Reverse flow injection for determination of clonazepam in pharmaceutical formulations using two different solid-phase reactors

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Abstract

Two simple, sensitive and economical reverse flow injection methods coupled with two different solid phase reactors were developed for the determination of clonazepam (CLO) in pharmaceutical formulations using a new strategy for preparation of two different solid-phase reactors; first method containing nanoparticles of PbO_2 (N-SPR) while the second method containing $FePO_4$ (F-SPR) immobilized in a polymeric matrix of cellulose acetate. The methods were based on oxidation of the 4-methoxyphenol reagent with PbO_2 or $FePO_4$ and then coupled with reduced CLO producing the colored product monitored at 570 and 608 nm for immobilized PbO_2 and $FePO_4$ respectively.

The calibration graphs were observed linear from 1-200 and 5-250 μg mL^{-1} of CLO with detection limits of 0.928 and 3.5 μg.mL^{-1} for N-SPR and F-SPR respectively. The statistical comparison of the obtained results with those obtained by the British pharmacopoeia procedure using the student t-test and variance ratio F-test shows a good agreement and indicates no significant difference in accuracy and precision at the 95% confidence interval.

Keywords: Reverse flow Injection, solid-phase reactor, clonazepam, immobilized lead dioxide nanoparticles, iron (III) phosphate.

Introduction

Clonazepam (CLO) is chemically known as 5-(o-chlorophenyl)-1, 3-dihydro-7-nitro-2H-1, 4-benzodiazepin-2-one [1,2]. It is a light-yellow crystalline powder CLO with molecular formula $C_{15}H_{10}ClN_3O_3$ and the chemical structure is shown in figure 1[3]. CLO is a benzodiazepine drug having anticonvulsant used for several kinds of seizures. It is seldom effective in generalized tonic-clonic or partial seizures. CLO activity leads to enhancement of GAMMA- aminobutyric acid receptor responses [4-6]. Because of the therapeutic importance of CLO, many methods have been developed for determination in pharmaceutical dosage forms and/or biological fluids. Literature survey reveals that various methods like Spectrophotometry [7-10], High performance liquid chromatography (HPLC) [11-16], Potentiometric methods [17] and Gas chromatography coupled with mass spectrometry (GC-MS)[18,19] and Flow injection procedures for the determination of clonazepam have also been reported[20].

The present work deals with an oxidative coupling reaction between reduced CLO and 4-methoxyphenol (MP) adopted as a basis to develop a reverse flow injection method using online solid phase reactor (rFI-SPR) containing nanoparticles of PbO_2 (N-SPR) or $FePO_4$ (F-SPR) immobilized on a polymeric matrix of cellulose acetate. The absorbance was measured at 570 and 608 nm for N-SPR and F-SPR respectively. The proposed methods are interesting strategy due to the advantages offered over the classical methods such as increased sensitivity and increased injection rate in addition of simplification of manifolds.

The rFI-SPR manifold contained one channel (Scheme 1). 100 μL of the reagent (MP) was injected into the stream of reduced CLO solution through the injection valve which then oxidized through the solid phase reactor (8 and 10 cm for N-SPR and F-SPR respectively with particles size of 1 mm) at a flow rate of 1.8 and 2.0 $mL.min^{-1}$ while the absorbance of colored product was measured at 570 and 608 nm for N-SPR and F-SPR respectively.

Material and Methods

Material: All chemical reagents were supplied in a pure form for the current study. Pure CLO was kindly provided from SDI, Samara, Iraq while tablets that contain CLO were obtained from market sources under the brand names.

Apparatus: Shimadzu UV/VIS 260 (Japan) digital double beam recording spectrophotometer was used for spectral and absorbance measurements with use of 1 cm path length flow cell and 50 μL internal volume. One-channel manifold was employed for the rFI spectrophotometric determination of CLO. A peristaltic pump (Shenchen, Lab M1, China) an injection valve (Knauer, Germany) was employed to provide appropriate injection volumes. Moreover, teflon tubes were used for making reaction coil (R.C) with an internal diameter of 0.5 mm.

Reduction procedure of CLO solution (500 μg ml^{-1}): About 0.0500 g of pure CLO was dissolved in 5 mL ethanol and then transferred into 50 ml volumetric flask and complete the volume to the mark with the same solvent, the previous solution was transferred into a beaker of 125 ml. The process was followed by addition of 20 ml of distilled water with 20 ml of concentrated hydrochloric acid (37%,

11.64 N, BDH, England)) and 3 g of zinc powder and the mixture was 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 then the volume completed to the mark with distilled water to obtain 500 μg ml^{-1} of reduced CLO solution [21]. The reduction solution was daily prepared. An appropriate dilution of stock solution using distilled water was performed to obtain a desirable working solution.

Procedure of pharmaceutical tablets: The preparation of 500 μg ml^{-1} stock solutions of CLO from the tablets was done by taking twenty tablets of the 2 mg commercial drug (Rivotril Hoffman-LaRoche Switzerland and Rivotril Roche Farma, S.A-Madrid, Spain) after weighing and pulverization, an amount of the powder corresponding to 50 mg of CLO was dissolved in 30 ml of ethanol, after shaking well and filtered into 50 ml volumetric flask to get rid of residue. The residue was washed with ethanol and finally the volume was made up to 50 ml with ethanol. Then the reduction procedure 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.

4-methoxyphenol reagent solution (0.1M) (MP): This MP solution was freshly prepared by dissolving about 0.6207 g of reagent (Hopkin and Williams, England) in 50 ml distilled water and stored in a dark bottle.

Procedure of solid-phase reactor containing nanoparticles of PbO_2 (N-SPR): The nanoparticles were prepared using sonication method. The ultra-sonication dispersed and then cracked the PbO_2 (Merck Chemicals Ltd., Germany) particles, thereby increasing the contribution of their surface area. An ultrasonic treatment resulting in an enhancement in the oxidation activity has been observed. Ultrasonic waves also have been found to inhibit the formation of PbO_2 particles larger than 150 nm [22]. The study for preparation of PbO_2 nanoparticles was carried out using 1g of PbO_2 and 50 ml of acetone as dispersion medium using 75 ml beaker in addition to use of constant sonication energy.

The immobilization of PbO_2(s) was similar to already reported[23]. Cellulose acetate (CA) (0.5g) was dissolved completely in 3 mL acetone solution and 0.5 mL of dimethyl formamide with continuous stirring. This was followed by addition of 4 g of nanoparticles lead dioxide powder to the solution. By manual stirring, the mixture was homogenized until an obvious increase in viscosity was observed. Ten minutes later the homogenized mixture was washed with water and rigid polyester was obtained. After air-drying, the polyester containing the immobilized nano PbO_2 was crashed into different sizes.

Procedure of solid-phase reactor containing immobilized $FePO_4$ (F-SPR): The new method for preparation of immobilized $FePO_4$ (Merck Chemicals Ltd., Germany) was successfully used for preparation of solid phase reactor which could be used in many oxidation methods. The immobilized steps for preparation of F-SPR were carried out by dissolving 0.5 g of cellulose acetate (CA) in 5 mL of acetone and 0.5 mL of dimethyl formamide with continuous stirring. And then 1.5 g of $FePO_4$ was added after manual homogenization by stirring until increase of homogenous mixture viscosity. A few minutes later the mixture was washed with water and rigid polyester was obtained.

After air-drying, the polyester containing the immobilized $FePO_4$ was crushed into different sizes. The suitable particle size for both reactors (N-SPR and F-SPR) was subsequently being selected (0.15 – 1.18 μM) by sieving on known mesh sieves. Finally, the SPR were prepared by packing the immobilized $FePO_4$ particles into glass tubes of different lengths (2 mm i.d.). Small pieces of sponge were inserted at the ends of the tubes to hold and prevent the packed particles in place.

Results and Discussion

The scanning of maximum absorption of the product was recorded between 200 and 900 nm. The obtained results showed that the maximum absorbance for the colored product was 570 and 608 nm for PbO_2 and $FePO_4$ respectively and will be used in the next experiments for N-SPR and F-SPR. Figure 2 shows the absorbance spectrum of CLO(30 μg mL^{-1}), reduced CLO and spectrum of colored product against reagent blank.

Preparation and characterization of PbO_2 nanoparticles: The pulsed sonication technique has been applied to the synthesis of nanoparticles PbO_2. Ultrasonic waves are generated in a liquid suspension either by immersing an ultrasound probe or "horn" into the suspension. The study for preparation of PbO_2 nanoparticles was carried out using 1g of PbO_2 and 50 ml of acetone as dispersion medium and using of constant sonication energy at room temperature. AFM (AA3000 Angstrom Advanced Inc). was used to characterize the formed nanoparticles PbO_2. The data obtained from AFM topography imaging were helpful to obtain information on the preparation mechanism of nanoparticles PbO_2. 50 μL of the sonicated PbO_2 solution was put on a cleaned glass slide and then the solid residual was used for AFM measurements. In figure 3, AFM images were recorded on the samples with shortest and longest sonication times (15-90 min).

For the 15 min sonication time, an average size of 103.5 nm was determined. On the other hand, for the sample obtained with longer sonication time (90min), the size distribution appears smaller (60.7 nm). The size distribution analysis confirmed that the nanoparticles size decreases with the increasing sonication time. Therefore, 90 min was chosen as the sonication time for producing PbO_2 NPs which will be used in all subsequent experiments for preparation of N-SPR.

Stoichiometry of the formed product and reaction mechanism: The stoichiometry of the reaction between CLO and MP was investigated in order to understand the reaction mechanism. The stoichiometry of the formed product was studied by mole ratio and continuous variation (Job's method) methods. The obtained results of both methods were plotted and indicated the existence of 1:1 (MP: CLO). The reduced drug (CLO), coupling with MP in presence of immobilized PbO_2 or $FePO_4$ leading to the form of oxidative coupled product is shown in scheme 2.

Preliminary investigations and optimization conditions for rFI-SPR procedure: Primary studies for the proposed method were directed towards the optimization of the experimental conditions to provide different reaction conditions for magnifying the absorbance signal generated by the reaction of CLO with MP in presence of SPR (immobilized PbO_2 nanoparticles, or $FePO_4$) as an oxidant. The optimum conditions were established by changing one factor at a time and keeping the others fixed. The experimental conditions were summarized in table 1 for the determination of CLO using the proposed rFI procedure.

Solid-phase reactor parameters: The proportion of oxidizer ($FePO_4$ and PbO_2 NPs): immobilized in CA has an important effect on the reactivity of the oxidant column. Different weight ratios of immobilized oxidizer in polyester resin were used in the preparation of the solid-phase materials: 2:0. 15, 2:0. 25, 2:0. 5, 4:0.15, 4:0.25, 4:0.5, 6:0.25 and 6:0.5 for N-SPR while 0.5:0.25, 0.5:0.5, 1:0.5, 1.5:0.5, 2:0.5 and 2:1 were used for F-SPR (oxidizer: CA, w: w, g). It was found that the ratio of 4:0.25 and 1.5:0.5 g for N-SPR and F-SPR respectively (Figure 4), provided the highest absorbance and good reproducibility for mentioned reactors which will be used in all subsequent experiments as optimum composition ratio.

The role of particles size on absorption was investigated and performed to identify a better balance between the magnitude and precision of analytical signals, therefore, the effect of particle size was studied in different sizes (0.15 - 1.18 mm) for immobilized oxidant agent. The desirable size particles were collected by passing through mesh sieves of known sizes. The results (Figure 5) show that the absorbance increased with increasing the particles size up to 1 mm (for N-SPR and F-SPR), then it decreased with increasing the particles size in the studied range. As a compromise of sensitivity and reproducibility in addition of the stability of baseline, the 1 mm particle size was chosen and used in all subsequent experiments for both N- SPR and F-SPR.

The effect of the reactor length on the absorbance and reactor performance was investigated by varying the reactor length in the range of 4-12 cm for each mentioned reactor (N-SPR and F-SPR). It was found that the highest analytical signal was obtained at use of 8 and 10 cm reactor length employed for N-SPR and F-SPR respectively as shown in figure 6. The reactor length of 4 cm gave a lower response owing to the short residence time. On the other hand, in the 12 cm reactor length, the absorbance was decreased probably due to the higher dispersion of the sample zone. Therefore, the lengths of 8 and 10 cm for N-SPR and F-SPR respectively were chosen and used in all subsequent experiments.

The effect of the packing degree (particles weight) of SPR on the absorbance was optimized using different amounts of the immobilized $FePO_4$ or PbO_2 nanoparticles on CA. The obtained results (Figure 7) indicate that the optimum weights of immobilized particles were 0.22 and 0.1 g for N-SPR and F-SPR respectively, above this weight the absorbance decreased since there was a resistance to sweeping the reaction plug through the reactor, because of the strong packing of SPR material causing an increase in the resistance against the flow of solution. Therefore, the optimum particle weights for each reactor (N-SPR and F-SPR) were chosen and used for further subsequent experiments.

Optimization of chemical and physical rFI-SPR parameters: The influence of MP concentration on the absorbance was investigated in the range from 0.005 to 0.09 M injected through carrier stream (reduced CLO) using the rFI-SPR manifold shown in scheme 1. It was observed (Figure 8) that the response increased with MP concentration increasing up to 0.03 M. On more than this concentration the absorbance signal was decreased. Therefore, 0.03 M of MP solution was chosen for each used reactor for further experiments.

Initially, the most appropriate flow rates were investigated since the life span of the SPR is directly related to the solution flowing through it. The effect of carrier stream flow rate on the absorbance was investigated from 0.5 to 2.2 $mL.min^{-1}$(Figure 9). The lower flow rate of carrier stream gave lower absorbance signals because of higher dispersion for sample zone whereas at higher flow rates more than 1.8 and 2 mL min^{-1} for N-SPR and F-SPR respectively were obtained. The absorbance signals decreased because of low contact between the sample zone and the immobilized oxidizer. In addition to, shorter residence time of sample and an excessive hydrodynamic pressure can occur. A flow rate of 1.8 and 2 $mL.min^{-1}$ for N-SPR and F-SPR was selected taking into account the magnitude of the analytical signal, stability of the baseline and increasing lifespan of the solid-phase reactor in addition to a sampling frequency of 34 and 30 for N-SPR and F-SPR respectively.

The sample volume injected into the carrier stream has a significant effect on the absorbance value, therefor its effect was evaluated by varying sample loop volume from 75 to 200 µL and injection of the optimum concentration of MP in CLO stream and then the mixture passed through the column with immobilized nanoparticles PbO_2 or $FePO_4$. The increase of the sample volume resulted in an increase of absorbance up to 100 µL whereas the absorbance was staying practically constant for larger volumes. This behavior can indicate that the quantity of MP in 100 µL for

these conditions is necessary for the formation of the colored product. 100 µL as sample volume showed better engagement between sensibility and analytical frequency for each reactor of N-SPR and F-SPR; additionally, increasing lifespan of the SPR and decreasing the consumption of chemicals Therefore, a sample volume of 100 µL was selected for further experiments.

The influence of the mixing coil length was studied in the range of 0 - 100 cm. The reaction coil was placed after SPR and then joined to a detector. It was observed that the absorbance decreases continuously with using of reaction coil; this fact can be explained by the higher effect of the sample zone dispersion. Therefore, the manifold without reaction coil was selected and used in the next experiment studies for N-SPR and F-SPR.

Solid-phase reactor life-time: To examine the efficiency of the N-SPR and F-SPR containing immobilized oxidizer on the CA, experiments were performed with injections of 0.03 M of MP. The column lifetime, in terms of its quantitative ability to give highly absorbance of the colored product, was investigated as a function of the sample volume of MP (100 µL) injected onto the reduced CLO stream. It was found that this solid reactor (N-SPR and F-SPR) could successfully be used for loading desirable number of reagent injections 36 and 32 with RSD% of 3.96 and 4.78 for N-SPR and F-SPR respectively. The obtained results indicated that the reproducibility (RSD ≤ 5) of reactors was good as well as life time [23]. In addition to the reactors material that was prepared gives high stability for more than one month.

Calibration graph: Under all optimum conditions, a series of CLO solutions were prepared in the range of 1-200 and 5-250 µg mL^{-1} for N-SPR and F-SPR respectively. The required concentrations were used by appropriate dilution of the CLO solution (500 µg mL^{-1}) to provide a final required concentration. Each measurement was repeated three times successively. The analytical values of statistical treatments for the calibration graph (Figure 10) are summarized in table 2. The accuracy and precision of the proposed methods (N-SPR and F-SPR) for the determination of CLO were applied under the mentioned optimum conditions. Table 3 indicated the values of percentage relative error (RE %), relative recovery (RC %) and relative standard deviation (RSD %) respectively for two replicates of each concentration using N-SPR or F-SPR. It can be seen from obtained results that both reactors successful gave a good accuracy and precision value for determination of CLO.

Analysis of pharmaceutical formulations: The proposed methods (N-SPR and F-SPR) were successfully applied for the determination of CLO using different tablets source of pharmaceutical formulation using standard addition method. The study was carried out using different concentrations for each sample (Table 4). Under the recommended procedure, the standard addition method was applied by preparing series of solutions for each sample (50 and 100 µg mL^{-1}) via transferring the required volume (1 and 2 mL, 500 µg mL^{-1}) of commercial dosage to five volumetric flask (10 mL) followed by the addition of 0 , 0.2, 0.5, 1 and1.5 mL from standard solution of CLO (500 µg mL^{-1}) in order to have the concentration range from 0 to 75 µg mL^{-1}.

The results were mathematically treated for standard additions method and the results were tabulated in table 4. In order to evaluate the efficiency and success of the N-SPR and F-SPR columns for the determination of CLO in pharmaceutical preparations, the obtained results by the proposed methods for commercial dosage forms were compared statistically using standard method[24] by means of the F-test and t-test at 95% confidence level. It can be seen that the calculated t- and F-values (Table 5) did not exceed the theoretical values which indicate that there is no significant difference between the methods in terms of accuracy and precision

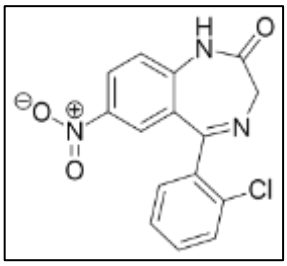
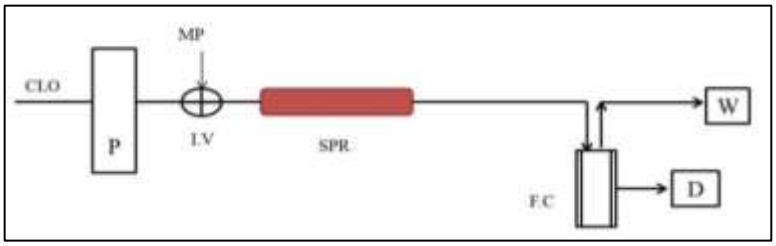


Figure 1: Chemical structure of clonazepam (CLO).



Scheme 1: Schematic diagram of rFI manifold; P, Peristaltic pump; I.V, Injection valve; F.C, Flow Cell; D, Detector; W, waste; CLO, reduced clonazepam and SPR, solid phase reactor.

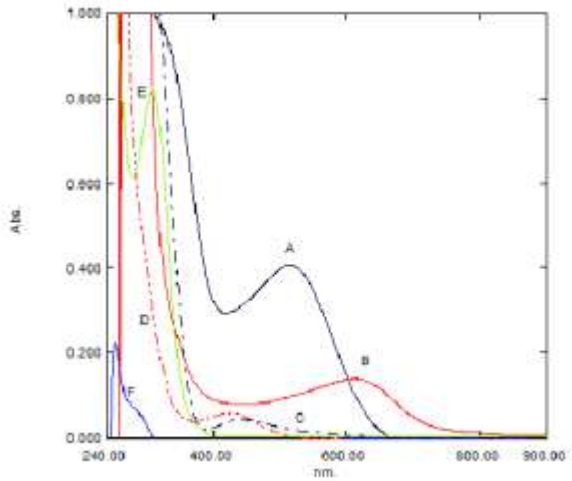


Figure 2: Absorption spectra of A: colored product using PbO_2 NPs, B: colored product using solid particles of $FePO_4$ measured against reagent blank (C and D for PbO_2 NPs and solid particles of $FePO_4$ respectively), E: CLO and F: reduced CLO.

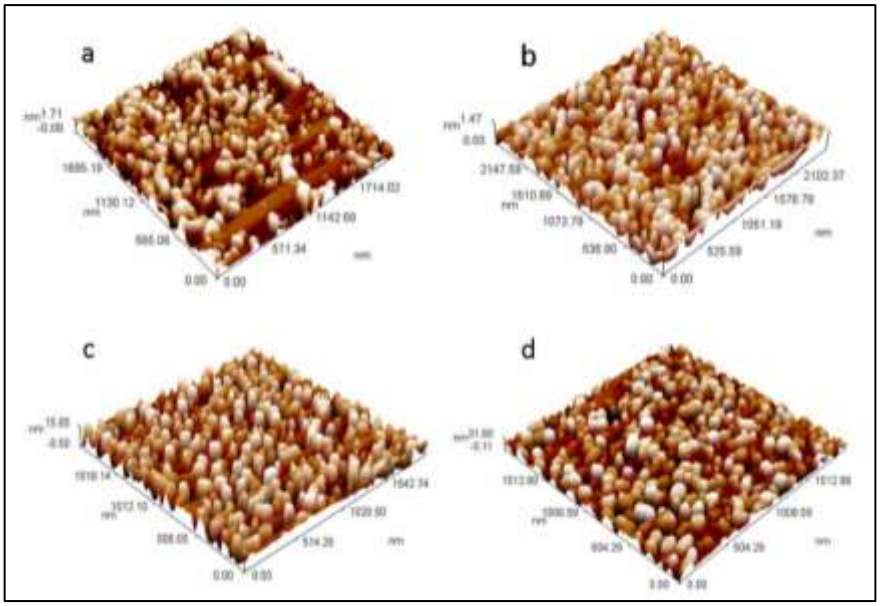
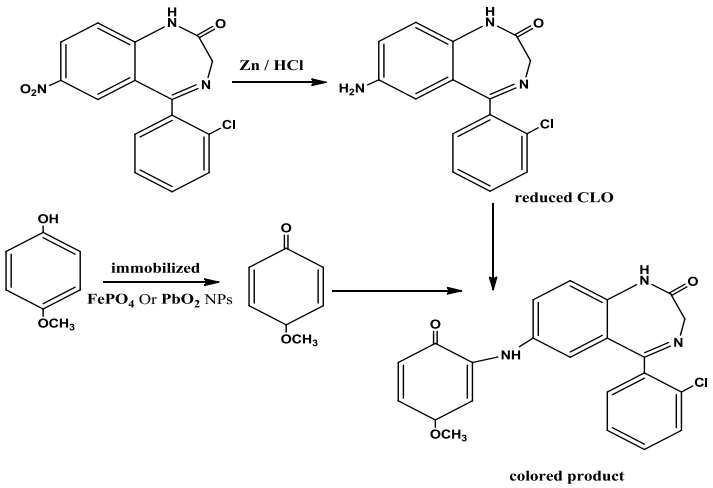


Figure 3: Characterization of PbO_2 NPs using AFM at different sonication periods; a:15, b: 30, c:60 and d: 90: min.



Scheme 2: The proposed reaction mechanism between CLO and MP in the presence of immobilized nanoparticles PbO_2 or $FePO_4$.

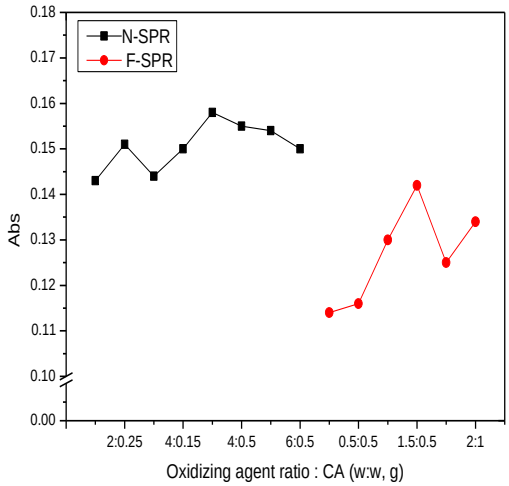


Figure 4: Oxidizing agent ratio immobilized in cellulose acetate (w:w, g) for methods N-SPR and F-SPR.

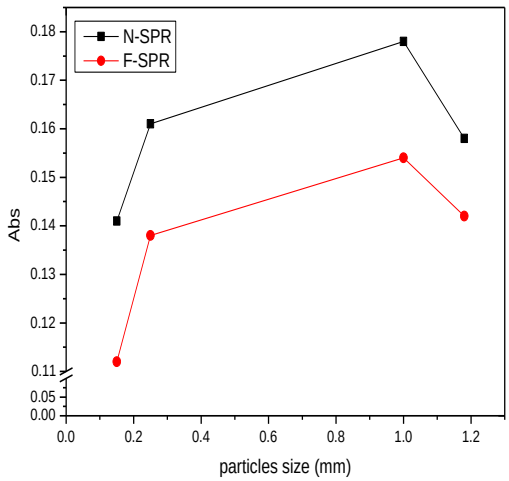


Figure 5: Particles size effect on formation of colored product.

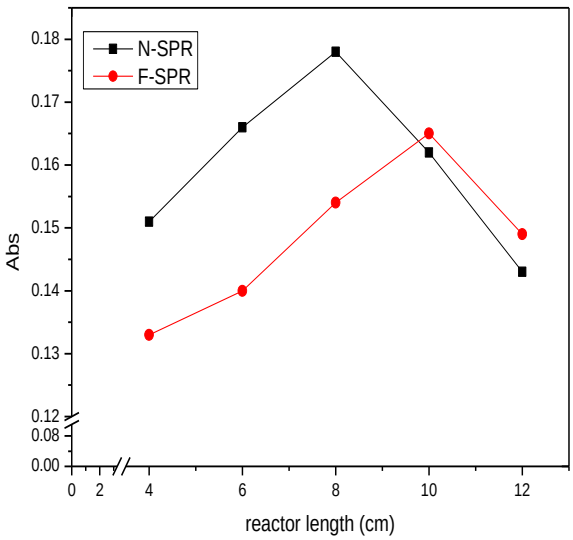


Figure 6: Reactor length effect for both methods.

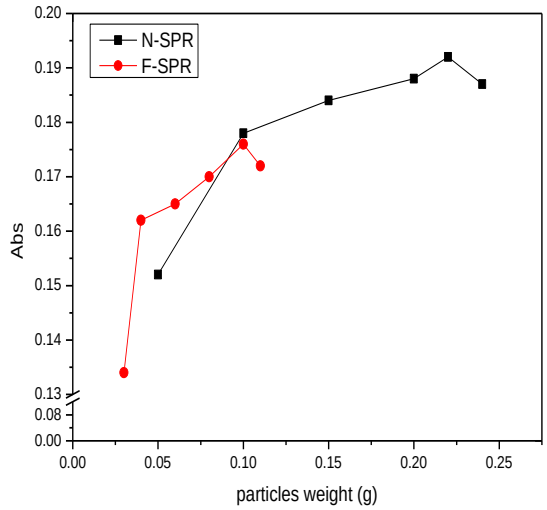


Figure 7: The effect of particles weight (degree of packing).

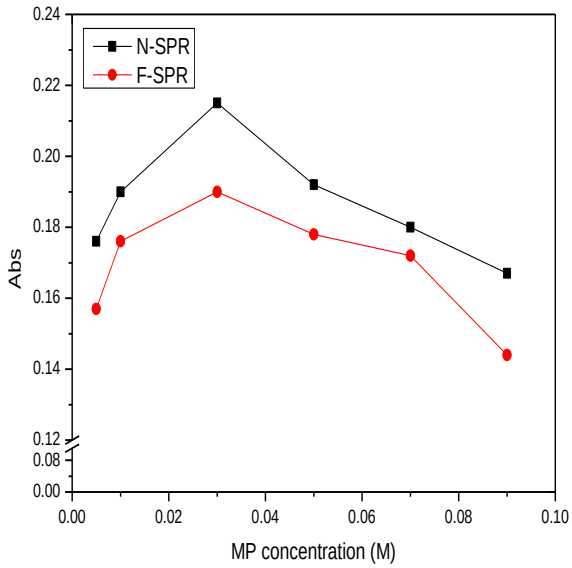


Figure 8: The effect of MP concentration on the formation of colored produt for both reactors

Table 1
The experimental conditions for the proposed rFIA procedure.

Parameter	Value	
	N-SPR	F-SPR
Concentration of CLO (μg mL^{-1})	100	100
Concentration of MP (M)	0.05	0.05
Total flow rate ($mL.min^{-1}$)	1.2	1.2
Sample volume (μL)	100	100
Ratio of oxidation agent: CA (w:w, g)	4:0.5	1:0.5
Reactor length (cm)	8	8
Particles size (mm)	1.18	1.18
Particles weight (g)	0.1	0.06
λ_{max} (nm)	570	608

Table 2
Analytical values of statistical treatments for the calibration graph

Parameter	N-SPR	F-SPR
	Value	
Regression equation	0.0021x + 0.0383	0.0018x + 0.0370
Correlation coefficient, r	0.9987	0.9979
Linearity percentage, r^2 %	99.74	99.58
Linear range (µg.mL^{-1})	1-200	5-250
Standard deviation of the residuals, $S_{y/x}$	0.0062	0.008
Standard deviation of the slope, S_b	3.1 x 10^{-5}	3.26 x 10^{-5}
Standard deviation of the intercept, S_a	0.003	0.0042
Limit of Detection (LOD) (µg.mL^{-1})	0.928	3.5
Sampling rate (per hour)	34	30

Table 3
The Accuracy and precision of the proposed method

CLO concentration (µg mL^{-1})		RC%	**RE%	RSD% n=6
Present	Found *			
N-SPR				
50	51.86	103.72	3.72	1.17
150	152.46	101.64	1.64	1.53
F-SPR				
50	52.16	104.32	4.32	1.62
150	155.13	103.42	3.42	1.75

* Average of two determinations and ** Relative error of mean.

Table 4
Application of the proposed method for determination of CLO in pharmaceutical preparations.

Sample	CLO concentration (µg mL^{-1})		RC%	RE%	RSD%
	Present	Found *			
	N-SPR				
1*	50	51.75	103.50	3.50	1.96
	100	102.86	102.86	2.86	2.28
2*	50	48.02	96.04	-3.96	1.90
	100	97.10	97.10	-2.90	2.30
1	**F-SPR**				
	50	47.83	95.66	-4.34	1.23
	100	98.20	98.20	-1.80	2.71
2	50	49.46	98.92	-1.08	2.45
	100	97.58	97.58	-2.42	1.86

* Where sample 1 and 2 are Rivotril (2mg) Hoffman-LaRoche Switzerland and Rivotril (2mg) Roche Farma, S.A-Madrid, Spain respectively.

Table 5
The comparison of the proposed method with standard method using t- and F-statistical tests.

Dosage form	N-SPR	F-SPR	Official method
	RC%	RC%	RC%
Pure CLO	102.68	103.87	100.59
Rivotril(2mg) Hoffman-LaRoche	103.18	96.93	99.47
Rivotril(2mg) Roche Farma, S. A	96.57	98.25	97.81
t_{cal}	0.67	0.17	**t_{tab} 2.776
F_{cal}	6.91	6.93	***F_{tab} = 19.0

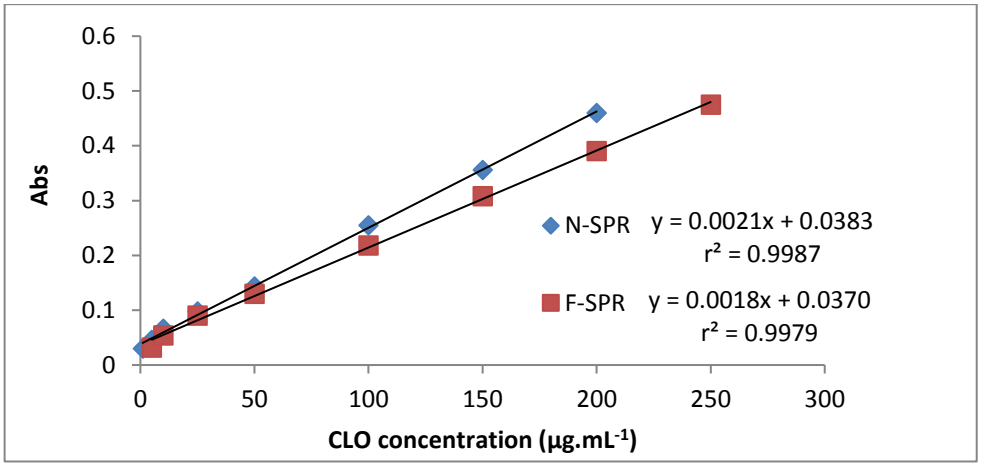


Figure 10: Calibration graph for determination of CLO using N-SPR or F-SPR.

Conclusion

The proposed methods studied about the application of solid-phase reactor containing immobilized nanoparticles PbO_2 or $FePO_4$ for spectrophotometric determination of CLO by rFIA and using 4-methoxyphenol as a new coupling reagent. The reproducibility, stability and high sensitivity in addition to more life time for the used reactors were active for loading desirable number of injections (36 and 32) for N-SPR and F-SPR respectively; these make the proposed methods an attractive alternative method for the determination of CLO in a flow system.

The proposed procedures were applied to the determination of CLO in commercially available tablets and the results obtained from the two sets of analyses were compared statistically. The student's t-values and F-values at the 95% confidence level did not exceed the tabulated values. And there is no significant difference between two methods regarding accuracy and precision. These methods may be suitable for routine determinations of CLO in pharmaceutical formulations specially the obtained results are in agreement with those obtained by the British Pharmacopoeia.

References

1. Lide D.R., Handbook of Chemistry and Physics, 88th ed., CRC Press, Taylor & Francis, Boca Raton, 3-120 **(2007)**

2. Martindale, The Complete Drug Reference, Pharmaceutical Press, 36th ed., 478-479 **(2009)**

3. Pinder R., Brogden R., Speight T. and Avery G., Metoclopramide: a review of its pharmacological properties and clinical use, *Drugs*, **12(2)**, 81-131 **(1976)**

4. Riss J., Cloyd J.G. and Collins S., Benzodiazepines in epilepsy: pharmacology and pharmacokinetics, *Acta Neurol Scand*, **118(2)**, 69–86 **(2008)**

5. Skerritt J.H. and Johnston G.A., Enhancement of GABA binding by benzodiazepines and related anxiolytics, *Eur J Pharmacol*, **89(3–4)**, 193–198 **(1983)**

6. Lehoullier P. and Ticku M., Benzodiazepine and beta-carboline modulation of GABA-stimulated 36Cl-influx in cultured spinal cord neurons, *Eur. J. Pharmacol*, **135(2)**, 235–238 **(1987)**

7. El-Brashy A., Aly F. and Belal F., Determination of 1,4-Benzodiazepines in drug dosage forms by difference spectrophotometry, *Microchim Acta*, **110(1-3)**, 55-60 **(1993)**

8. Salem A., Barsoum B. and Izake E., Determination of bromazepam and clonazepam in pure and pharmaceutical dosage forms using chloranil as a charge transfer complexing agent, *Anal. Lett*., **35(10)**, 1631-1648 **(2002)**

9. Salem A., Barsoum B. and Izake E., Spectrophotometric and fluorimetric determination of diazepam, bromazepam and clonazepam in pharmaceutical and urine samples, *Spectrochim Acta*, **60(4)**, 771-780 **(2004)**

10. Hind H., Spectrophotometric Determination of Clonazepam in Pure and Dosage forms using Charge Transfer Reaction, *Iraqi J. Pharm Sci*., **24(1)**, 25-32 **(2015)**

11. Patil P., Wankhede S. and Chaudhari P., A Validated Stability–Indicating HPLC Method estimation of ClonazepamIn the bulk drug and Pharmaceutical Dosage Form, *Pharm Anal Acta*, **6**, 332 **(2015)**

12. Chaves A., Leandro F., Carris J., Eugênia M. and Queiroz C., pharmacopoeia Microextraction in packed sorbent for analysis of antidepressants in human plasma by liquid chromatography and spectrophotometric detection, *J. Chromatography B*, **878(23)**, 2123–2129 **(2010)**

13. Katrine M., Nielsen K., Johansen S. and Linnet K., Pre-analytical and analytical variation of drug determination in segmented hair using ultra-performance liquid chromatography–tandem mass spectrometry, *Forensic Science International*, **234**, 16–21 **(2014)**

14. Cavedal L., Mendes F., Domingues C., Patni A., Monif T., Reyar S., Pereira A., Mendes G. and Nucci G., Clonazepam quantification in human plasma by high-performance liquid chromatography coupled with electrospray tandem mass spectrometry in a bioequivalence study, *J. Mass Spectrom*, **42(1)**, 81-88 **(2007)**

15. Gandhi S., Dhavale N., Jadhav V. and Sabnis S., Spectrophotometric and reversed-phase high performance liquid chromatographic methods for simultaneous determination of escitalopram oxalate and clonazepam in combined tablet dosage form, *J AOAC Int*., **91(1)**, 33-38 **(2008)**

16. Ibrahim F., El-Enany N., Shalan S. and Elsharawy R., Micellar High-Performance Liquid Chromatographic Method for Simultaneous Determination of Clonazepam and Paroxetine HCl in Pharmaceutical Preparations Using Monolithic Column, *J Chromatogr Sep Tech*., **7**, 331 **(2016)**

17. Nie L., Liu D. and Yao S., Potentiometric determination of diazepam with a diazepam ion-selective electrode, *Pharm Biomed Anal*., **8**, 379-383 **(1990)**

18. Cavedal L., Mendes F., Domingues C., Patni A. and Monif T., Clonazepam quantification in human plasma by high-performance liquid chromatography coupled with electrospray tandem mass spectrometry in a bioequivalence study, *J. Mass Spectrom*, **42**, 81-88 **(2007)**

19. Pujadas M. et al, A simple and reliable procedure for the determination of psychoactive drugs in oral fluid by gas chromatography-mass spectrometry, *J Pharm Biomed Anal*., **44**, 594-601 **(2007)**

20. Al-Abachi M.Q. and Maysaa A.H., The use of Spectrophotometric Batch and Flow injection Estimation of Clonazepam drug in Pure and Pharmaceutical Preparations, *Iraqi J. of Science*, **56(3B**), 2115-2125 **(2015)**

21. Hassan S.M., Belal F., El-Din M.S. and Sultan M., Spectrophotometric determination of some pharmaceutically important nitro compounds in their dosage forms, *Analyst*, **113(7)**, 1087-1089 **(1988)**

22. Ghasemi S., Mousavi M., Shamsipur M. and Karami H., Sonochemical-assisted synthesis of nano-structured lead dioxide, *Ultrasonics Sonochemistry*, **15**, 448–45 **(2008)**

23. Zhang Z. and Tang Y., Solid-phase reactor flow-injection on-line oxidizing spectrofluorimetry for determination and dissolution studies of folic acid, *Anal. Bioanal. Chem*., **381**, 932 **(2005)**

24. *British Pharmacopeia*. On CD, **5**, 7-118 **(2005)**.