CLOUD-POINT EXTRACTION AND SPECTROPHOTOMETRIC DETERMINATION OF CLONAZEPAM IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT. Aneco-friendly, simple and very sensitive method was developed for preconcentration and determination of clonazepam (CLO) in pharmaceutical dosage forms using cloud point extraction (CPE) technique. The method is based on cloud point extraction of product from oxidative coupling between reduced CLO and phloroglucinol (PHG) using Triton X-114 as surfactant. The product was extracted by micelles of the non-ionic surfactant (TritonX-114) and subsequently detected spectrophotometrically at specific 543 nm. Under optimal conditions, the linearity of calibration curve was in the range of 0.3-25 μg/mL of CLO (r = 0.9994). Average recoveries for samples were determined to be between 96-101%. The relative standard deviation (RSD) for 3.0 μg/mL of CLO was (2.5%). The limits of detection and quantification were 0.11 and 0.37 μg/mL respectively. The suggested method was applied successfully for determination of CLO in commercial tablets.

KEY WORDS: Clonazepam, Cloud point extraction, Triton X-114

INTRODUCTION

Clonazpam, (chemically named; 5-(o-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one) is a benzodiazepine, which in general prescribed for epilepsy, anxiety and insomnia. Further uses include treatment for alcohol withdrawal, skeletal muscle spasms, tardive dyskinesia, and sedation prior to dental and medical procedures [1-3].

Various methods have been reported for determining CLO in either pharmaceutical forms or biological fluids, including RP-HPLC [4-6], automated solid phase extraction-liquid chromatograghy-tandem quadruple mass spectrometry LC-MS/MS in blood [7], chemiluminescence [8], capillary electrophoresis [9], voltammetry [10], silver nanoparticles/multi walled carbon nanotubes modified electrode [11] and flow injection spectrophotometry techniques [12-14]. Spectrophotometric technique combined with cloud point extraction (CPE) is still provided attractive features in routine analyses of metals and organic compounds in many matrices as an alternative of using an expensive instrumentations mentioned above [15, 16].

CPE technique (green method) has become the best extraction techniques in comparison with other extraction methods because of its advantages like simplicity, good recovery, high enrichment factor, low consumption of organic solvents and in addition possibility to extraction a wide range of compounds and metals. Surfactant and micelles are increasingly utilized in analytical methods mainly in extraction and preconcentration procedures. CPE (in comparison with other liquid–liquid extraction) exhibits good environmentally friendly properties. The small volumes of extracted solvents or surfactants instead of toxic organic solvents made the CPE more safely methods, in addition the reducing of consumption of the solvents reduces the costs of method and extraction time [17, 18].

To the best of our knowledge, there is no work reported in literatures relating to the estimation of CLO drug in any matrices utilizing CPE-spectrophotometric method as a result, the present work is an attempt to establish a new method for the extraction and preconcentration

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of CLO drug based on the oxidative coupling reaction and in drug formulations using CPE followed their determination using spectrophotometry.

EXPERIMENTAL

The absorption spectra and absorbance measurements of analyte were carried out by using a digital double beam spectrophotometer a type of Shimadzu UV–VIS 260 (Shimadzu, Kyoto-Japan). The absorbance measurements were carried out using 1 cm path length of quartz matched cells (Cecil, 50 μL internal volume) for cloud point extraction measurements, and for the measurements of absorbance, a matched (1 cm) silica cells had been used. A thermostatic water bath expert (England) was used of CPE experiments. A Hettich, EBA 21 model centrifuge with 50 mL calibrated centrifuge tubes were used to achieve and separation process.

Reagents and materials

Reagents and chemicals used in this work were ordinary of analytical grade, and the distilled water used all the time. CLO (Pharmaceutical grade) and excipients were received from state company for Drug Industries and Medical Appliance, SDI, Samara-Iraq. Pharmaceutical dosage forms that containing the studied drug (CLO), were purchased from local commercial suppliers and subjected to analysis : Rivotril® 2 mg (Hoffman LaRoche, Switzer-land) and Rivotril® 2 mg (Roche Farma, Madrid, Spain). All reagents used in this study were of analytical reagent grade.

Solution of CLO (500 μg/mL). Stock solution of reducing CLO was prepared [19] by dissolving 0.0500 g of CLO in 50 mL of ethanol, which then transferred into beaker of 125 mL and a 20 mL of distilled water, 20 mL of 11.7 M of HCl, and 3 g of reducing agent (zinc powder) were added. The reduction process was completed by allowed the beaker to stand for 15 min at room temperature (25 $^{\circ}$C), then the solution was filtered into 100 mL volumetric flask, washed the residues with distilled water, and finally diluted to the mark with distilled water. Working solution was prepared by appropriate dilutions in water.

Phloroglucinol solution (0.005 M). PHG (M.wt 126.1 g/mol, purity 99.0%, BDH) solution was freshly prepared by dissolving 0.0631 g of PHG in distilled water and diluting to 100 mL with the same solvent in volumetric flasks and then transferred in a brown bottle.

Sodium periodate solution (0.1 M). $NaIO_4$ (M.wt 213.9 g/mol, purity 99.9%, Merck) solution were prepared by dissolving 2.14 g of $NaIO_4$ and diluting to 100 mL with distilled water in volumetric flask.

Triton X-114(10% v/v). This solution was prepared by dissolving 10 mL of Triton X-114 (purity > 99.9%, Fluka) in distilled water and diluted to mark in 100 mL volumetric flask with the same solvent.

General CPE procedure for CLO

In a 10 mL volumetric flask, an amount of CLO standard or sample solution matched within calibration range, 1.5 mL of PHG solution (0.005 M), 0.1 mL of oxidant (0.1 M), and 1 mL of Triton X-114 (10% v/v) were added, mixed and dilute with to mark with water. The content of the flask was transferred into a 10 mL centrifuging tube and then kept for 20 min in the thermostatic bath at 65 $^{\circ}$C. The separation of two phases was conducted by centrifugation (3500 rpm for 5 min) and there after cooled down in an ice bath (in order to increase the viscosity of

the surfactant-rich phase). The aqueous phase was easily poured by inverting the tube. The surfactant-rich phase that contains the complex was dissolved with 0.5 mL of ethanol and the absorbance of the complex measured at 543 nm against a reagent blank prepared under similar conditions.

Samples preparation of pharmaceutical for CLO detection

Thirty tablets containing CLO (Rivotril-2 mg) were exactly weighted and powdered then an amount of the powder that equivalent to 50 mg of CLO was dissolved in 30 mL of ethanol. The solution was filtered and the residue was washed with ethanol and finally the volume was diluted into a 50 mL volumetric flask to the marked with the same solvent to obtain 1000 μg/mL of CLO. This solution later was transferred into 125 mL beaker and was reduced as previously described.

RESULTS AND DISCUSSION

Absorption spectra

Spectrophotometric investigations were carried out to study the formation of coupling product between CLO and PHG in the presence of the $NaIO_4$ [20]. This reaction was adopted to determine CLO using CPE procedure. The absorption spectra of the colored product and the blank were recorded before and after extraction between 200 and 750 nm (Figure 1). It was observe that there is a significant absorption bands with shoulder occurred at 543 nm, indicating the formation of a complex between CLO drug and PHG reagent. Whilst pure CLO solution gave absorption maximum at 273 nm, therefore, the wavelength maximum of 543 nm for color complex was chosen throughout this study.

The mechanism of reaction summarized by reduction the nitro group of CLO compound in first step (using zinc powder with concentrated HCl) followed by oxidative coupling between reduced drug and PHG in the presence of sodium periodate as oxidizing agent [20] as show in Scheme 1.
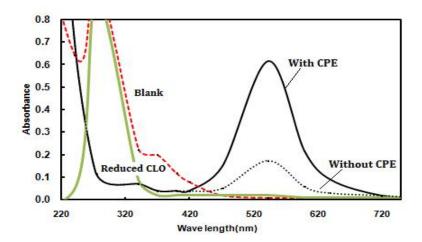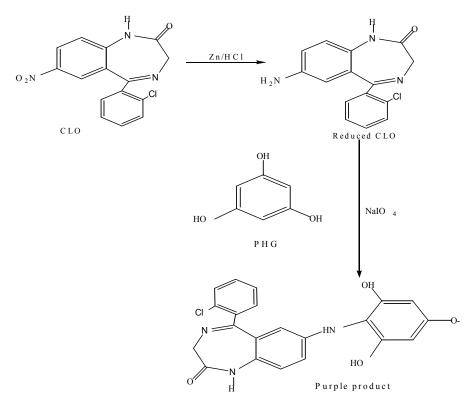

Figure 1. Absorption spectra of 20 μg/mL of reduced CLO treated as described under procedure (pink product) measured against reagent blank with and without CPE, and the reagent blank and reduced CLO measured against distilled water.

Scheme 1. The mechanism of the reaction between CLO and PHG.

Optimization of CPE method

The effects of the important parameters that affecting the efficiency of the cloud point extraction were studied by using a series of experiments. All the variables that may affecting the sensitivity and stability of the colored product were optimized by variable one parameter with the time, and keeping others fixed. A solution of 100 μg of reduced CLO in a 10 mL final volume (i.e.10 μg/mL) was used for the optimization all conditions, with measuring absorbance at 543 nm against the blank.

Effect of reaction medium

The reaction carried out without addition any acidic or basic solution and the reaction medium is acidic (produced from reduction process) and to examine the effect of increase the acidic medium, varying volume of 0.5 M HCL (from 0.0–2.5 mL) was added to the reactants and examined by measuring the absorbance at 543 nm. The results indicated that the slightly acidic of the reaction medium was enough for development of the color. More acidic solution was decreased the absorbance (Figure 2).
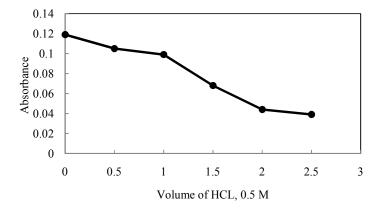
Figure 2. Effect of volume of 0.5 M HCl.

Effect of phloroglcinol concentration

The effect of PHG concentration on the formation of colored product in Triton X-114 medium was investigated using different volumes in the range (0.5- 4.0 mL) of 0.005 M PHG, the results are presented in Figure 3. It was pointed out the absorbance increased with increasing PHG volume, and reached a maximum at 1.5 mL then suddenly decreases at high volume. Therefore, a volume 1.5 mL of 0.005M PHG was selected as the optimal for complete formation complex.
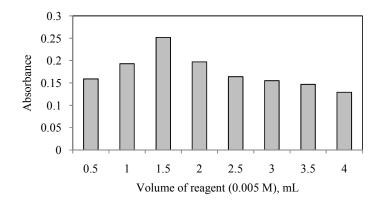

Figure 3. Effect of 0.005 M PHG reagent.

Effect of oxidant concentration

The results indicated that $NaIO_4$ is a useful oxidizing agent for the proposed reaction, other oxidizing agents have also been tested, but none give product when tried instead of $NaIO_4$. Therefore, the effect of oxidant volume was investigated in the range (0.05-2.0 mL) of (0.1 M) $NaIO_4$. The results showed that maximum absorbance was maintained if the color is developed using 0.1 mL $NaIO_4$, and it was used in all the following experiments (Figure 4).
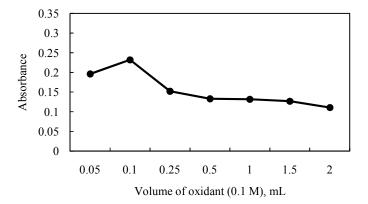
Figure 4. Effect of 0.1 M NaIO$_4$ oxidant.

Effect of surfactant concentration (Triton X-114)

Different kinds of surfactant were studied and it was found that Triton X-114 is the best one used in CPE for the present method. Concentration of the Triton X-114 has an important influence on the extraction efficiency under the cloud point extraction method, due to its important effect to increase extraction efficiency through diminish the phase volume ratio. The effect of the Triton X-114 concentration on the absorbance of the extracted complex was studied within the surfactant volume range of (0.25-3.5 mL) of 10% (v/v) Triton X-114, the concentration and volume used for Triton X-114 very high, so they must be used under heating at a temperature about 50 ºC due to a high viscosity. The results are presented in Figure 5. Indicated that a volume of 1.0 mL of 10% Triton X-114gave the maximum intensity and was used in all the following experiments.
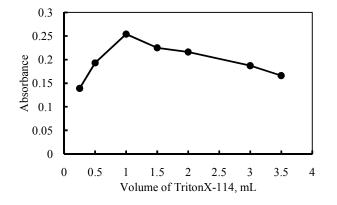

Figure 5. The effect of Triton X-114 concentration.

Effect of temperature and time of extraction

In order to achieve an efficient phase separation and easy preconcentration, it is essential to optimize the extraction temperature and incubation time. To study the effect of temperature on the extraction of colored product, a varying of the incubation temperature in the range from 25 to 85 °C at incubation time for 20 min were applied. It was shown that the maximum absorbance for complex was achieved at 65 °C (Figure 6). Therefore, an equilibration temperature of 65 °C was chosen for further experiments.
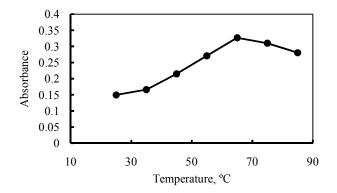

Figure 6. The effect of temperature.

CPE method usually needs a sufficient time to reach to the equilibrium between surfactant-rich and aqueous phases. The dependence of extraction efficiency upon incubation time was studied in the range of 5.0-40 min at 65 °C. An incubation time of 20 min was sufficient for the separation process. A centrifuge time period of 5.0 min was selected as optimum, as complete separation occurred within this time and no appreciable improvements were observed for longer periods.

Analytical characteristics

Under optimum conditions for CLO estimation, the calibration curve was constructed. To the sequence of 10mL standard flasks, an increasing amounts of sample containing 3–250 μg of reduced CLO was transferred (cover the range 0.3-25 μg/mL) then a volume of 1.5 mL of 0.005 M PHG solution, 0.1 mL of 0.1 M of solution of $NaIO_4$, and 1 mL of 10% (v/v) Triton X-114 were added and applied the previous extraction procedure. Linear relationships between the absorbance measured and the concentration of the CLO in solution were obtained. The regression equation was derived using the least-squares method [21]. The intercept, slope, correlation coefficient, and molar absorptivity values in addition to values of analytical statistical treatments [22-24, 27] for the calibration curve are calculated and listed in Table 1. Results listed in Table 1 indicate good sensitivity with linearity. A very small values of obtained standard deviation of the residual ($S_{y/x}$), intercept (S_a), and slope (S_b) referred to the precision of the suggested method, and low scattering of points of calibration curve. The results obtain from applied CPE procedure were compared with those without extraction which have been done by the same authors [20].

The preconcentration factor was calculated by dividing the aqueous phase volume by the final volume of the preconcentrated phase, is 20, whereas the enrichment factor   value,

calculated as the ratio of the slope of the calibration curve of the analyte after preconcentration to the slope of the calibration curve of the analyte before pre-concentration, is 3.

Table 1. The statistical data of CLO estimation with and without CPE.

Parameter	Value	
	With CPE	Without CPE [20]
Regression equation	y = 0.0274x + 0.0573	Y = 0.0088x - 0.0097
Correlation coefficient, r	0.9994	0.9972
Linearity percentage, % r^2	99.9	99.4
Dynamic range (µg/mL)	0.3-25	4-45
Molar absorptivity, ε (L/mol cm)	8650.6	2778
Sandell sensitivity (µg/cm^2)	0.0365	0.1136
Slope, b (mL/µg)	0.0274	0.0088
Intercept, a	0.0573	-0.0097
$S_{y/x}$	0.0063	0.0070
S_b	0.0002	0.0002
S_a	0.0028	0.0044
Preconcentration factor	20	--
Enrichment factor	3	--
Limit of detection, LOD (µg/mL)	0.11	0.70
Limit of detection, LOQ (µg/mL)	0.37	2.33

Accuracy and precision

To evaluate the accuracy and precision of suggested method, two different concentrations of CLO solutions were analyzed according to suggested method in five replicates. The resulting analytical values are summarized in Table 2. The small values of percentage RSD as precision and percentage relative error as accuracy indicated that the proposed method show a good accuracy and precision.

Table 2. Accuracy and precision of the proposed method.

	CPE			
Sample	Added conc. (µg/mL)	Found conc. (µg/mL)	Recovery (%)[*]	RSD (%)[*]
1	5	4.82	96.3	2.13
2	10	9.98	99.8	0.99

[*]Average of three determinations.

Interference studies

In order to examine the usefulness of the method, the studied drug (CLO) was determined in the presence of excipients (or additives) [28] which frequently attend to CLO in its dosage forms. The experiment accomplished by measuring the absorbance of solution containing 10 µg/mL of CLO in the presence of fivefold of excipient (50 µg/mL). The good percentage recoveries were obtained indicating no interference was observed from any of these excipients in determining the CLO in its dosage forms.

Determination of CLO in pharmaceutical products

The developed method was applied to the CLO estimation in two pharmaceutical drugs containing CLO as an active ingredient. The results are given in Table 3. In order to assess the success and competence of a current method, the obtained results were usually compared with resulted maintained from applying the standard method (UV method according BP [3]. The

obtained results were compared statistically by using two common tests (t-and F-tests at confidence level of 95%) [25-27]. In all cases, the calculated values were less than the theoretical one, which indicated absence of any considerable difference between each method in accuracy and precision in the estimation of CLO in its medical product.

Table 3. Application of the current and official methods to the determination of CLO in different dosage forms.

Dosage form	Suggested method					Official method [3]
	Taken conc. (µg/mL)	Found conc. (µg/mL)	Recovery (%)*	Mean recovery (%)	RSD (%)*	Mean recovery (%)*
Rivotril (2 mg) Hoffman-LaRoche-Switzerland	1	0.96	96.0	96.7	1.89	98.3
	5	4.85	97.0		1.94	
	10	9.72	97.2		1.02	
Rivotril (2 mg) RocheFarma, S.A-Madrid, Spain	1	1.03	103.0	101.7	1.90	98.5
	5	5.06	101.2		1.73	
	10	10.10	101.0		1.14	
t (2.78)^c	0.32					
F (19.0)^c	4.09					

*Average of four determination; c = theoretical value; conc., concentration; RSD = relative standard deviation.

CONCLUSION

In this work, we introduced an easy and sensitive green method, named cloud point extraction procedure, as safe and inexpensive method for the preconcentration and determination of clonazepam spectrophotometrically. The method validation yielded good results and included linearity, repeatability and sensitivity as compared with other methods. The proposed method can be applied to the determination of clonazepam in tablet.

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