Spectrophotometric Determination of Clonazepam in Pure and Dosage forms using Charge Transfer Reaction

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Abstract

A rapid, sensitive and without extraction spectrophotometric method for determination of clonazepam (CLO) in pure and pharmaceutical dosage forms has been described. The proposed method was simply depended on charge transfer reaction between reduced CLO (n-donor) and metol (N-methyl-p-aminophenol sulfate) as a chromogenic reagent (π- acceptor). The reduced drug, with zinc and concentrated hydrochloric acid, produced a purple colored soluble charge-transfer complex with metol in the presence of sodium metaperiodate in neutral medium, which has been measured at λmax 532 nm. All the variables which affected the developed and the stability of the colored product such as concentration of reagent and oxidant, temperature and time of reaction were investigated and optimized. The linearity of the method was observed within a concentration range 5-40 μg ml^{-1} CLO and with a correlation coefficient not less than 0.998, while the molar absorbitivity and sandell sensitivity were 3.473×10^3 $L.mole^{-1}.cm^{-1}$ and 0.0909 μg cm^{-2} respectively. The present work includes also the usage of the Benesi–Hildebrand equation for the evaluation of the association constant and molar absorptivity of the colored complex .Finally the proposed method was successfully applied for the determination of CLO in tablets.

Keywords: Spectrophotometric ; Clonazepam ; Metol ; Charge-transfer reaction.

التقدير الطيفي للكلونازيبام في الاشكال النقية والصيدلانية باستخدام تفاعل انتقال الشحنة

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الخلاصة

يتضمن البحث طريقة طيفية بسيطة سريعة وبدون استخلاص لتقدير عقار الكلونازيبام في الشكل النقي والمستحضرات الصيدلانية باستخدام المطياف الضوئي .تعتمد الطريقة المقترحة ببساطة على تفاعل انتقال الشحنة للكلونازيبام (من نوع مانح n)المختزل بواسطة الخارصين وحامض الهيدروكلوريك المركز مع كاشف الميتول(كبريتات N-مثيل-بارا-أمينو فينول) (مستقبل من نوع π) بوجود ميتابيرايودات الصوديوم كعامل مؤكسد وفي وسط متعادل حيث يتكون ناتج ارجواني مستقر وذائب في الماء أعطى أعلى امتصاص عند طول موجي ٥٣٢ نانوميتر. جميع المتغيرات المؤثرة على استقرار وتطور الناتج الملون تم تثبيتها وايجادها. يشير الرسم البياني الخطي للامتصاص مقابل التركيز بأن الخطية كانت ضمن مدى التركيز ٥- ٤٠ جزء بالمليون وبمعامل ارتباط مالا يقل عن ٠,٩٩٨ وكانت قيمة الامتصاصية المولارية مساوية إلى ٣,٤٧٣ × ١٠^{٣} لتر.مول$^{-١}$.سم$^{-١}$ وقيمة حساسية ساندل ٠,٠٩٠٩ ٠مايكروغرام .سم$^{-٢}$. يتضمن البحث ايضا استخدام معادلة بينيسي هيلدبراند لاستخراج ثابت تجميع المعقد وامتصاصه المولاري. أخيرا تم تطبيق الطريقة بنجاح على الحبوب الحاوية على الكلونازيبام.

الكلمات المفتاحية: التقدير الطيفي، الكلونازيبام، الميتول ، تفاعل انتقال الشحنة.

Introduction

Clonazepam (CLO) which is chemically known as 5-(o-chlorophenyl)-1, 3-dihydro-7-nitro-2H-1, 4-benzodiazepin-2-one (Figure1) is a very important drug. It is and medically considered as an anticonvulsant drug which is broadly used in the controlling of epilepsy. The most effective action of CLO is treatment of absence seizures, myoclonic seizures, and infantile spasms [(1, 2)]. For the importance of CLO compound, the literature review contains several researches that deal with its estimation. Different methods for CLO identification have been reported in all dependant pharmacopeia [(3-6)] such as IR spectroscopy method, whereas for the assay purposes a potentiometric and HPLC methods have been reported.

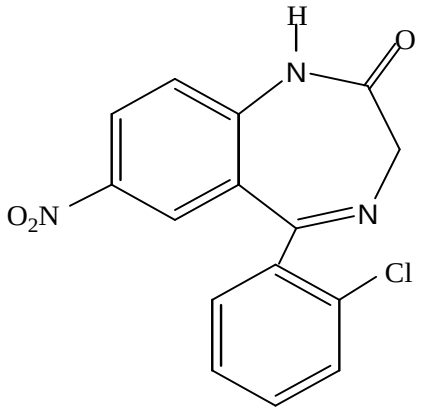

Figure (1) Clonazepam

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Many researches involved different methods for the determination of clonazepam in different matrices (biological fluids and pharmaceutical forms) have been reported.

These methods include chromatography (High performance liquid chromatography) [7, 8], voltametry using carbon nanotubes modified electrode[9], electrochem-iluminescence sensors [10, 11] , derivative spectrometry[12,14], gas and liquid chromatography-mass spectroscopy [15, 16].

From the literature search it was very clear that there are only a few spectrophotometric works for the determination of CLO. Therefore the present work describes the development of a cheap, simple and fast spectrophotometric method based on charge transfer (C.T) reaction between CLO as donor and metol as acceptor in the presence of sodium metaperiodate. The purple charge transfer complex was measured at a maximum wave length of 532 nm. All the factors affected the reaction or the product were studied and optimized, and the method was satisfactorily applied for determination of CLO in tablets.

Experimental

Apparatus

The spectral and absorbance measurements in this work were performed using a digital double beam recording spectrophotometer (Shimadzu UV-Visible 260) using 1-cm quartz cells.

Materials and reagents

All chemical reagents were supplied in a pure form and utilized for the current study. Highly pure clonazepam (CLO) was obtained from the state company for Drug Industries and Medical Appliance (SDI/Iraq), while tablets that contain CLO were obtained from market sources under the brand names (Rivotril 0.5 and 2mg Clonazepam/Roche Farma-Spain).

- ***Reduction solution of clonazepam (500 μg ml^{-1})***

About 0.0500 g of pure CLO was dissolved in a small volume of ethanol and then transferred into 50 ml volumetric flask and complete the volume to the mark with the same solvent. Into a beaker of 150 ml, the previous solution was transferred. Then a 20 ml of distilled water with 20 ml of concentrated hydrochloric acid (37%, 11.64 N), and 3 g of zinc powder were added and the mixture was mixed then allowed to stand for 15 min at ambient temperature (25 ºC).Finally the reduction mixture was filtered and transferred into 100 ml volumetric flask, and diluted to the mark with distilled water to obtain 500 μg ml^{-1} of CLO reduced solution [17]. The reduction solution is stable more than one week if kept in refrigerator. An appropriate dilution of stock solution using distilled water was performed to obtain a working solution.

- ***Blank solution***

This solution was prepared exactly as the previous reduction solution prepared but without presence of CLO. This solution was added to the all blanks prepared in all experiments to minimize the error.

- ***Metol (N-methyl-p-aminophenol sulfate) reagent solution(BDH) (3×10^{-2}M)***

This aqueous solution was freshly prepared by dissolving about 1.0331 g of reagent (M.wt=344.38 g/mol) in 100 ml distilled water, and stored in dark bottle.

- ***Sodium metaperiodate (SPI) solution (0.1M)***

The oxidant solution in this concentration was obtained by dissolving 2.1360 g of SPI and diluting to 100 ml with distilled water in volumetric flask.

- ***Solutions of pharmaceutical tablets***

The preparation of a 500 μg ml^{-1} stock solutions of CLO from the tablets was done by taking twenty tablets of the commercial drug after weighting and pulverization, an amount of the powder corresponding to 50 mg of CLO was dissolved in 30 ml of ethanol, shaked well and filtered into a 50 ml volumetric flask. The residue was washed with ethanol and finally the volume was made up to 50 ml with ethanol. Then the reduction procedure which was described earlier was accomplished by transferring this solution into 125 ml beaker and followed the previous steps. Further appropriate diluted solutions of pharmaceutical tablets were made using distilled water.

- ***Solution of blank pharmaceutical tablets***

Similar procedure was followed for drug free tablets, to exclude the interference that may occur with excipient of tablets.

Recommended procedure and calibration curve

Into a sequence of 25 ml standard flasks (7 flasks), increasing volumes (0.25-2 ml) from reduced standard CLO stock solution were transferred to cover the calibrated range (5-40 μg ml^{-1} of CLO). To each flask a volume of 1.0 ml of 3×10^{-2}M metol solution, and 0.5 ml of 0.1M sodium metaperiodate solution were added and the contents of the flasks were diluted to the mark with distilled water and mixed well. The absorbances of resulting solutions were measured after 10 min at 532 nm at ambient temperature (25°C) against reagent blank containing all materials except CLO. Alternatively the corresponding calibration curve and regression equation were

constructed. For the optimization of conditions and in all later experiments, a solution of 500 µg of CLO was used in a final volume of 25 ml (i.e. 20 ppm).

Stoichiometry of charge transfer reaction using Job's method and mole ratio method

The stoichiometry of charge transfer reaction was calculated using equimolar of reduced CLO and metol (1.584×10^{-3} M) at constant oxidant concentration adopting Job's method of continuous variation [18], and mole ratio method. In job's method a series of solutions was prepared in which the total volume of drug and reagent was constant (10 ml). While in mole ratio method an increasing volume of metol (0.25, 0.5, 1, 1.5, 2 ml) was added to 1 ml of reduced CLO at constant oxidant concentration. In various proportions of both drug and reagent, the solutions were mixed and diluted with distilled water in 25 ml volumetric flask, then the absorbance was measured at optimum wavelength and under optimal time and temperature against a reagent blank. The results obtained from figure 2 and 3 show that a 1:1 (drug to reagent) charge-transfer complex formed between CLO and metol.

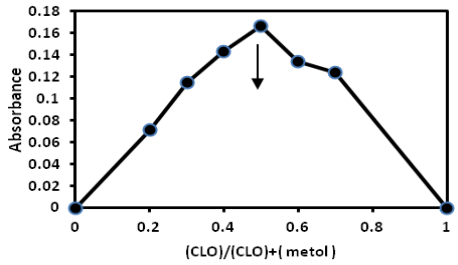
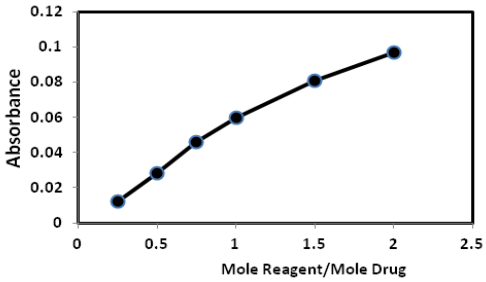


Figure (2): Job's method



Figure(3): Mole ratio method

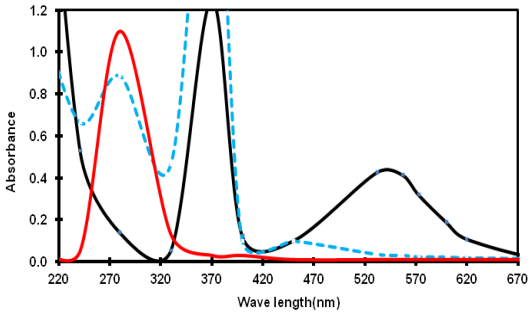
Estimation of association constant

Into a series of 25-ml volumetric flasks (7 flasks) an increasing volume of 0.25–1.5 ml of 1.584×10^{-3} M standard solution of CLO were transferred followed by 1.0 ml of 30 mM metol solution, and 0.5ml of 0.1M sodium metaperiodate solution. The detailed procedure under Section 3 was then followed for this method. Alternatively the association constant and standard free energy were calculated dependant on Benesi–Hildebrand equation.

Results and Discussion

Absorption spectra

Among different reactions used for spectrophotometric determination of drugs, charge transfer reactions considered as a very sensitive and adequate method of analysis. Based on formation a charge-transfer complex between reduced CLO as electron-donor with selected π-acceptor (metol) in aqueous medium, a simple and rapid spectrophotometric method for CLO determination was adopted in the present work. Electron donor and acceptor compounds produce a new band of absorption at a specific maximum wavelength belong to the new complex. The reaction of reduced CLO with metol results in the formation of a charge transfer complex of the *n*–π* type which absorbs at 532 nm (Figure 4).



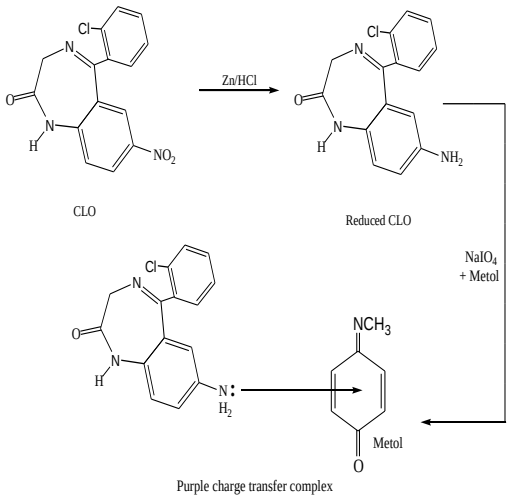
Figure(4): Absorption spectra of (1) 40 µg/ml of CLO treated as described under procedure and measured against blank (2) the reagent blank measured against distilled water and (3)40 µg/ml of solution of reduced CLO only.

Reaction mechanism

Depending on the result obtained from job's method about the stoichiometry of the reaction which showed that 1:1 (drug to reagent) charge-transfer complex has been formed between reduced CLO and metol reagent at 532 nm, therefore, the formation of product possibly occurs as illustrated in Scheme 1[19].

Optimization of charge transfer reaction conditions

The experimental factors (reagents concentrations, order of addition, temperature and stability time) that affecting on development, stability and sensitivity of charge transfer complex were carefully considered. As we mentioned previously all experiments were done using 500 µg of CLO in final volume 25 ml (i.e. 20 µg ml^{-1}) and the absorbance measurements were carried out after 10 min of mixing of the reagents at laboratory ambient temperature (25 ± 2°C).



Sheme1: Proposed mechanism of the reaction

Effect of reagent volume

To examine the effect of volume of reagent, various concentrations of metol were added (0.5-2.0 ml of 30mM) to a fixed amount of reduced CLO solution. A 1 ml of 30 mM solution was found enough to develop the color to its full intensity and gave a maximum absorbance of product (Figure5), and was considered to be optimum for the next experiments.

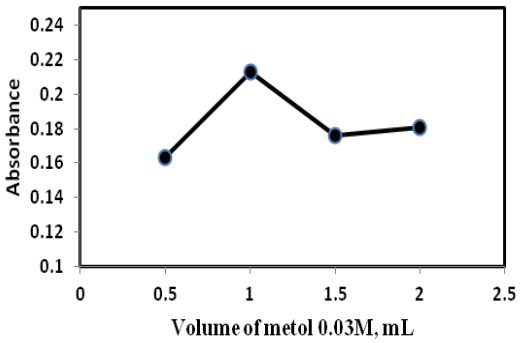


Figure (5): Effect of volume of metol

Effect of sodium metaperiodate concentration

The effect of sodium metaperiodate ($NaIO_4$) concentration were studied using different volumes over range (0.5-2.5 ml) from 0.1M of $NaIO_4$ solution which were added to a reduced CLO solution in the presence of 1 ml of 3×10^{-2}M of metol, it was found that 0.5 ml of this oxidant was enough to give a maximum absorbance and full intensity (Figure 6). The absorbance was decreased with increase of the volume of oxidant that added to the mixture of reaction, this may be due to the increase in the absorbance of the blank with each addition of sodium metaperiodate.

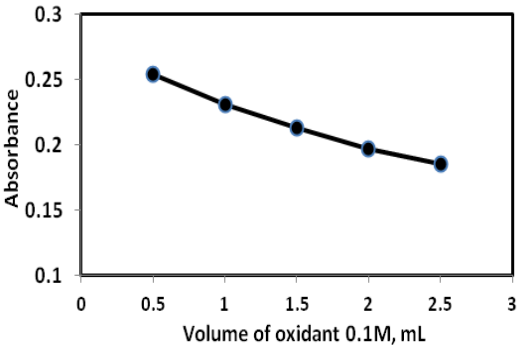


Figure (6): Effect of volume of oxidant

Effect of reaction medium and order of addition

The charge transfer product was only formed in acidic medium, while a precipitate had been formed in a basic medium. It is found that the acidic media used for reduction of CLO was enough for reaction to proceed. Usually a blank for CLO solution containing all the contents of reduction material except CLO, was used as a blank of reaction.

Different orders of addition of reagents were experimented (Figure 7) and it was found that the order of addition of reagents cited under general procedure (CLO+Metol+$NaIO_4$) was used in all following experiments, and was efficient in producing the aimed results and reaction progression.

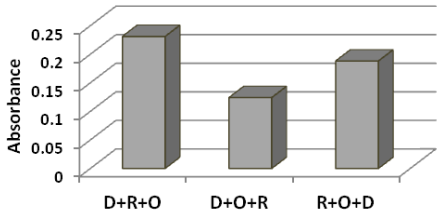


Figure (7): Effect of order of addition (D: Drug, O:Oxidant, R:Reagent)

Effect of temperature and reaction time

Different temperatures were examined to study the effect of temperature on the color intensity of the charge transfer product. The experiment showed that the charge transfer complex formed between reduced CLO and metol is formed immediately at an optimum ambient temperature of (25°C±2), furthermore the absorbance decreased with decreasing temperature (0°C), while at high temperature (60°C) the complex had been damaged.

During following the progress of color intensity of the charge transfer complex with the time, the experimental results revealed that the color intensity reach a maximum after the reduced CLO solution had been reacted with

metol and $NaIO_4$ for 10 min, therefore, a 10 min development time was suggested as the optimum reaction time and remain stable for 90 min (Figure 8).

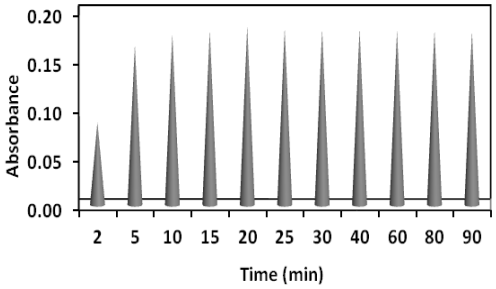
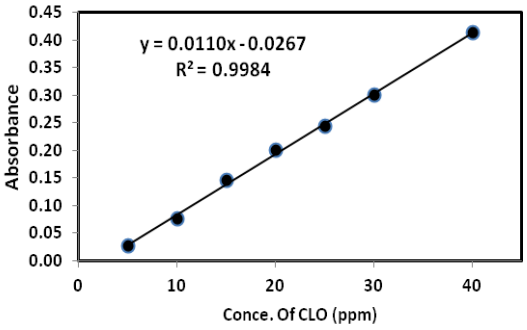


Figure (8): Stability of charge transfer complex with the time

Analytical performance

Calibration curve and linearity

After employing the optimum conditions described earlier under general procedure, linear absorbance-concentration plot (Fig.9) over the concentration range of 125-1000 µg/25 ml or 5-40 µg ml^{-1} of CLO with a correlation coefficient of 0.9984 were obtained. The visual characteristics such as molar absorpativity, limit of detection (LOD), limit of quantification (LOQ) and sandell's sensitivity [20] were summarized in Table 1.



Figure(9): Calibration curve

Table (1): Analytical data obtained from proposed method

Parameter	value
Regression equation	Y= 0.0110X - 0.0267
Linear range (µg ml^{-1})	5-40
Correlation coefficient(R^2)	0.9984
LOD (µg ml^{-1})	3.2427
LOQ (µg ml^{-1})	10.8089
Reproducibility (%)	0.7-2.6
Average of recovery(%)	99.1-104.1
Molar absorbativity ($L.mole^{-1}.cm^{-1}$) (ε=b×M×1000; M= M.wt (g mol^{-1}))	3.473×10^3
Sandell's sensitivity (µg.cm^{-2}), S (S = M/ ε)	0.0909

Accuracy and precision

In order to establish the accuracy and precision of the method, three different concentrations of standard solutions of CLO were analyzed in four replicates. The results presented in Table (2) indicate that the proposed method gave a reasonable precision and accuracy.

Table (2): Accuracy and precision of the proposed method

Conc. of CLO, µg ml^{-1}		Error %*	Rec .%*	RSD %*
Present	Found			
20.00	20.82	4.11	104.11	4.32
30.00	29.73	-0.89	99.10	1.17
40.00	40.54	1.35	101.35	1.07

*Average of four determinations.

Method specificity (Study of pharmaceutical additives)

The proposed charge transfer reaction is carried out only between donor compound (reduced CLO) and other acceptor, therefore tablets excipients such as talc, starch, lactose, polyvinyl pyrrolidone and magnesium stearate did not interfere with the assay due to absence of acceptor characteristic. Experimental obtainable recoveries of spiked pure drug with previous excipients were ranged between (99.6-101.2%) and that means no interferences were observed in present work.

Analytical applications

In order to examine if the proposed method is adequate for CLO quality control, the proposed method was applied to the quantitative determination of CLO in its dosage forms. Two doses of tablets containing 0.5 and 2mg of CLO have been analyzed and the results in Table 3 (good precision <2.6 and high recoveries best than 98%) showed obviously compatibility of the proposed method for drug analysis.

The new method was compared effectively with the British pharmacopeia's standard method (ultra violet method) [4], which depends on t- and F-tests respectively [20]. The obtained results tabulated in Table 4 (calculated values <<tabulated values) showed that there was no significant differences in accuracy or precision between two methods at 95% confidence level.

Table (3): Application of the proposed methods for determination of CLO in pharmaceutical tablet forms

Drug form	Conc. of CLO, µg ml^{-1}		Error%*	Rec.%*	RSD%*
	Present	Found			
Rivotril® (0.5mg, Roche Farma, Spain)	20.00	19.88	-0.62	99.38	1.86
	30.00	30.12	0.41	100.41	0.71
Rivotril® (2.0 mg, Roche Farma, Spain)	20.00	19.83	-0.86	99.15	2.60
	30.00	29.42	-1.92	98.08	2.57

*Average of four determinations.

Table (4): The comparison of the proposed method with standard method using t- and F-statistical tests

Drug form	Proposed method		Standard method[4]	
	Rec.% $(Xi)_1$	$(Xi-\bar{X})_1^2$	Rec.% $(Xi)_2$	$(Xi-\bar{X})_2^2$
CLO pure	101.52	2.274	100.50	0.322
Rivotril/0.5mg	99.90	0.013	99.80	0.018
Rivotril/2.0mg	98.62	1.952	99.50	0.188
S**	1.091(S_1^2=2.119)		(S_2^2=0.263)	
t (2.776)*	0.088		$(n_1 + n_2 - 2) = 4$	
F (19.000)*	8.047		n_1= 3 , n_2=3	

* indicates table value. **s = pooled standard deviation= $\sqrt{\frac{(n_1-1)S_1^2+(n_2-1)S_2^2}{n_1+n_2-2}}$, t = $\frac{|\bar{X}_1-\bar{X}_2|}{s\sqrt{\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}}$, $S_1^2 = variation = \frac{\sum(Xi-\bar{X})_1^2}{n_1-1}$ and $S_2^2 = \frac{\sum(Xi-\bar{X})_2^2}{n_2-1}$

In addition a paired t-test [20] was conducted between the samples from three sources by either method of analysis i.e. charge transfer method with standard method as shown in Table 5. A t-value for n-1 degree of freedom = 4.303. Calculated t-value=0.124 for n-1 at α 0.05 (95%), two tailed indicate that since 0.142<<4.303, therefore it can be regarded that there is no difference in using the two methods.

Table (5): Paried t-test for proposed method with classical method

Sample	Amount found		Xd	$\bar{X}$ d	σ_{n-1}	$t_{cal} = \bar{X}\sqrt{n}$ /σ_{n-1} at 95%	t_{tab} at 95%
	Proposed method	Classical method					
CLO pure	101.52	100.50	1.020	0.078	0.953	0.142<<4.303	
Rivotril/0.5mg	99.90	99.80	0.100				
Rivotril/2.0mg	98.62	99.50	-0.885				

Xd: difference between two methods, $\bar{X}$d:Difference mean, σ_{n-1}:Difference SD, $t_{critical} = t_{tab} = t_{\frac{\alpha}{2},n-1} = 4.303$, n=no of sample.

Association constant (Benesi–Hildebrand equation)

The most important factors that associated with the stability of the complex and should be calculated during the study of the charge transfer reactions are the association constant and standard free energy change of product complex. These can be obtained by applying the Benesi–Hildebrand equation [21, 22] which depends on reacting two species one of them is in large excess concentration, in order that its concentration will be unchanged on the construction of the complex.

$$\frac{[A_\circ]}{A^{AD}} = \frac{1}{\varepsilon^{AD}} + \frac{1}{\varepsilon^{AD}K_C^{AD}} \times \frac{1}{[D_\circ]}$$

Where $[A_o]$ is the total concentrations of acceptor, $[D_o]$ is the total concentrations of donor, A^{AD} is the absorbance of the charge transfer complex, $\boldsymbol{\varepsilon}^{AD}$ and K_C^{AD} are the molar absorptivity and association constant of the of the complex respectively. In order to obtain the association constant, molar absorpativity and standard free energy of the charge transfer complex product, a simple plotting of $[A_0]/A^{AD}$ versus $1/[D_0]$ (Fig. 10) was adopted. The intercept and slop of a straight line obtained were used to calculate the corresponding wanted factors (Table 6). The value of the standard free energy change (**ΔGº**) of the product complex is determined by the following equation: $\Delta G^o = -2.303RT \text{ Log } K_C^{AD}$ where R is the universal gas constant (8.314 J mol^{-1} deg^{-1}) and T is the absolute temperature in Kelvin and **ΔGº** were calculated to be -18.1418 KJ/mol as presented in Table 6. The negative charge of ΔG^o value refers to the spontaneously of the reaction.

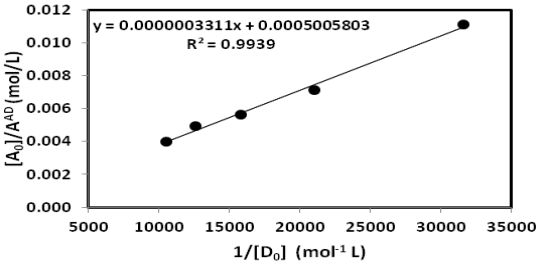


Figure (10): Benesi–Hildebrand plot for CLO and metol complex

Table(6): Association constant, molar absorptivity values and calculated free energy from Benesi–Hildebrand plots for the complexes formed

Intercept	slope	ε^{AD} (L.mol^{-1}.cm^{-1})	K_C^{AD}	Log K_C^{AD}	ΔGº, J/mol (ΔGº=-2.303RT Log K_C^{AD})	ΔGº, KJ/mol
0.000501	3.311E-07	1997.681	1511.87	3.1795	-18141.8	-18.1418

Conclusions

Charge transfer reaction is considered as one of the most important reaction used for drug analysis. The present work describes a simple and cheap method for determination of CLO in its dosage forms depend on this kind of reaction. The literature contained very little researches of spectrophotometric method for CLO determination. The method described here have many advantages such as simplicity, specificity, sensitivity and in addition it does not need expensive apparatus or extraction steps or need any expensive solvents. After studying all the chemical conditions of the method and optimized, the method applied successfully for estimation of CLO in tablets and with good recoveries. The proposed method was compared with classical method of CLO determination in British pharmacopeia and by using two statistical methods (t- and F tests and paired t-test) and both methods proved accuracy and precision of the method, and in addition the proposed method can be used routinely in quality control studies.

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