form oxidative coupled products [61].

Interference: To evaluate the selectivity of the proposed method to be applied on pharmaceutical preparations of selected drugs. The effect of additives in the pharmaceutical preparations containing clonazepam and nitrazepam were studied by adding separately excess amounts about (10:1) from excipients to (20 $\mu\text{g mL}^{-1}$) clonazepam and nitrazepam solutions under optimum reaction conditions followed in the calibration curves. No effect of excipients are found on the proposed methods (Table-4).

Pharmaceutical applications: The proposed methods were applied successfully on pharmaceutical preparation solutions for clonazepam and nitrazepam. The proposed methods have

good accuracy and precision for determination pharmaceutical tablets containing clonazepam and nitrazepam (Table-5).

The standard method for estimation of clonazepam and nitrazepam in British pharmacopoeia was applied on pure and pharmaceuticals preparation for studied drugs and compared with proposed methods for evaluating the results for estimating clonazepam and nitrazepam drugs. The results summarized in Table-6 showed no significant differences were found between the two methods.

Conclusion

A rapid and selectivity spectrophotometric methods has been developed, which do not require any temperature control or the solvent extraction step, and also no affected by excipients. The method is successfully applied for the determination of trace amounts of clonazepam and nitrazepam drugs in pure

Scheme-II: Proposed reaction mechanism of reduced nitrazepam, clonazepam with Phy-HCl in the presence of KBrO_3 as oxidant agent

TABLE-4
DETERMINATION OF (20 $\mu\text{g mL}^{-1}$) FROM CLONAZEPAM AND NITRAZEPAM IN THE PRESENCE OF EXCIPIENTS

Excipient	Clonazepam			Nitrazepam		
	Conc. found ($\mu\text{g mL}^{-1}$)	Error (%)	Recovery* (%)	Conc. found ($\mu\text{g mL}^{-1}$)	Error (%)	Recovery* (%)
Pvp	19.63	-1.85	98.15	20.20	1	99.00
Lactose	20.15	0.75	100.75	19.96	-0.2	99.90
Starch	20.05	0.25	100.25	19.85	-0.75	99.25
Mg stearate	19.87	-0.63	99.37	20.09	0.45	100.56

*Average of five determinations

TABLE-5
APPLICATION OF PROPOSED METHODS ON PHARMACEUTICAL PREPARATION FOR CLONAZEPAM AND NITRAZEPAM

Clonazepam (Rivotril)					Nitrazepam (Zipex)			
Concentration ($\mu\text{g mL}^{-1}$)		Error (%)	Recovery (%)	RSD (%)	Concentration ($\mu\text{g mL}^{-1}$)		Error (%)	Recovery (%)
Taken	Found				Taken	Found		
10	10.03	0.378	100.378	1.069	10	9.907	-0.925	99.074
20	20.04	0.205	100.205	0.582	20	20.10	0.544	100.544
30	30.08	0.295	100.295	0.199	30	29.86	-0.466	99.530

TABLE-6
APPLICATION OF F AND t TEST FOR COMPARISON BETWEEN PROPOSED AND STANDARD METHODS

	Pharmaceutical preparation	Proposed method		Standard method	
		Recov.* (%) (x_{i1})	$(x_{i1} - \bar{x}_1)^2$	Recov.* (%) (x_{i2})	$(x_{i2} - \bar{x}_2)^2$
Clonazepam	Pure clonazepam	100.64	0.0306	99.85	1.1342
	Rivotril	100.29	0.0306	101.98	1.1342
		$\bar{x}_{i1} = 100.465$	$\Sigma = 0.0612$	$\bar{x}_{i2} = 100.915$	$\Sigma = 2.2684$
	F-value (experimental) = 0.0269, Critical F-value (19.000); t-value (experimental) = -0.417, Critical t-value (4.303)				
Nitrazepam	Pure nitrazepam	100.04	0.0262	99.96	0.0009
	Zipex 5	99.72	0.0249	99.90	0.0009
		$\bar{x}_{i1} = 99.878$	$\Sigma = 0.0511$	$\bar{x}_{i2} = 99.93$	$\Sigma = 0.0018$
	F-value (experimental) = 5.677, Critical F-value (19.000); t-value (experimental) = -0.3198, Critical t-value (4.303)				

and pharmaceutical formulations based on the oxidative coupling reaction between benzodiazepine drugs and phenylephrine-HCl as a new chromogenic reagent in the presence of KBrO_3 as an oxidation agent.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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