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Batch and Flow Injection Spectrophotometric Methods for the Determination of Clonazepam in Pharmaceutical Preparations via Oxidative Coupling with Pyrocatechol

Mouayed Q. Al-Abachi, Maysaa Abdulwaha Hammoudi*
Department of Chemistry, College of Science, University of BaghdadBaghdad , Iraq.

Abstract

A simple, fast, and sensitive batch and flow injection spectrophotometric methods have been developed for the determination of clonazepam(CZP) in pure form and in pharmaceutical preparations. The proposed methods are based on the oxidative coupling reaction of the reduced clonazepam using Zn powders and conc. HCl with payrocatechol and in the presence of ferric sulphate. The resulting reddish colored product had a maximum absorbance at 515 nm. The optimum reaction conditions and other analytical parameters have been evaluated . The linear ranges for the batch and FI methods determination of CZP were 0.5-32, 50-400 µg mL^{-1} and the detection limits were 0.193, 22.60 µg mL^{-1} for both methods respectively. Statistical analysis of the results and comparison with results by the British Pharmacopoeia method are also reported.

Keywords: Spectrophotometry, Flow Injection, clonazepam, pyrocatechol, oxidative coupling organic reaction

استخدام الطرق الطيفية(الدفعة والحقن الجرياني) لتقدير دواء كلونازيبام في المستحضرات الصيدلانية من خلال تفاعل الازدواج التاكسدي مع البايروكاتيكول

مؤيد قاسم العبايجي، ميساء عبد الوهاب حمودي*
قسم الكيمياء، كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة:

تم تطوير طرق طيفية بسيطة وسريعة وحساسة تعتمد على طريقة الدفعة والحقن الجرياني لتقدير دواء كلونازيبام في شكله النقي و في المستحضرات الصيدلانية. تعتمد الطريقتين على تفاعل الازدواج التأكسدي للدواء المختزل بالزنك و بوجود حامض الهيدروكلوريك مع كبريتات الحديديك المائية وبايروكاتيكول حيث يتكون ناتج احمر ذائب في الماء يمتلك أقصى امتصاص عند طول موجي ١٥نانومتر ً١. تم تثبيت المتغيرات الكيميائية والفيزيائية للحصول على أفضل حساسية و تطابقيه للنتائج. تم الحصول على منحنيات معايرة خطية من ٠.٥-٣٢و٥٠- ٤٠٠مايكرو غرام مل$^{-1}$ مع قيم حد كشف ٠.١٩٣ و ٢٢.٦ مايكرو غرام مل$^{-1}$ لكل من طريقة الدفعة والحقن الجرياني على التوالي. طبقت الطريقتين بنجاح في تقدير الدواء في المستحضرات

*Email:maysaa_hammoudi@yahoo.com

الصيدلانية و كانت النتائج المستحصلة متوافقة مع النتائج المستحصلة من الطريقة القياسية المعتمدة من قبل دستور الأدوية البريطاني.

Introduction

Clonazepam (CZP) [5-(2-Chlorophenyl)-1, 3-dihydro-7-nitro-2H-1, 4-benzodiazepin-2-one] a white to light yellow crystalline powder [1]. It is a member of the benzodiazepine series of drugs, having various properties [2] such as anxiolytic ,anticonvulsant, sedative, muscle relaxant, and hypnotic. It is classified as a high potency nitro-benzodiazepine and is sometimes used as a second-line treatment of epilepsy to prevent seizures or intravenous infusions in status epilepticus [3] and neonatal convulsions [4] and is useful in various types of attacks in both children and adults [5]. CZP, like as all benzodiazepines, is also a benzodiazepine receptor agonist [6-8]. Several analytical methods such as spectrophotometry [9-11], liquid chromatography [12–15], gas chromatography [16] ,

chemiluminescence[17] and electrochemiluminescent [18] have been reported for the analysis of CZP. Most of spectrophotometric and chromatographic methods usually suffer from either extensive sample preparation involving extractions [19] and long-time analysis [20] or expensive equipment [21], so they are not suitable for routine works; nevertheless, the spectrophotometry and FI are very simple, highly sensitive, highly selective and less expensive in comparison to the above mentioned methods In this paper, both batch and FI methods using spectrophotometric detection at 515 nm are described for the determination of clonazepam via oxidative coupling reaction. The methods are depends on the formation of reddish product between CZP and pyrocatechol (PC) in presence of ferric sulphate. The proposed methods have been successfully applied to the determination of CZP in pharmaceutical preparations.

Materials and Methods

Apparatus

All spectral and absorbance measurements were performed on a Shimadzu UV -VIS 260 (Tokyo, Japan) digital double-beam recording spectrophotometer using 1 cm quartz cells. The FI system comprised a peristaltic pump (Ismatec, Labortechink-Analytic, CH-8152, glatbrugg-zurich, Switzerland, six channels) with poly vinyl chloride flow tubes of 0.8 mm i.d., an injection valve (Rheodyne, Altex 210, Supelco-USA), a 50 µL flow cells and Shimadzu UV-VIS 260 spectrophotometer (Tokyo, Japan) as the detector. Flexible Teflon tubes of 0.5 mm i.d. were used for reaction coils and to transport reagents solutions. T-link was also used to mix two streams of reagents.

Reagents

All chemicals were of analytical reagents grade.

1- All chemicals used were of analytical reagent grade and pure clonazepam CZP [$C_{15}H_{10}ClN_3O_3$, M.wt =315.7 g.mol^{-1}] drug sample was kindly provided from state company for Drug Industries and Medical Appliance, SDI, Samara. Iraq .Dosage forms (Rivotril from Roche-Hoffmann, Switzerland 2 and 0.5 mg tablets) were obtained from commercial sources .

2- Stock solution of reduced CZP (500 µg mL^{-1}). This solution was prepared by dissolving 0.0500 g of clonazepam in ethanol then transferred into 50 mL volumetric flask, and diluted to the mark with the same solvent. The solution was transferred into beaker of 125 mL. A 20 mL of distilled water, 20 mL of concentration hydrochloric acid (11.64 N), and 3 g of zinc powder from[Fluka] as reducing agent were added. The beaker was allowed to stand for 15 min at room temperature, then the solution were filtered into 100 mL volumetric flask, washed the residues with distilled water, and diluted to the mark volume with distilled water to obtain 500 µg mL^{-1} of clonazepam reduction solution. More dilute solutions were prepared daily by appropriate dilutions using distilled water.

3- Pyrocatechol (PC) is[$C_6H_6O_2$, M.wt =110.11g.mol^{-1}] from (Sherman Chemicals Ltd.,Downham Mills, Tottenham,London). The solutions of PC is (5 and 10mM) for batch and FI procedure respectively. These were freshly prepared by dissolving (0.055 and 0.1101 g) of PC and diluting with distilled watr in 100mL volumetric flasks.

4- Hydrochloric acid (HCl) Purity%= 36.0 (w:w) from(BDH).The solution (2M)HCl was prepared by diluting 43.1 mL of concentrated hydrochloric acid (Merck) (11.64N)with distilled water in 250mL volumetric flask.
5- Ferric sulfate [$Fe_2(SO_4)_3.9H_2O$] M.wt =561.7 g.mol^{-1}] from(H& W). This solution was prepared by dissolving 0.5619 g of ferric sulphate and diluting to 100 mL with distilled water in volumetric flasks to obtain(10 mM) [$Fe_2(SO_4)_3).9H_2O$] for batch method.
6- Ferric sulfate with (2M)HCl as oxidizing agent was prepared by dissolving 4.2128 g Ferric sulphate solution with 2M HCl and diluting to the marked with the same solvent in 250 mL volumetric flask to obtain 30mM for FIA procedure[Figure-1].
7- samples preparation: Tablets were accurately weighted and finely powdered. An amount of the powder equivalent to 50 mg of CZP was dissolved in 30 mL of ethanol. The solution was filtered into a 50 mL volumetric flask, the residue was washed with ethanol and diluted to volume with the same solvent .This solution was transferred into 125 mL beaker and was reduced as described above to obtain 500 µg mL^{-1} of CZP. Further appropriate solutions of pharmaceutical preparations for batch and FI procedures were made by using distilled water.

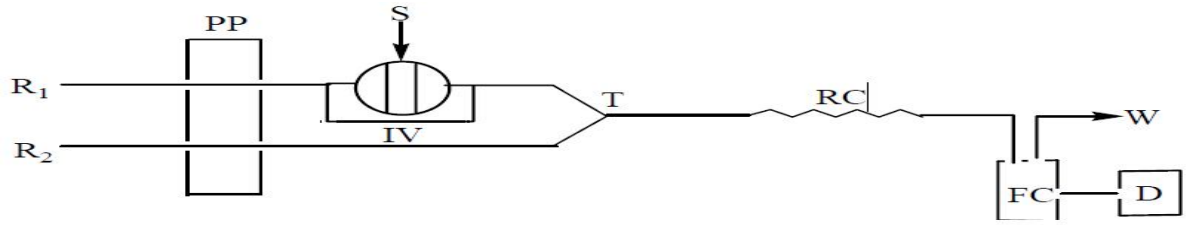
Procedure

General batch procedure

In a 25 ml calibrated flask, transfer increasing volumes of CZP (reduction solution) working solution (100 µg.ml^{-1}) to cover the range of the calibration graph 0.5- 32 µgmL^{-1} of CZP. Add 2 ml of 5m M (PC) and 2 ml of 10m M of ferric sulphate solution and shake well .Dilute the solution to the mark with distilled water and allow the reaction mixture to stand for 10 min at room temperature. Measure the absorbance at 515 nm against a reagent blank prepared in the same way but containing no CZP. The colour of the formed product is stable for 120 min. For optimization of conditions and in all subsequent experiments, a solution of CZP in a final volume of 25 ml (i.e.400µg) was used.

General FI procedure

Different concentrations of reduced CZP (50-400µ g mL^{-1}) were injected into the carrier stream of solution (PC,10 mM).which was mixed then with the oxidizing solution (30 mM $Fe_2(SO_4)_3.9H_2O$ in 2M-HCL)as shown in figure-1. Calibration graphs were prepared by plotting the absorbance versus CZP concentration.



Figuer 1- FI manifold for determination of clonazepam .Where: R1 =10 mM pyrocatecol, R2 =30mM $Fe_2(SO_4)_3.9H_2O$+HCl , PP = Peristaltic Pump, IV = Injection Valve, T = T-link, RC = Reaction Coil, FC = Flow Cell, D = Detector and W = Wast.

Statistical analysis

Statistical analysis was conducted with Statistical Product and Service Solutions (SPSS) 16.0 for Windows (SPSS Inc., U.S.A.). Differences in application methods (Batch and FI) of pharmaceutical formulations containing clonazepam were compared with standard method of BP using independent-samples t-test at a 95% confidence level or $P < 0.05$.

Results and Discussion

Preliminary Studies

When a very dilute aqueous solution of CZP was mixed with PC reagent and Ferric sulfate solutions , an intense red colure product formed. This product has a maximum absorption at 515nm [Figure- 2], in contrast to the reagent blank which shows no absorption at the same wavelength.

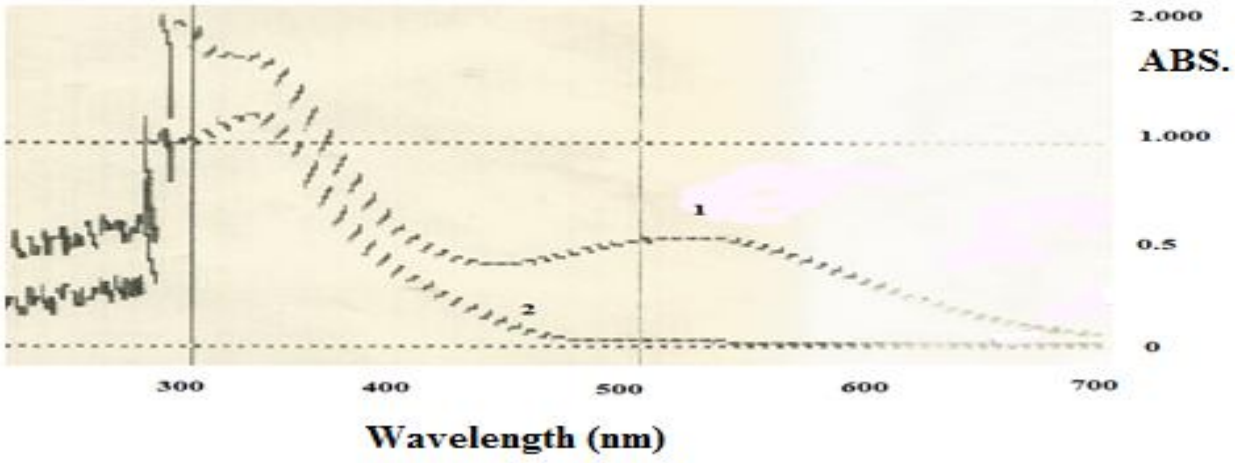


Figure 2- Absorption spectra of the(1) product obtained by the reaction of PC with 16 µg mL-^{1} of reduced drug of CZP in the presence of ferric sulfate, all versus reagent blank, (2)reagent blank(PC and ferric sulfate) versus distilled water

Optimization of the experimental conditions

The effects of various parameters on the absorption intensity of the formed product were optimized.

Batch method

In the subsequent experiments, 400 µg of clonazepam was taken in 25 mL final volume (i.e. 16 µgmL^{-1}) and the absorbance of a series of solutions were measured by varying one and fixing the other parameters at 515 nm versus reagents blanks.

The effects of different volumes (0.5-4mL) of 5mM PC and (0.3-4mL) Of 10mM $Fe_2(SO_4)_3.9H_2O$, were examined on the maximum absorbance of the formed product. Figure-3 shows that 2 mL of 5mM PC, and of 10mM $Fe_2(SO_4)_3.9H_2O$ were enough to obtain the maximum absorbance.

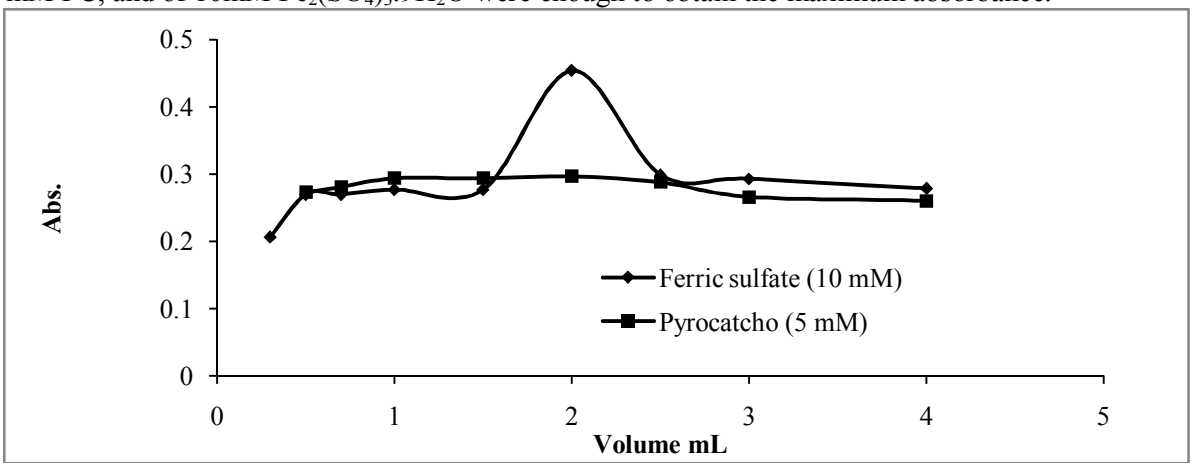


Figure 3- Optimum conditions of batch procedure for determination of CZP

The order of addition of reagents should be followed as given under the procedure, otherwise a loss in colour intensity and stability was observed. The effect of temperature on the colour intensity of the product was studied. In practice, high absorbance was obtained when the colour was developed at room temperature (25^{0} C) that when the calibrated flasks were placed in ice bath (5 ^{0}C) or in water bath (45 ^{0}C). The stoichiometry of the product was investigated using the mole ratio method. The

results obtained Figure-4 show that a 1:1 complex was formed between CZP (D) and PC (R). Therefore, the formation of the product probably occurs according to the following equation (scheme-1):

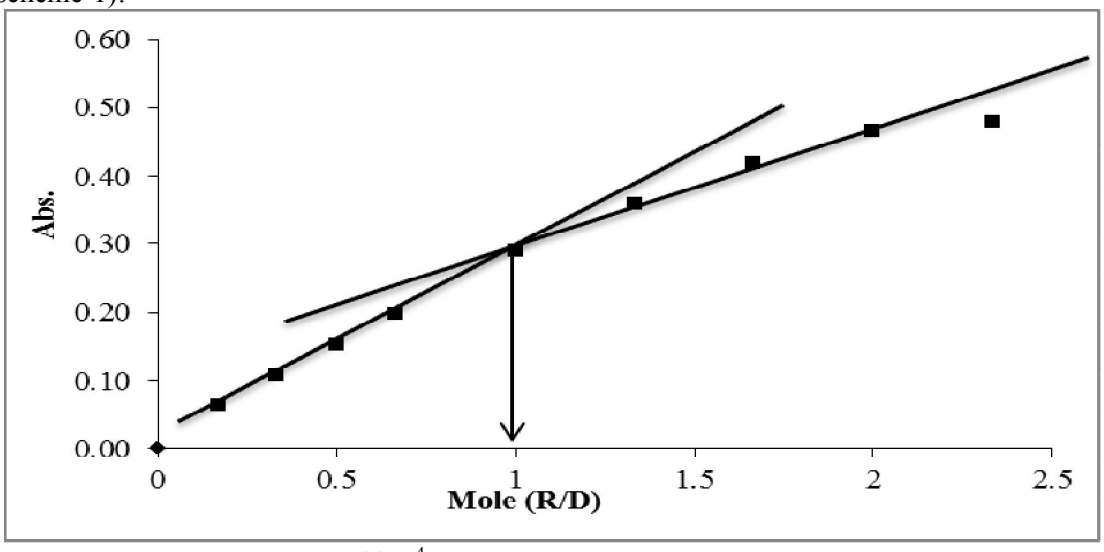
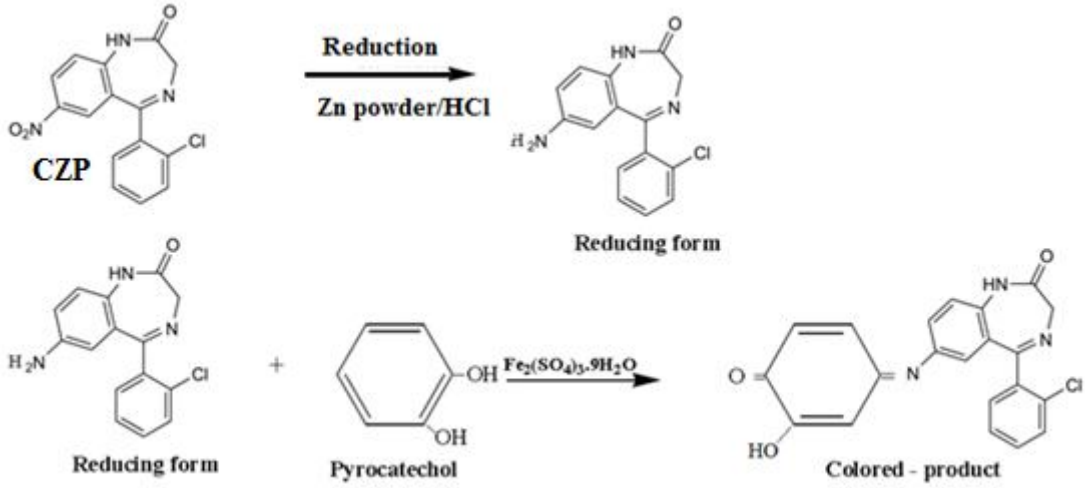


Figure 4- Mole ratio plot using 7.9×10^{-4}M for both D, R and 2mLof (10mM) $Fe_2(SO_4)_3.9H_2O$

The reduced drug[22] of CZP, by virtue of the strong electron donating ability, coupling with PC (oxidized to o-benzoquinone by ferric sulfate), leading to the formation of oxidative coupled products[23], as shown in scheme (1).



Scheme 1-Reaction path

The apparent stability constant was calculated by comparing the absorbance of a solution containing stoichiometric amount of CZP and PC reagent. The stability constant(K) of the product in water under the described experimental conditions was (3.07×10^4L mol^{-1}).

In order to assess the possible analytical applications of the proposed method, the effects of some common excipients frequently found with CZP drugs in pharmaceutical formulations, such as lactose, starch, talc, magnesium stearate and poly vinyl pirrolidone (PVP) were studied. A sample solution containing 20µg mL^{-1} of CZP with excess amounts (10-fold excess) of each excipient were analysed. The results showed that none of these substances interfered seriously (Table 1).

Table 1- Determination of 20µ g mL^{-1} of CZP in the presence of excipients.

Excipient	**Concn. Ofclonazepam**		
(200µ g mL^{-1})	**20µgmL^{-1}**	**E%**	**Rec%**
Starch	20.579	2.898	102.898
Mg-stearate	20.231	1.159	101.159
PVP	20.420	2.100	102.100
Talc	20.568	2.840	102.840
Lactose	20.286	1.434	101.434

FI method

The manifold used for the determination of CZP is shown in Figure-1. A two-channel FI system was applied, in which the sample was injected into the PC stream, which was then mixed with a stream of oxidizing solution. The reagent and the oxidizing solution streams were pumped at the same flow rate to achieve effective mixing of the sample and reagents solutions. The chemical and physical parameters were optimized by the unvitiated method with the purpose of maximizing the analytical frequency and reproducibility. According to the results of preliminary spectrophotometric studies concerning the effect of acidic medium on the absorbance of the product, hydrochloric acid was used for the FI method.

Chemical variable

The effects of various concentrations (1-35 mM) of ferric sulfate were studied, and it was found that 30mM gave the best results .Different concentrations of PC (1-20mM) were also investigated and 10mM was optimum(Figure-6a). It was found that hydrochloric acid was essential for the reaction between drug and PC , therefore, the effect of various concentrations of hydrochloric acid were studied in the range (0.5-3 M) . From the Figure-6b it was found that 2M was gave a maximum absorbance.

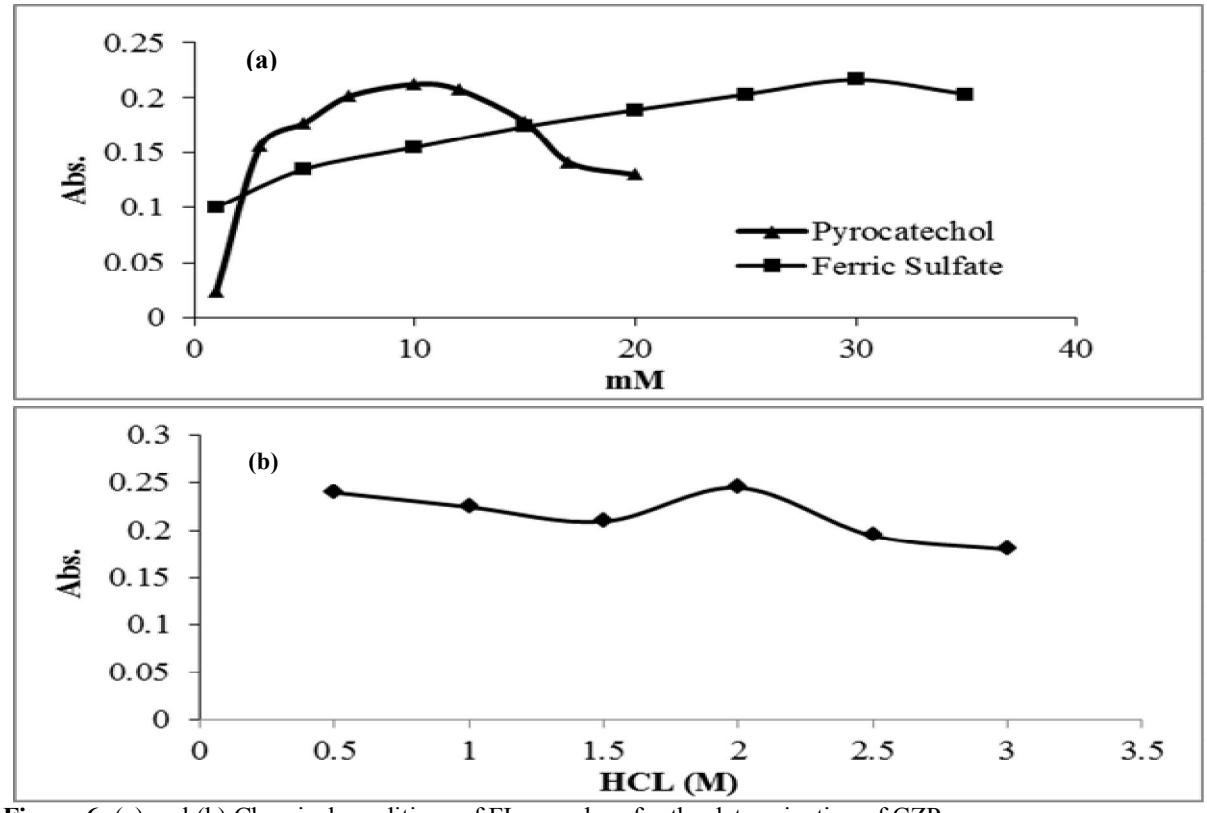


Figure 6- (a) and (b) Chemical conditions of FI procedure for the determination of CZP

Physical variables

The effects of flow rate in the analytical response was studied over the range 0.75-5 mL min^{-1}. Figure-7 shows that the absorbance increased up to 1.65mL min^{-1} and then decreased ,therefore, this flow rate was selected.

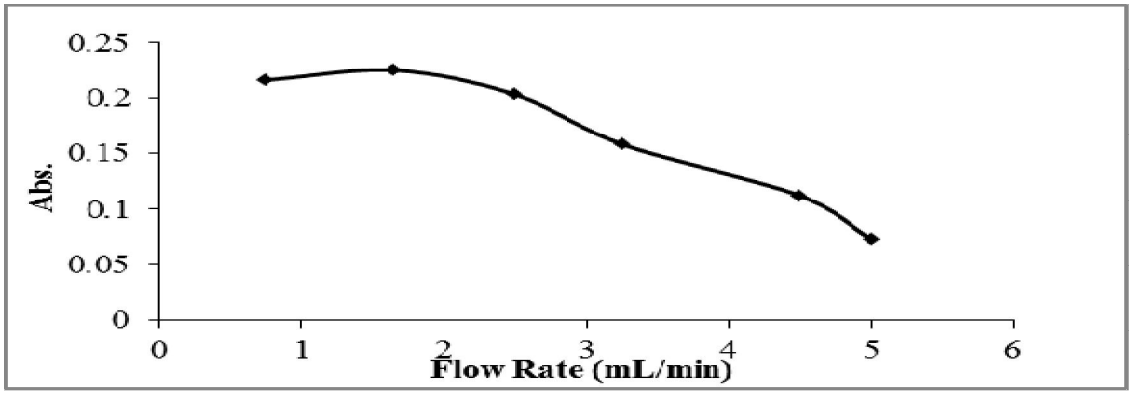


Figure 7- The effects of flow rate in the analytical

The reaction coil length is very essential parameter that effected on the sensitivity of the coloured reaction product ,therefore, it was investigated in the range(25-250)cm. The results obtained showed that a coil length of 75 cm gave the highest absorbance as shown in Figure- 8 and was used in all subsequent experiments. The injected sample volume in the range (100-250) µL was evaluated by changing the length of sample loop in the injection valve, while other variable remained fixed. The absorbance increased with increasing the volume of sample injected up to 200 µL(Figure- 8), which was chosen. The flow system selected provided a sampling rate of 64 samples h^{-1}.

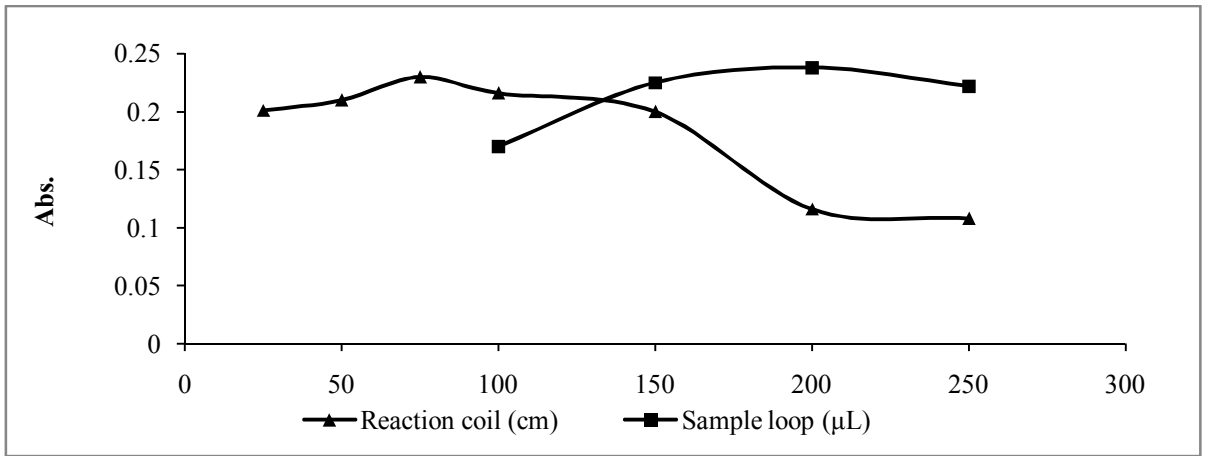


Figure 8- Physical conditions of FI procedure for determination of clonazepam

Analytical characteristics for both batch and FI methods were obtained. The calibration graphs for both procedures were determined using a series of standard solutions analysed in triplicate. The slope (a), intercept (b), correction coefficient (r) and correlation of determination (r^2) were evaluated by a least squares regression analysis and are included in Table 2. Statistical evaluation [24] of the regression line gave the values of standard deviations for residuals ($S_{y/x}$), slope (S_a) and intercept (S_b) at 95% confidence are shown in the same table. These small figures point out to the high precision of the proposed methods.

Table 2- Data for calibration graphs for clonazepam using the proposed methods

Parameter	Value	
	Batch method	**FI method**
Linearity range (μg mL^{-1})	0.5-32	50-400
R	0.9997	0.9997
r^2	0.9995	0.9995
a ($mL\mu g^{-1}$)	0.0059	0.0136
B	0.0169	0.0018
$S_{y/x}$	0.0049	0.0046
S_a	0.0024	0.0097
S_b	0.00014	1.2764E-05
E%*	-0.1479**	1.1111***
RSD%*	1.3597**	1.7562***

***Average of five determination.**
****For 20μ g mL^{-1} of clonazepam**
*****For 100μ g mL^{-1} of clonazepam**

Accuracy and precision of the batch and FI spectrophotometric methods

The accuracy and precision of the two methods were tested by analysing five replicate samples of CZP by batch and FI spectrophotometric methods. The low values of percentage errors (E%) and standard deviation (RSD%) summarized in Table 3 indicated the high accuracy and precision of the two methods.

Table 3- Accuracy and precision of the proposed methods

Drug (CZP)	Conc., μgmL^{-1}		E%	Rec.%	RSD%
	Present	Found			
Batch	8.00	8.05	0.67	100.67	1.11
	20.00	19.97	-0.15	99.85	1.36
	28.00	27.92	-0.27	99.73	1.04
FIA	50.00	49.89	-0.22	100.22	4.46
	100.00	100.44	0.44	99.56	2.08
	200.00	200.11	0.06	100.06	1.45

Pharmaceutical applications

The two proposed methods were applied successfully for the analysis of different pharmaceutical formulations containing CZP and the results are summarized in Table 4. For all formulations examined, the assay results of both methods were in good agreement with the declared content. The results obtained by two proposed methods were compared with BP method [25].

Tables (5 and 6) showed the statistical analysis according to t-test. The F-value was 6.29 and 3.088 for Batch and FI comparing with BP respectively at 95% confidence level. The batch and FI methods were not significant ($P > 0.05$) corresponding to BP standard method.

Table 4- Pharmaceutical applications for clonazepam using the proposed methods

Method	Pharmaceutical preparation	Conc. of clonazepam (μg mL^{-1}) Presence	Found*	E,%	Recovery,%	RSD,%
Batch	Rivotril 2mg	8	7.891	-1.361	98.639	2.626
		20	20.139	0.696	99.305	1.610
		28	27.416	-2.083	102.082	1.682
	0.5 mg	8	8.329	4.114	95.886	2.530
		20	19.813	-0.932	100.932	0.626
		28	28.387	1.385	98.615	0.958
FI	Rivotril 2 mg	50	49.236	-1.528	101.528	1.356
		100	102.800	2.804	97.196	2.784
	0.5 mg	50	48.700	-2.600	97.400	3.949
		100	99.899	-0.101	99.900	3.609

Table 5- Mean values of the application methods of Batch, FI and BP

Group Statistics								
	Method	N	Mean	SD	Method	N	Mean	Std. Deviation
Recovery	Batch	3	99.88	0.2948	FI	3	99.27	0.602
	BP	3	101.51	1.4781	BP	3	101.6	1.478

SD: standard deviation; N: sample number;

Table 6- Comparison of the two methods with BP method for determination of pharmaceutical preparations

		F	t	df	*P*	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
Recovery	&BP Batch	6.291	-1.875	4	0.134	0.870	-4.048	0.784
	FI&BP	3.088	-2.431	4	0.072	0.921	-4.798	0.319

Significantly different ($P < 0.05$), df: Degrees of freedom

These confirming that there are no significant differences between the two proposed methods with BP method with respect to precision and accuracy in the determination of clonazepam in pharmaceutical preparations.

Conclusions

Two simple, accurate and sensitive batch and FI spectrophotometric methods have been developed for the determination of clonazepam in pharmaceutical preparations. The developed procedures based on oxidative coupling reaction of CZP with PC reagent in presence of ferric sulfate. The proposed methods require neither temperature control nor solvent extraction step. The methods were successfully applied to a different pharmaceutical preparation samples.

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