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Spectrophotometric Estimation of Clonazepam as Pure Form and in its Pharmaceutical Formulation (Tablet) Using Alizarin Red S

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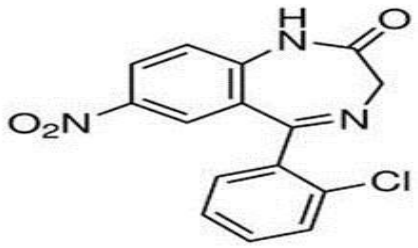
ABSTRACT

An easy and sensitive spectrophotometric method has been suggested for the assay of clonazepam (CLZM) as pure form and in its formulation as tablets. The present method based on the reaction of CLZM with alizarin red S reagent (ALRS) via proton transfer reaction to form a colored solution with a maximum absorption at wavelength of 528 nm and the optimal conditions for the reaction were studied. The linearity was within the concentration range from 1 to 30 µg/ml, with an R^2 value (determination coefficient) of 0.9882 and the sensitivity express via the molar absorption of $7.04x10^3$ l $mol^{-1}.cm^{-1}$, while Sandell's sensitivity index has been determined and equal to 0.0448 µg/ cm^2.The percentage of relative standard deviation (RSD%) which express the precision of the method, percentage of relative error (RE%) express the accuracy, the LOD and LOQ also have been estimated. The application of the suggested method to the determination of CLZM in tablet gave satisfactory results.

Keywords: clonazepam, alizarin red S reagent, spectroscopy, proton transfer.

INTRODUCTION

CLZM chemically has the scientific name 5-(2-chlorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one Fig. (1) with slightly yellowish, crystalline powder and practically insoluble in distilled-water but slightly soluble in ethanol and methanol (European Pharmacopoeia, 2019).



$C_{15}H_{10}ClN_3O_3$, M.wt. 315.7 g/mol

Fig. 1: The chemical formula and structure of CLZM.

CLZM is a nitrodiazepine, and it is a class of psychoactive drugs that have been poorly used at the present time. Clonazepam is a benzodiazepine derivative that is widely used as a treatment for sleep disorders, anxiety, muscle relaxant, sedative, and epileptic seizures. It has side effects such as drowsiness and fatigue when low doses lead to dizziness, mood swings, and memory loss at high concentrations (Gurusamy and Thangadurai, 2019; Degreef *et al*., 2021; Qriouet *et al*., 2019).

Several analytical methods have been reported for assay of CLZM in different samples including diazo coupling spectrophotometric methods (Hajir and Kassim, 2020; Ameen and Al-Badry, 2005), Cloud point extraction (CPE) combined with diazo-coupling (Abdullah, 2017; Mahdi and Kadhim, 2020), oxidative coupling (Saddam and Kadhim, 2019; Mahdi and Kadim, 2018), charge transfer (Hadi, 2015; Zankanah and Dikran, 2017; Salem *et al.*, 2002), Prussian blue complex (Fadhel and Khamees, 2018), UV-spectrophotometry (Kakde and Satone, 2009; Karajgi *et al.*, 2016), spectrofluorometric (Patel and Chhalotiya, 2017; Belal *et al.*, 2017; Salem *et al.*, 2004).

Various chromatographic methods were also used to estimate CLZM whether in pharmaceutical preparations or biological body fluids such as HPLC (Gadge *et al*., 2020; Spell and Stewart, 1998; Isse and Mahmoud, 2021; Naidu *et al.*, 2018), UPLC/MS-MS (Xu *et al.*, 2019), GC-MS (Santhosh *et al.,* 2019; Fang *et al*., 2020) as well as to other analytical methods such as voltammetry (Lotfi and Veisi, 2019; Sachdeva *et al*., 2021; Honeychurch *et al.,* 2016), electrophoresis (Seyfinejad *et al*., 2021), and normal reverse flow injection (Malik *et al*., 2018).

EXPERIMENTAL

Apparatus

A double-beam spectrophotometer Jasco V-630 UV-Visible (Japan), using glass cell (1 cm), and a TRANSE BP3001 professional pH meter were used in present investigation.

Chemicals used

Chemicals used are in a high degree of purity.

Solutions used

Clonazepam Standard Solution (100 µg/ml)

Dissolving 0.0100 g of clonazepam (supplied by the General Company for Pharmaceuticals and Medical Appliances Samarra - Iraq) in 70 ml of absolute ethanol, then supplementing the volume with distilled water to 100 ml in calibrated flask. The container was kept away from light and the stability of solution not more than three days.

Alizarin Red S Solution (0.1%)

0.1 g of the ALRS supplied by BDH company is dissolved in distilled water in 100-ml volumetric flask, and kept in an opaque and airtight bottle, and it is stable for at least a week.

Formulation (Tablet) Solution (100 µg / ml)

Crushing 10 tablets (after weighting each tablet, 0.1696 g), contains 2 mg of CLZM), weighed 0.8481g of crushed powder equivalent to 0.0100 g of clonazepam, then dissolved in 70 ml of absolute ethanol (warm), then after filtration the volume was completed to 100 ml using distilled water.

Procedure and Calibration Curve

Increasing volumes of 0.1 - 3.0 ml of CLZM (100 µg / ml) have been added to a set of 10-mL volumetric flask to cover a concentration of 1 to 30 µg/ml, followed by the addition of 1.25 ml of 0.1% ALRS reagent to each flask and a waiting period of 5 minutes and then dilution of flasks with distilled water to the mark level. The absorbance of the colored product formed measured against the blank solution at the wavelength 528 nm after 5 minute of dilution. The standard curve was obtained by drawing the relationship between the absorbance and concentration as in Fig. (2), which shows that there is a linear relationship between the concentration of CLZM and the absorbance for the colored products up to the concentration of 30 µg/ml, and a negative deviation from Beer's law is obtained above 30 µg/ml. The molar absorption was 7.04 x 10^3 l mol^{-1} cm^{-1}, the value of Sandell's sensitivity index was equal to 0.0449 µg/ cm^2, LOD and LOQ were also calculated, and their values were 0.177 and 0.590 µg / ml, respectively.

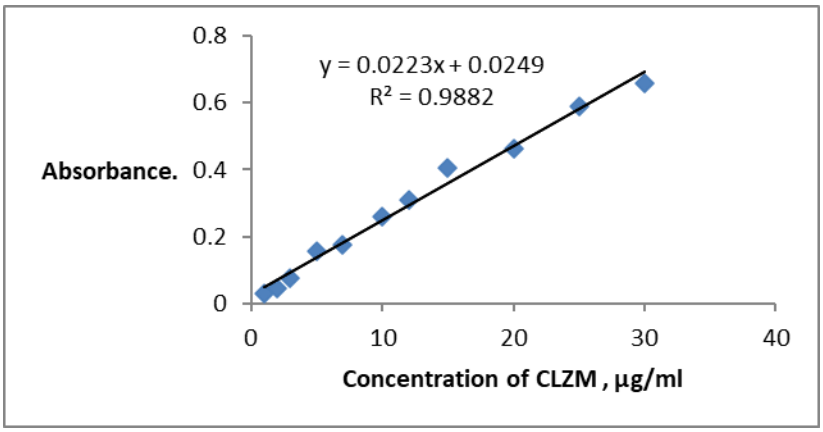


Fig. 2: The calibration curve for estimating CLZM via application of the proposed method.

RESULTS AND DISCUSSION

All parameters that affect the absorbance of the resulting-colored product have been studied using 1 ml of CLZM (100 µg/ml) in a final volume of 10-ml.

Study of the Amount of the ALRS Reagent

Different quantities of ALRS solution from 0.5 to 3.0 ml were added to 10 ml volumetric flask, each containing 1 ml of CLZM, then left for 10 minutes and then diluted with distilled water to the mark. The absorbance of the resulting solution is measured at 528 nm versus to the blank-solution. Although 2.0 ml and 3.0 ml of the reagent gives higher absorbance but due to the turbidity of the solution after 10 minutes of dilution and it was found that the highest determination coefficient (R^2) achieved at the volume of 1.25 ml of the used reagent, so this volume was chosen in the subsequent studies, (Table 1).

Table 1: The effect of the amount of reagent ALRS on the absorbance of the colored product.

ml of ALRS (0.1 %)	Absorbance /µg CLZM ml^{-1}				
	1	3	7	10	R^2
0.5	0.012	0.022	0.074	0.147	0.9513
1	0.027	0.070	0.179	0.255	0.9935
1.25	0.033	0.089	0.200	0.277	0.9997
2.0*	0.055	0.091	0.206	0.289	0.9955
3.0*	0.064	0.102	0.214	0.302	0.9957

* Turbid after 10 minutes from dilution.

The Effect of pH

Increasing volumes of 0.1-0.4 ml of each of dilute HCl and NaOH in equal concentration (0.001M) were added to a number of 10-ml volumetric flasks each one contains 1 ml of CLZM and 1.25 ml of 0.1% ALRS reagent, wait for 10-minutes, and then dilution with distilled water to the mark. The absorbance was measured and drew against the pH of solution. The results cited in Fig. (3) indicate that the highest resulting absorbance is for a solution that does not contain either an acid or a base (pH = 4.78), so that it isn't recommended to add acid or base.

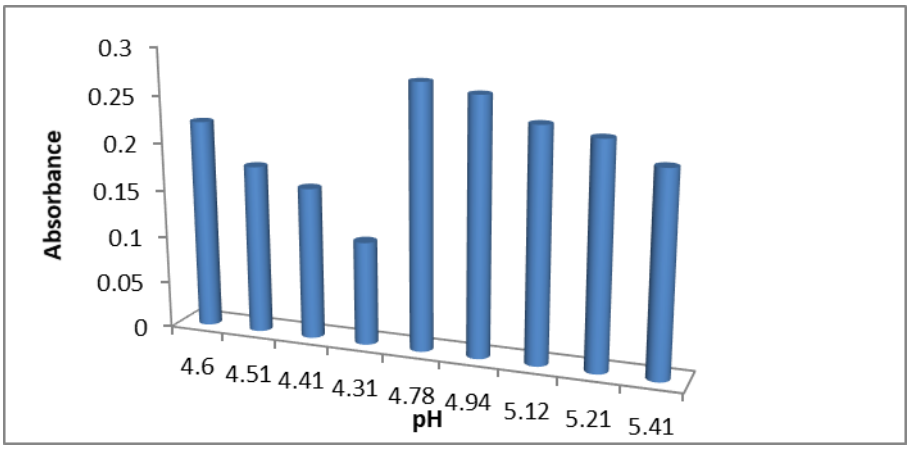


Fig. 3: Effect of pH on the absorbance of the complex formed.

Choosing a Buffer Solution

The pH of drug solution is 5.98 and after adding 1.25 ml of alizarin red reagent S, the pH reduced to 4.78, so that buffer solutions with pH 4.78 and 5.0 were prepared. From the results obtained in Table 2, it is clear that buffer solutions have a negative effect on the absorbance of the colored product solution, and therefore they were not recommended to use in subsequent experiments.

Table 2: Effect of the acidity function.

pH	Buffer solutions	Absorbance
4.78	Acetic acid, phosphoric acid ,boric acid ,sodium hydroxide	0.022
5.00	Potassium hydrogen phthalate, sodium hydroxide	0.034

Absorbance without buffer solution = 0.272.

Effect of Solvent

1 ml of CLZM, and 1.25 ml of ALRS reagent were taken. A number of organic solvents, as well as water used in dilution to the mark, with a final volume of 10 ml, and the results are cited in Fig. (4) and (Table 3).

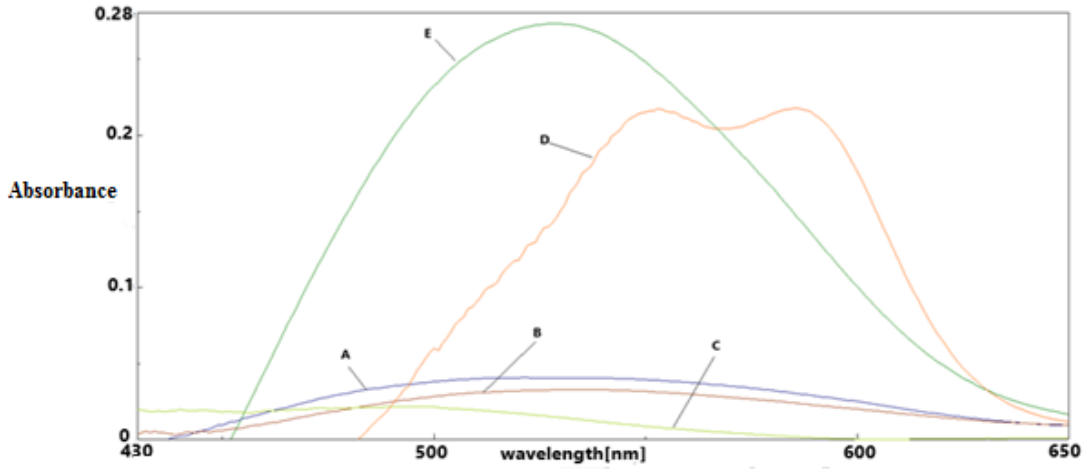


Fig. 4: Effect of solvents on the absorption spectrum of the formed product.

Table 3: Various analytical parameters of the colored product using various solvents.

Curve symbol	Solvent	Abs.	λmax (nm)	ε $l.mol^{-1}.cm^{-1}$
A	Methanol	0.040	521	1.2 x10 ^{3}
B	Ethanol	0.033	533	1.0 x10 ^{3}
---	1-Propanol	-----	No peak	-----
C	n-Butanol	0.0216	496	0.6 x10 ^{3}
D	Acetone	0.217	585	6.8 x10 ^{3}
E	Water	0.279	528	8.8 x10 ^{3}

From the results of the absorption spectrum of the solvents used in the final dilution, and from the observation of the change in both the absorbance and the greatest measurement wavelength, although the red shift caused by dilution with acetone, but the highest absorbance was observed using distilled water, and it was kept as a solvent used in subsequent experiments.

Study the time required to complete the reaction before dilution

1.25 ml of ALRS reagent was added to a number of volumetric flasks containing the same volume (1 ml) of CLZM, then the flasks left for different periods of time (Table 4) before diluting the sample solution with distilled water in a final volume of 10 ml and reading absorbance of solutions versus their blank solutions.

Table 4: Study of the standing time before dilution.

Standing time (min.)	0	5	10	15	20
Absorbance	0.212	0.275	0.271	0.269	0.261

From the results in (Table 4), it is clear that the best absorbance after dilution is to wait for a period of 5 minutes and then dilute the resulting-colored solution with distilled water.

The stability of the formed compound

The stability of the colored product was studied via measuring the absorbance at different time intervals, by taking 10 and 20 µg/ml of CLZM and dealing as mentioned in procedure and calibration method (see the results in Table 5).

Table 5: Compound stability study.

Standing time (min.)	Absorbance/µg of CLZM. ml^{-1}	
	10	20
Immediately	0.209	0.486
5	0.276	0.502
10	0.276	0.501
15	0.274	0.503
20	0.276	0.507
25	0.274	0.505
30	0.274	0.506
35	0.274	0.507
40	0.275	0.506
45	0.270	0.499
50	0.270	0.495
55	0.268	0.492
60	0.266	0.492

The results in (Table 5) show that reaction completed after approximately 5 minutes and the colored product stay stable for at least 55 minutes after dilution. Therefore, 5 minutes was recommended in the subsequent experiments.

Accuracy and precision of the method

The recovery percentage (Recovery %) and the relative standard deviation (RSD%) were calculated via applying the optimal reaction conditions to estimate three different concentrations of the drug compound CLZM. The results shown in (Table 6) indicate that the method has good accuracy according to the relative error values (RE %) which is from -2.9 to 0.7 as well as a good value of the relative standard deviation values (RSD%), which was not more than 2.6%.

Table 6: Accuracy and precision of the present method.

Amount added µg/ml	Recovery* %	RE %	RSD* %
5	98.0	-2.0	2.60
10	100.7	0.7	0.98
15	97.1	-2.9	1.20

* Average for five determinations.

Study of the Nature of the Reaction Product of ALRS with Clonazepam CLZM

The continuous variation method (Job's methods) (Kenner,1979) was applied in order to know the molar interaction ratio between CLZM and ALRS, as equal concentrations of both were prepared. At a concentration of $1.583x10^{-2}$ M and for different volumes of both solutions (CLZM and ALRS) but, the total volume of them was equal to 1 ml and diluted to final volume of 10 ml Fig. (5a). While Fig. (5-b) shows the molar ratio method plot (Kenner, 1979) by adding increasing volumes of the organic reagent ALRS 0.05-0.3 ml and at a concentration of $1.583x10^{-2}$ M to 1 ml of CLZM solution, with the same concentration of the used reagent, with a total volume of 10 ml.

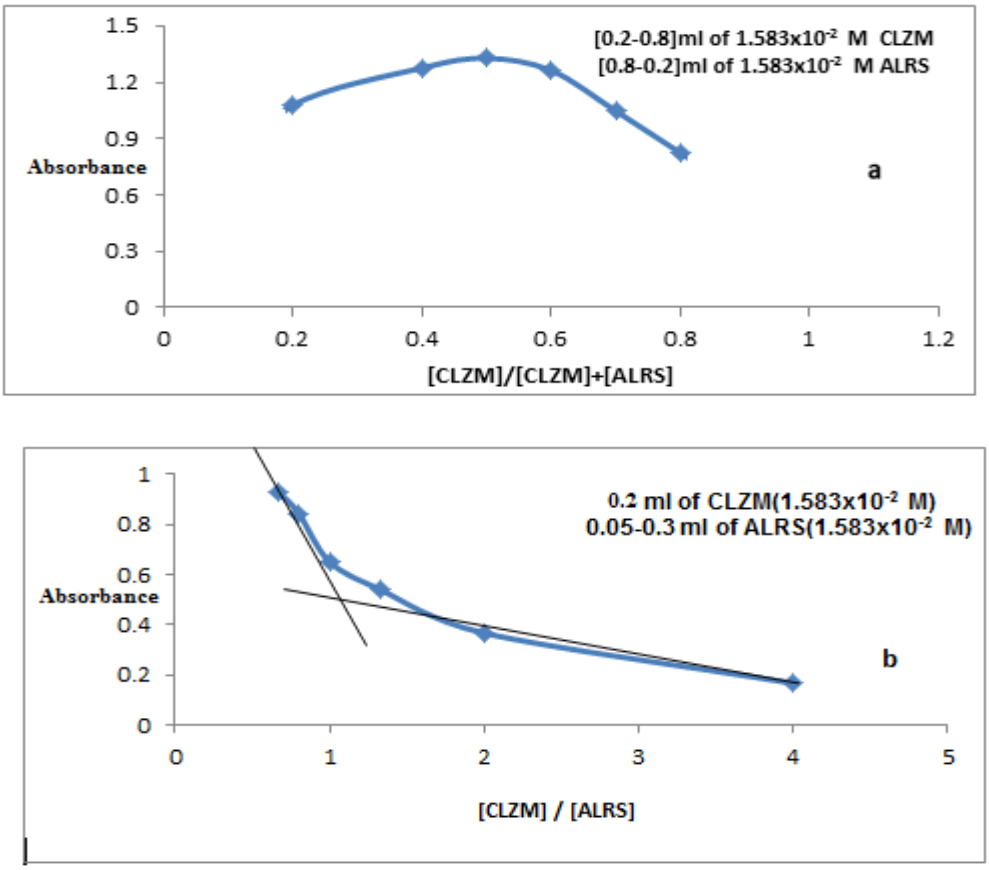


Fig. 5: Job's method curve (a) and molar ratio curve (b) for the product of the reaction of CLZM with ALRS.

It can be seen from Figures.5a and 5b that the molar reaction ratio is 1:1 (CLZM: ALRS). The mechanism of interaction between CLZM and ALRS can be undergo proton transfer reaction (Al-Neaimy and Al-Delymi, 2013). Fig. (6) shows the proposed mechanism for the resulted colored product.

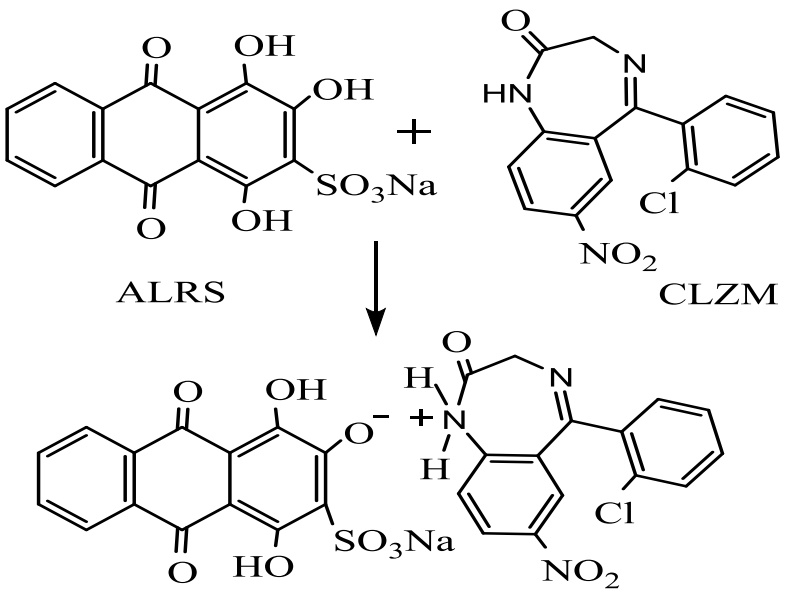


Fig. 6: The suggested mechanism for resulting colored product [CLZM-ALRS].

Final Absorption Spectrum

1.25 ml of 0.1% ALRS reagent was added to a volumetric flask of 10 ml containing 2.5 ml of clonazepam, waiting for a period of 5 minutes, followed by dilution with distilled water and also waiting for 5 minutes, then a spectrum scan was taken from 200 to 800 nm and Fig. (7) shows that the highest absorbance of the colored product is at 528 nm.

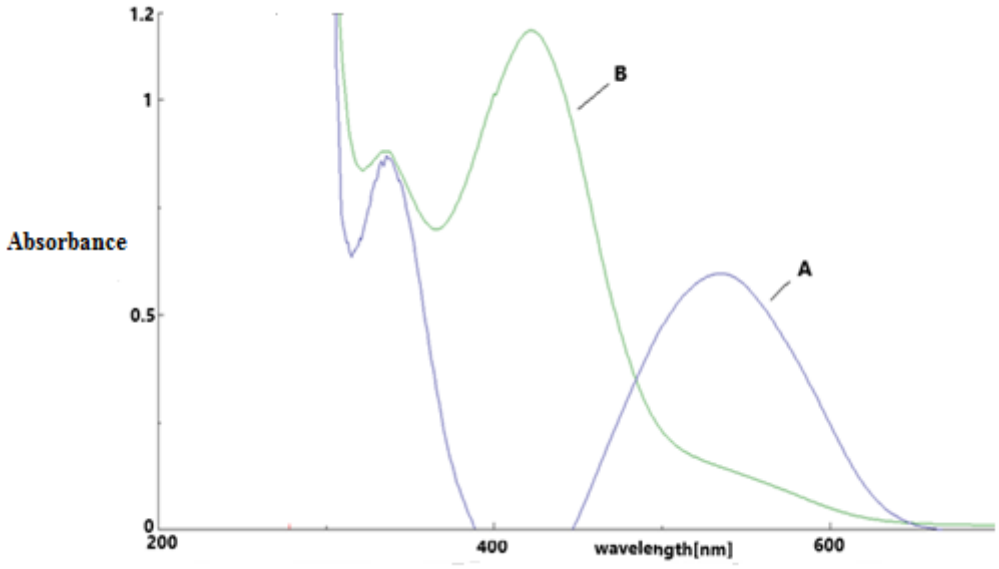


Fig. 7: Absorption spectra of: A: 25 µg/mL CLZM proceeded according to the proposed method vs. blank solution, B: Blank solution vs. distilled water.

Applications of the Method

The applicability of present method to estimate CLZM in the form of tablets formulations was tested using three different concentrations (7, 15 and 20 µg/ml) after completing the additions on optimal amounts mentioned before using 10 ml volumetric flask (Table 7).

Table 7: Results of estimation of CLZM in pharmaceutical preparation.

Drug	CLZM amount µg/ml	Recovery*%	RE%	RSD*%	Drug content measured(mg)
Rivotril 2mg/tablet Roche/Spain	7	96.0	-4.0	1.28	1.920
	14	97.9	-2.1	1.31	1.958
	20	103.1	3.1	3.40	2.062

* Average of four determinations.

The results in Table (7) indicate that the mean recovery was 99% and the relative standard deviation value not more than 3. 4 % while the main drug content was 1.98 mg, which indicates the success of the present method in assaying CLZM.

Standard addition method

For the purpose of proving that the proposed method is free from the interference of additives used in the preparation of dosage forms, the standard addition method was applied, and according to the results obtained in Fig. (8) and (Table 8), the method is agreed well with the direct method of determination and is free from the interactions of excipients.

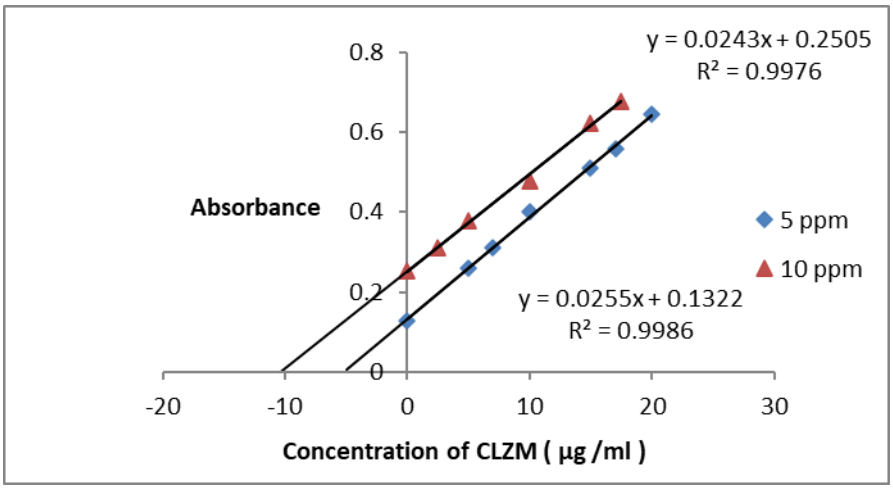


Fig. 8: The standard addition curve of CLZM estimation.

Table 8: Various parameter resulting from applying standard addition method.

Drug	Amount taken (µg/ml)	Amount found (µg/ml)	Recovery* %	RE %	RSD %	Drug content measured (mg)
Rivotril 2mg/tablet Roche/Spain	5	5.18	103.6	3.6	2.10	2.072
	10	10.30	103.0	3.0	3.40	2.060

* Average of four determinations.

Comparison of the Methods

By comparing the current proposed method for the determination of CLZM using ALRS reagent with other spectrophotometric methods via taking some analytical variables for different interactions between the drug compound CLZM and different reagents as shown in Table (9).

Table 9: Comparison of analytical variables for the proposed method with another method for estimating CLZM.

Parameters	Present method	Literature method (Hadi,2015)
Temperature(◦C)	Room temperature	Room temperature
λ_{max} (nm)	528	532
Reagent	Alizarin Red S	Metol
Development time(min.)	10	Without standing
Type of reaction	Proton transfer	Charge transfer
Beer's law range(µg/ml)	1-30	5-40
RSD %	≤2.6	≤4.32
Molar absorptivity ($l.mol^{-1}.cm^{-1}$)	7.04×10^3	3.473×10^3
Sandell's sensitivity µg/ cm^2	0.0449	0.0909
Color of the product	Red	Purple

It was found that the proposed method is more sensitive and has a low value of RSD% compared with the method in our comparison.

CONCLUSION

A spectrophotometric method was proposed for the assay of Clonazepam as pure form and in its formulation (Rivotril Tablet) and through its interaction with the alizarin red S reagent to give a red colored product with maximum absorbance at 528 nm. The method was successfully applied to the determination of clonazepam in pharmaceutical preparation in tablet form of Rivotril (2 mg/ tablet). The reaction takes place in an aqueous medium and the reaction does not need heating or extraction of product to an organic solvent.

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التقدير الطيفي للكلونازيبام بشكله النقي وفي مستحضره الصيدلاني (الأقراص) باستخدام الاليزارين الاحمر S

الملخص

تم إقتراح طريقة طيفية بسيطة وحساسة لتقدير الكلونازيبام CLZM في شكله النقي وفي المستحضر الصيدلاني (على شكل أقراص). تعتمد الطريقة على تفاعل CLZM مع كاشف الاليزارين الاحمر S، حيث يظهر محلول ملون بأقصى طول موجي 528 نانومتر وتمت دراسة الظروف المثلى للتفاعل وكانت الخطية في حدود 1 إلى 30 مايكروغرام/ مللتر، مع معامل تقدير 0.9882 وبمعامل امتصاص مولاري 7.04×10^3 لتر. مول$^{-1}$. سم$^{-1}$ ، بينما بلغت دلالة حساسية ساندل 0.0449 مايكروغرام/ سم^2، وقد تم تقييم الطريقة إحصائيًا عن طريق حساب النسبة المئوية للخطأ النسبي (RE%) التي يمكن أن تعبر عن الدقة والنسبة المئوية للانحراف القياسي النسبي (RSD%) والتي يمكن ان تعبر عن التوافق. كما تم حساب LOD و LOQ، وتم تطبيق الطريقة المقترحة بنجاح بتقدير الكلونازيبام في مستحضره الصيدلاني على شكل أقراص.

الكلمات الدالة: الكلونازيبام، الاليزارين الاحمر S، الطيفية، انتقال البروتون.