Modified Simplex - Spectrophotometric Determination of Clonazepam via Charge-Transfer Complexation

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Abstract

A simple, sensitive, accurate, and precise spectrophotometric method for the determination of clonazepam (CLNZ) was developed. The method is based on charge transfer reaction between CLNZ and *p*-Bromanil (*p*-Br) to form a colored complex. The optimum conditions of complex formation were investigated by (1). Unvariable method, for the optimization of reagent concentration, base concentration, temperature, and time. (2). Multivariable simplex method including the effect of three experimental factors via; reagent concentration, concentration of NaOH and time. The linearity range of CLNZ was (1-30) $\mu g.mL^{-1}$ at 378 nm under condition established via simplex method with molar absorptivity ($1.9069x10^4$) $L.mol^{-1}.cm^{-1}$, Sandell's sensitivity index (0.0165) $\mu g.cm^{-2}$, detection limit of $0.2957\mu g.mL^{-1}$, quantification limit $0.9858\mu g.mL^{-1}$ and association constant of the formed complex (2333.3). The proposed method has been successfully applied for the determination of CLNZ in pure form and pharmaceutical preparations.

Keywords: Clonazepam, charge transfer complexation, *p*-Bromanil, spectrophotometry.

Introduction

Clonazepam (CLNZ) is a benzodiazepine, has the IUPAC name (5-(2-Chlorophenyl)-7-nitro-1, 3-dihydro-2*H*-1, 4-benzodiazepin-2-one) [1] (Figure 1), medically considered as an anticonvulsant drug which is broadly used in the controlling of epilepsy. It affects chemicals in the brain that may be unbalanced [2, 3].

For the importance of CLNZ compound, several researches have been conducted to deal with its estimation of that compound. In this respect, different methods for CLNZ identification have been reported in all dependent pharmacopeia, such as IR and TLC [1]; potentiometric and HPLC methods [4,5]. Different analytical methods have been used for the determination of CLNZ such as (HPLC) [6, 7], (TLC) [8, 9], (LC) [10, 11], capillary electrophoresis [12] and spectrophotometry [13-15].

Simplex optimization of experimental parameters was first introduced by Spendley [16] and then modified by Nelder [17] and Aberg [18]. A simplex is a geometric figure in which there are n +1 vertices, where (n) represents the number of variables [19]. The method found a lot of applications in field of analytical chemistry [20-23], because it offers the capability of optimizing several factors simultaneously depending on a statistical design search to find out the maxima or minima of response, by rejecting the point producing the worst response and a replacement of it by the new point which is obtained statistically.

The aim of this work is to develop an inexpensive, simple and fast spectrophotometric method based on charge transfer (CT) reaction between CLNZ as donor and Bromanil (*p*-Br) as acceptor in alkaline medium.

Experimental

Materials

All the chemicals and reagents used in the present study were of analytical grade. CLNZ was provided by the State Company for Drug Industries and Medical Appliances, Samara-Iraq (SDI). Absolute ethanol and acetonitrile were supplied from Aldrich while *p*-Br was supplied from Merck. Rivotril tablets (Switzerland) labeled to contain 0.5 mg of CLNZ per tablet was purchased from commercial source.

Preparation of stock and working standard solutions: A stock solution of CLNZ (250 $\mu g.mL^{-1}$) was prepared by dissolving accurately 0.0625g of CLNZ in 250 mL ethanol. The stock solution was further diluted to get working concentrations of (1.0, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0) $\mu g.mL^{-1}$.

***p*-Bromanil ($1x10^{-2}$ M) solution:** Prepared by dissolving 0.4237g of *p*-Br in 40 mL acetonitrile and the solution was diluted to final volume of 100 mL with acetonitrile.

Sodium hydroxide (~ 0.4M) solution: Prepared by dissolving 1.6000 g of NaOH in 30 mL distilled water and diluted to 50 mL in volumetric flask with distilled water.

Potassium hydroxide (~0.4 M) solution: Prepared by dissolving 2.2444 g of KOH in 50 mL distilled water and diluted to 100 mL in volumetric flask with distilled water.

Preparation of sample solution: Fifteen of Rivotril tablets were weighed and finely powdered. A quantity of powder equivalent to 0.7545g of CLNZ (an equivalent amount of five tablets) was dissolved in 25 mL ethanol, left to stand for 5 min and transferred to a 50.0 mL volumetric flask and diluted with ethanol to obtain 50$\mu g\ .mL^{-1}$ CLNZ. The solution was filtered by using Whatman filter paper No.41 to avoid any suspended or undissolved material before use.

Apparatus

All spectrophotometric measurements were performed using Shimadzu 1800 UV-Vis; with match silica cells, a Sartorius BL 210S balance and water bath memmert W-200 RING were used throughout the work.

General procedure for the determination of clonazepam

i. Under condition established by univariate method:

To a series of 25mL volumetric flasks, 5.0 mL of standard solution containing (25.0-750.0) µg of CLNZ were transfered. Then, 5.0mL of $5.0x10^{-3}$M *p*-Br solution was added to each flask with shaking followed by the addition of 5.0mL of 0.2M NaOH. The flasks were allowed to stand in water bath for 25 minutes at 70 ^{o}C, and completed with the ethanol .The absorbance was measured at 384.0 nm against the reagent blank.

ii. Under condition established by simplex method:

A series of calibrated 25 mL volumetric flasks, 5.0 mL aliquots of standard solutions containing (25.0-750.0) µg of CLNZ and 5.0 mL of $1.0x10^{-3}$M *p*-Br solution different amounts and 5.0 mL of 0.12M KOH were shaking, The solution were then allowed to stand in water bath for 15 minutes at 70 ^{o}C . The volume of each flask was then completed to the mark with ethanol and the absorption value of the resulted product was measured against the corresponding reagent blank at 378.0 nm.

Results and discussion

Selection of wavelength

The absorption spectra of the product solution versus reagent blank and for reagent blank versus ethanol were recorded (Figure 2-I). The product shows a maximum absorption at 384.0 nm under primary test.

Optimization studies

Optimization of reagent concentration

The effect of *p*-Br concentration on the colour intensity of the product was examined in the range of $1.0x10^{-3}$ to $9.0x10^{-3}$M. However, 1 mL of 5.0 x 10^{-3} M *p*-Br is found to be suitable for quantitative determination of CLNZ as well as for attainment of maximum and reproducible colour intensity (Figure 3-a).

Optimization of KOH concentration

The effect of different concentrations in the range of (0.04 - 0.40M) of KOH on the absorbance has been investigated. Figure 3-b shows that 1.0 mL of 0.2M KOH solution was optimum and it was recommended for the subsequent experiment.

Optimization of the base type

The effect of 0.2 M solution of different bases (NaOH, KOH and Na_2CO_3) was investigated (Table 1). It was found that NaOH solution gives the maximum absorption intensity of the colored complex, which is used for the following experiments.

Optimization of heating time

The absorbance of the developed colored complex with respect to different time intervals (1-35 minutes) was investigated (Figure 4-a). Constant absorbance value was obtained of the 25 min of heating, which is used in the subsequent experiments.

Optimization of temperature

The effect of different heating temperatures (10 to 100 °C) was examined and the results are depicted (figure 4-b). The absorbance attains maximum colour intensity at temperature 70 °C, while higher temperatures gave no satisfactory results because the product solution starts to show a slight turbidity.

Optimization of solvent type

Different organic solvents (ethanol, methanol, acetone, DMSO, benzene, toluene and acetonitrile) have been tried for dissolving the reagent by studies on the absorbance. Acetonitrile was found to be the most suitable solvent and also gives optimum stability of the absorbance values of the formed charge transfer complexes (Table 2).

Optimization of diluting solvent

The effect of solvent on the absorptivity of the CLNZ-Br complexes was studied by using different solvents (absolute ethanol, ethanol: H_2O (1:1), H_2O and acetonitrile) for diluting the reaction mixture. Ethanol was proved to be the most suitable diluting solvent (Table 3).

Sequence of addition

Different orders of addition of reagents were experimented (Figure 5-a). It was found that the order of addition: CLNZ + *p*-Br + Base was efficient in producing the obtained results.

Stability

The effect of time on formed charge transfer product was investigated by allowing standing for varying times. The results showed that the complex remains stable at least for 60 minutes (Figure 5-b).

Simplex method

Simplex method is used to confirm the optimum conditions, which were obtained by the univariate procedure. The simplex procedure (Table 4) optimized three major parameters: reagent concentration, concentration of NaOH and heating time. After setting the boundary conditions for each variables (Table 5), four (n + 1) arbitrary experimental conditions were chosen, within specified boundaries for each, at which they affected the measured absorption signal of the colored product (experiments 1 - 4 in Table 4). The absorbance of these four experiments was fed into the modified multisimplex program, which starts to reflect the worst point through the centroid of other points to obtain a new point 5. An experiment was then performed utilizing the variable setting as a reflected point; because this value was better than that at point 3, the latter was rejected and replaced by point 5. A measured absorption signal was fed again to the program and the process was repeated successively until optimum conditions were obtained similarly to those obtained by the univariate method.

Final absorption spectra

Figure 2-II shows the final spectra of charge transfer product which exhibits maximum at 384 nm, 378nm under the univariate conditions and simplex method respectively.

Validation of Beer's law (Linearity, accuracy and precision)

According to the optimum conditions, linear calibration graph was obtained by plotting absorbance versus varying concentration of CLNZ and Beer's law is valid over the concentration range of 1.0-30.0 $\mu g.mL^{-1}$ for both univariate and simplex methods (Figures 6-a &b) at 384nm and 378 nm respectively. Table (6) shows the different analytical parameters obtained such as slope, intercept, correlation coefficient, Sandell's sensitivity، molar

absorptivity (ε), standard deviation, limit of quantification, limit of detection and relative standard deviation.

The precision and accuracy of the univariate and simplex methods were evaluated by performing three replicate analyses on pure drug solutions at three different concentration levels within the Beer's law limits. The percent error (RE %) and relative standard deviation (RSD) values presented in Table (7) reveal the high accuracy and precision of the methods.

Stoichiometric ratio

The stoichiometric ratio of the reactants was determined by employing Job's method of continuous variation [24] and molar ratio method [25]. The results indicated that the interaction occurs between equimolar solutions of CLNZ and *p*-Br and the complex was formed in the ratio of 1:1 as illustrated in Figures (7 –a & b) and Scheme 1.

Association constant (Benesi–Hildebrand equation)

The association constants of CLNZ- *p*-Br complex has been calculated via Benesi-Hildebrand equation [26]:

$$\frac{[A^o]}{A^{CT}} = \frac{1}{\varepsilon^{CT}} + \frac{1}{\varepsilon^{CT} K_C^{CT}} * \frac{1}{[D^o]}$$

Where [A^o] is the initial concentration of acceptor, [D^o] is the initial concentration of donor. A^{CT} is the absorbance of the charge transfer complex, ε^{CT} and K^{CT} are the molar absorptivity and association constant of the complex respectively.

A straight line was obtained by plotting [A^o]/A^{CT} versus 1/ [D^o] (Figure8) and the calculated parameters are presented in Table (9).

Interferences study

The results showed that no interferences were found in the presence of 10 μg.mL^{-1} of the studied excipients (lactose, sucrose and glucose) in the determination of clonazepam. (Table 10).

Application of the method to pharmaceutical preparation (commercial tablet)

The proposed method was applied successfully to determine CLNZ in the commercial dosage form as tablets (0.5 mg/tablet) and the obtained results are given in Table (11).

The recommended method was statistically compared with other methods, no significant differences were found between the calculated and theoretical values of t- test at 95% and 90% and F- test at 95% confidence limit (Table 12).

Analytical application by standard additions method (SAM)

Standard addition method has been followed to check the validity of the proposed method. Good recoveries suggesting non-interference were obtained as presented in Figure (9- a and b).

Conclusion

A charge-transfer complexation between CLNZ with *p*-Br reagent occurred with a 1:1 stoichiometry and maximum wavelength of absorption at 378 nm. The proposed method is beneficial over univariate method due to its sensitivity, accuracy, low relative standard deviation and high percentage of recovery and therefore it can be used in rapid quantitative determination of CLNZ in both pure and dosage form.

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Table (1): Effect of type of bases.

Type of bases(0.2M)	Absorbance
NaOH	0.5521
KOH	0.5333
$LiOH.H_2O$	0.3748
Na_2CO_3	0.3509

Table (2): Effect of different types of solvent on the absorbance of 10µg.mL^{-1} CLNZ.

Solvent	Absorbance
Acetonitrile	0.6327
Methanol	0.4370
Acetone	0.2142
Ethanol	0.5633
DMSO	0.6515
Benzene	Immiscible
Toluene	Immiscible

Table (3): Effect of different diluting solvents on the absorbance of 10µg.mL^{-1} CLNZ.

Solvent	Absorbance
Acetonitrile	0.6845
H_2O	0.2460
Ethanol	0.6327
Ethanol: H_2O	0. 4998

Table (4): Boundary of Simplex independent variables for determination of CLNZ.

Variable	Minimum boundary	Maximum boundary	Step size
Con. of *p*-Br (M)	$1.0x10^{-3}$	$9.0x10^{-3}$	$1.0x10^{-3}$
Con. of NaOH (M)	0.04	0.40	0.04
Time(minute)	0.0	35.0	5.0

Table (5): Simplex method for determination of 10µg.mL^{-1} CLNZ.

Exp.No	Factors			Absorbance at 378 nm
	Con.of *p*-Bromanil (M x 10^{-3})	Con. Of NaOH (M)	Heating time (minute)	
1	1.0	0.08	5.0	0.6643
2	6.0	0.32	20.0	0.5840
3	5.0	0.04	1.0	0.3294
4	2.0	0.04	11.0	0.6877
5	1.0	0.20	20.0	0.6485
6	1.0	0.04	0.0	0.6600
7	2.0	0.04	0.0	0.2826
8	1.0	0.12	15.0	0.7015
9	1.0	0.16	25.0	0.6568
10	1.0	0.08	15.0	0.6667
11	1.0	0.12	25.0	0.6544
12	1.0	0.08	10.0	0.6911
13	1.0	0.12	10.0	0.6481

Table (6): Optical characteristics and statistical data for the determination of CLNZ by univariate and simplex methods.

Parameter	univariate method	Simplex method
λmax (nm)	384.0	378.0
Colour	Purple	Yellow
Linearity, (µg. mL^{-1})	1.0-30.0	1.0-30.0
Regression equation	Y=0.0567X +0.0329	Y=0.0604X +0.0544
Slope (mL. μg^{-1})	0.0567	0.0604
Intercept	0.0329	0.0544
Correlation coefficient (r)	0.9983	0.9986
Molar absorptivity ($L.mol^{-1}.cm^{-1}$)	ε =17963.967	ε =19068.956
Sandell's sensitivity($\mu g.cm^{-2}$)	0.0176	0.0165
*Detection limit ($\mu g.mL^{-1}$)	0.0632	0.0594
**Quantification limit ($\mu g.mL^{-1}$)	0.2108	0.1979

*LOD = 3.3 σ /S , **LOQ = 10 σ/S

Table (7): Evaluation of accuracy and precision via intra-day and inter-day.

Method	Taken Con. (µg. mL^{-1})	Intra-day accuracy and precision			Inter-day accuracy and precision		
		*Found (µg. mL^{-1})	RE%	RSD%	Found* (µg. mL^{-1})	RE%	RSD%
Univariate	5.00	4.975	-0.500	1.6470	4.890	-2.200	1.930
	12.00	12.046	0.300	1.0242	12.110	0.900	1.545
	20.00	19.880	-1.000	1.5180	19.604	-1.978	1.848
Simplex	3.00	3.020	0.670	0.3340	3.042	1.334	0.827
	15.00	15.145	0.967	0.1285	15.170	1.130	0.289
	25.00	24.861	0.563	0.2060	24.890	0.440	0.244

*Average of three measurements.

Table (8): Analytical parameters for the analysis of CLNZ by the proposed and other methods.

Methods	Linearity (µg.mL^{-1})	λ_{max} (nm)	Correlation Coefficient (R)	Recovery%	RSD%	Ref.
Proposed method	1.0-30.0	378	0.9986	96.50 - 97.90	0.846-1.253	-
Spectrophotometric	5.0-40.0	532	0.9984	99.10 - 104.11	1.07 - 4.32	[13]
spectrofluorometric	0.1-0.5	383	0.9999	99.33 - 101.55	0.776	[14]
Spectrophotometric	0.32 – 4.1	425	0.9985	97.28 - 103.12	1.53 - 3.39	[15]
RP-HPLC	20.0-120.0	------	0.9992	99.41-99.95	-------	[6]
HPLC	5.0 – 25.0	254	0.9993	99.00-101.00	0.2222- 0.5810	[7]

Table (9): Parameters from Benesi–Hildebrand plot for the formed complex.

Parameter	value
Intercept	7E-05
Slope	3E-08
Correlation coefficient (r)	0.9973
$^{*}\varepsilon^{CT}$ (L.mol^{-1}.cm^{-1})	14285.7
$^{**}K^{CT}$	2333.3
Log K^{CT}	3.367
$^{***}\Delta G^{o}$, J/mol	-22112.7
ΔG^{o},KJ/mol	-22.1127

$^{*}\varepsilon^{CT}$ = 1/Intercept
$^{**}K^{CT}$ = Intercept/Slope
*** (ΔG^{o}=-2.303RT Log K^{CT})

Table (10): Percent recovery for 10 µg.mL^{-1} of CLNZ in the presence of 10µg.mL^{-1} of excipient.

Excipients	Con. found µg.mL^{-1}	Recovery%
Sucrose	10.56	107.00
Glucose	10.71	105.00
Lactose	10.66	106.00
Starch	10.29	102.89

Table (11): Determination of CLNZ in pharmaceutical tablet.

	Found amount mg	Conc. taken µg.mL^{-1}	Conc.* found µg.mL^{-1}	Recovery* %	S.D*	RSD* %
Clonazepam (0.5mg tablet) Switzerland	0.4825	5.0	4.8250	96.50	0.0408	0.8457
	0.4879	10.0	9.7593	97.65	0.1223	1.2533
	0.4883	20.0	19.5802	97.90	0.1874	0.9571

*Average of three determinations.

Table (12): T- and F- values for the analysis of 10 µg.mL^{-1} CLNZ in pharmaceutical compound.

$\bar{X}$	N	S.D	t- values[a]	F-values[b]	Ref.
19.580	3	0.1874	3.8799	----------	Proposed Method[c]
19.880	4	0.3698	1.266	3.8961	[13]
20.139	5	0.3242	2.6763	2.9945	[27]
19.970	4	0.1997	2.6192	1.1362	[28]

a. Theoretical value for t-test: N=2(4.303), N=3(3.182) and N=4(2.776) at 95% confidence limit.
b. Theoretical values for F-test **:** N = (3,2), at 95% is (19.16) and N= (4,2) at 95% is (19.25)
c. Clonazepam 0.5mg/tablet, Switzerland.

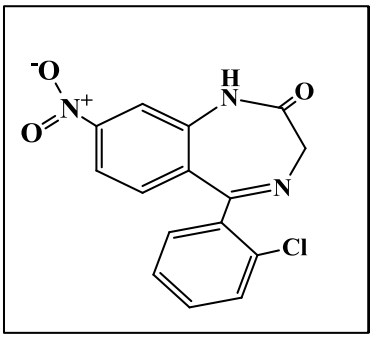


Figure (1): Chemical structure of clonazepam.

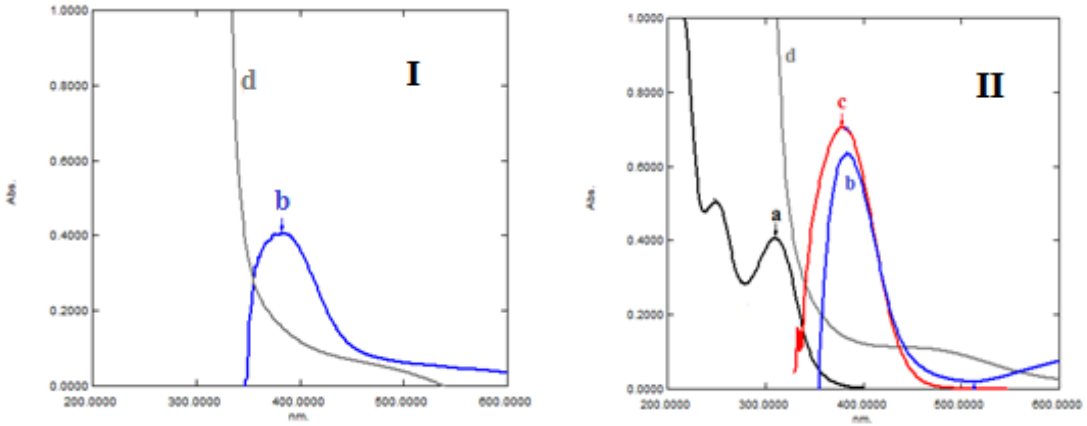


Figure (2): Absorption spectra a-(II) 10 µg.mL.$^{-1}$ of clonazepam only against ethanol.
b. 10 µg.mL.$^{-1}$ of clonazepam against reagent blank: (I)-under primary test.
(II) -under the optimum conditions
c. 10 µg.mL.$^{-1}$ of clonazepam under simplex conditions
d. The reagent blank measured against ethanol.

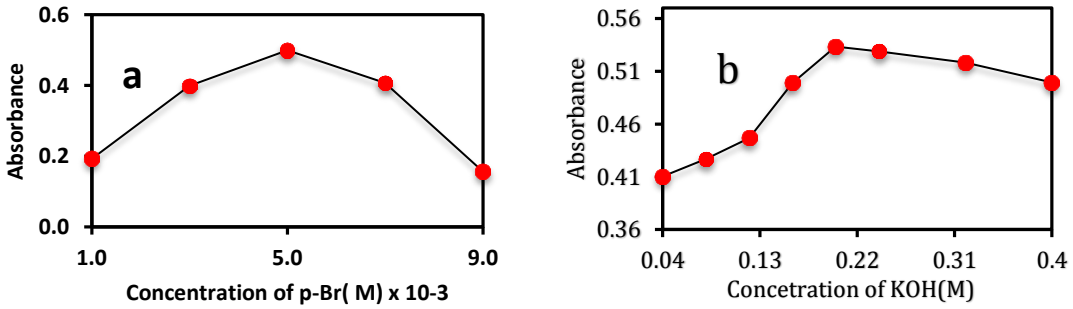


Figure (3): (a) Effect of *p*-Br concentration. (b) Effect of KOH concentration on the absorbance of 10µg.mL^{-1} of CLNZ.

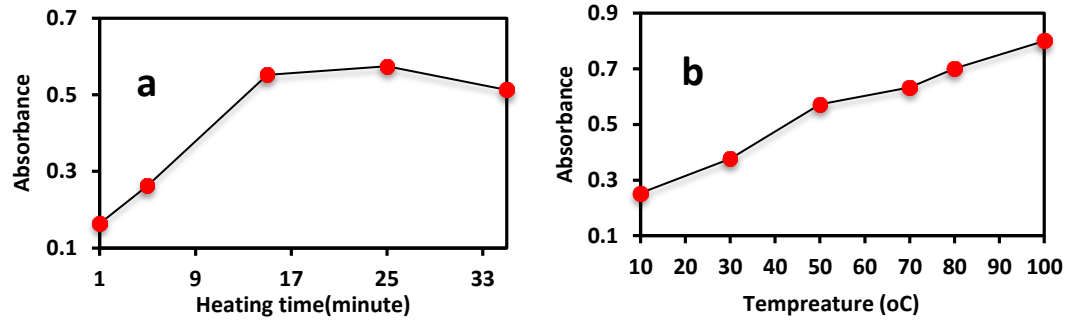


Figure (4): (a) Effect of heating time. (b)Effect of temperature on the absorbance of 10µg.mL^{-1} of CLNZ.

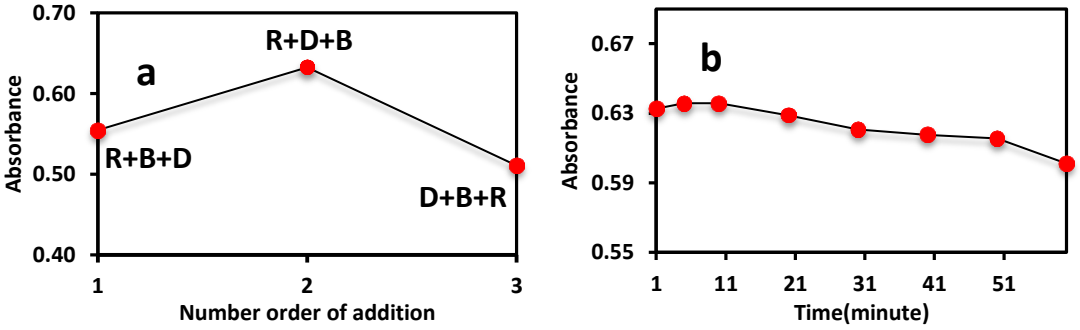


Figure (5): (a) Effect of order of addition (D: Drug, R: Reagent, B: Base) (b) Effect of time on the stability of CLNZ- *p*-Br complex (10 µg mL^{-1}).

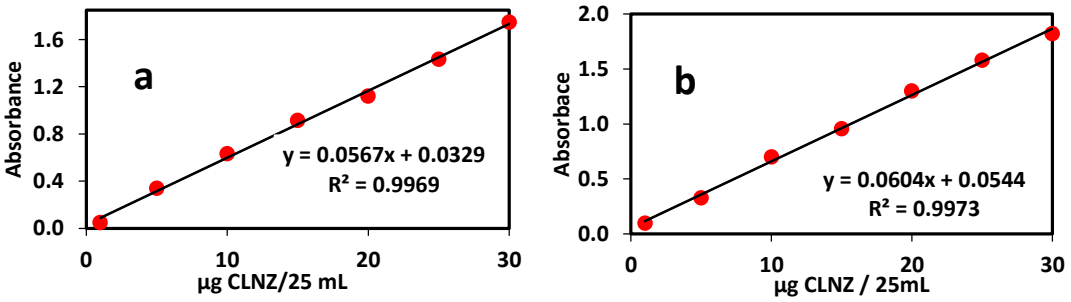


Figure (6): Calibration graph for CLNZ by (a) univariate method (b) the simplex condition.

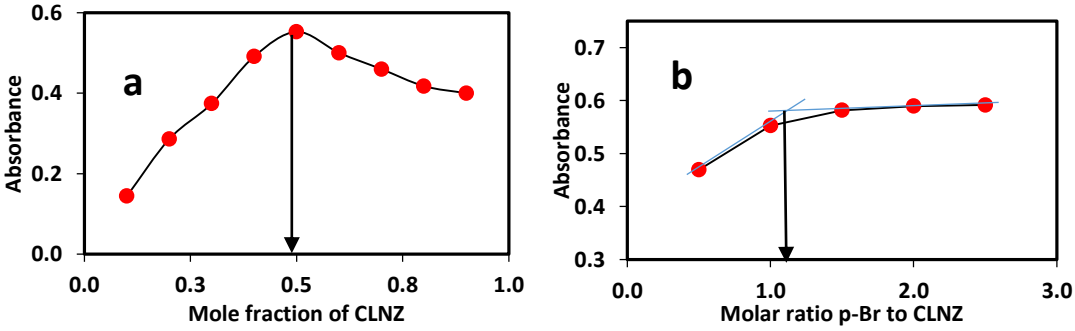


Figure (7): (a) Job's method of continuous variation (b) Mole ratio method ([CLZ], [*p*-Br] = 1.5x10^{-5}M, λmax=387nm).

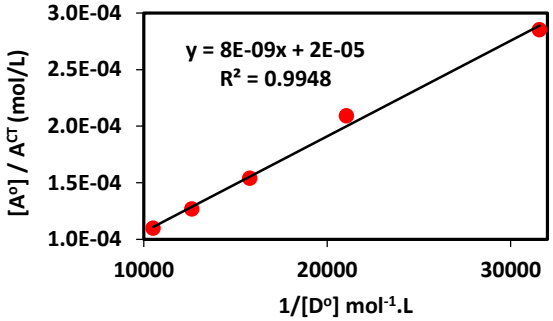


Figure (8): Benesi–Hildebrand plot for CLNZ –*p*-Br complex.

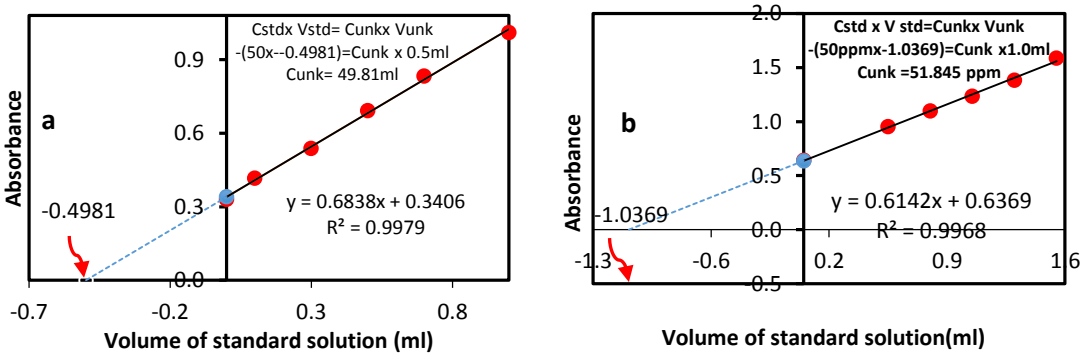
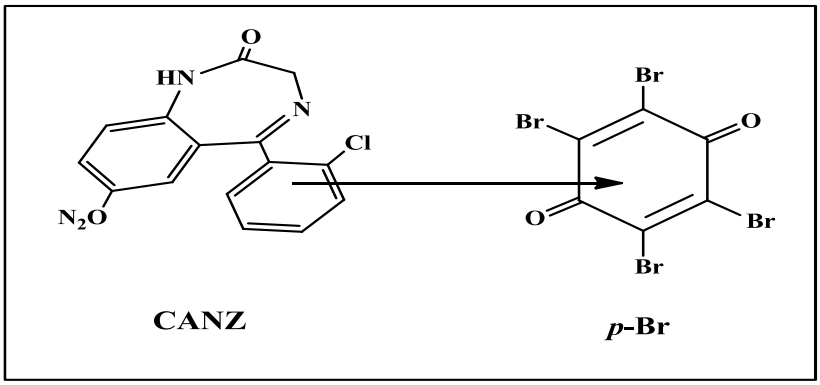


Figure (9): Determination of CLNZ in tablet (Rivotril) by SAM (a) (0.5 mL from 50 µg.mL^{-1}). (b) (1.0 mL from 50 µg.mL^{-1}).



Scheme (1): Charge Transfer complex formed between CLNZ and *p*-Br.