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Short Communication

FIA-SPECTROPHOTOMETRIC DETERMINATION OF NITRAZEPAM BY OXIDATION WITH A SOLID-PHASE REACTOR AND COUPLING WITH 2,2'-DIHYDROXYBIPHENYL REAGENT

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ABSTRACT. A new reverse flow injection system combined with a solid-phase reactor containing PbO_2 was suggested for spectrophotometric determination of nitrazepam in pharmaceutical tablets. Nitrazepam was measured by coupling with a new reagent (2,2'-dihydroxybiphenyl) oxidized by forced through the reactor containing PbO_2 immobilized in a polymeric matrix. The absorbance of the resultant blue colored product was measured at 635 nm, and the calibration graph for nitrazepam was linear in the 10 to 300 µg/mL concentration range with RSD of less than 2.5% (n = 38) and a sample throughput of 40 h^{-1}. The influence of the variables of flow system and solid-phase reactor such as composition, size of particles and length of the reactor, were studied. The proposed method was applied for determining nitrazepam in two commercial tablets without any interferences.

KEY WORDS: Reverse flow injection, Nitrazepam, PbO_2 reactor, Oxidative coupling

INTRODUCTION

Nitrazepam (7-nitro-5-phenyl-1,3-dihydro-2H-1,4-benzo-diazepin-2-one, $C_{15}H_{11}N_3O_3$) belongs to the class of medications called benzodiazepines. It is used for treatments of sleeping (insomnia), early awakening during the night to manage myoclonic seizures [1]. Its therapeutic relevance and importance have encouraged the development of several methods for its determination in pharmaceutical dosage forms and biological samples.

Several methods are also reported for nitrazepam (NIT) determination such as reverse phase high pressure liquid chromatography (RP-HPLC) [2], liquid-liquid microextraction followed by HPLC [3], chemiluminescence [4], capillary electrophoresis [5], and ultra high performance liquid chromatography–tandem mass spectrometry [6, 7]. Moreover, new trends employing the spectrophotometric and flow injection analysis (FIA) procedures have been suggested for trace analysis of drugs [8-13]. It is still supplied an attractive features in routine analyses of drugs as an alternative of using an expensive instrumentations mentioned above. FIA gives many benefits, such as high precision, high sample throughput, and minimum interference. Among different types of FIA techniques, reverse flow injection analysis (rFIA) is used to reduce the consumption of reagent, minimized sample dispersion, and improve sensitivity.

The present work deals with a new strategy for exploiting the use of oxidant solid phase reactors [14] for the direct determination of different substances especially pharmaceuticals. The proposed single line flow injection manifold includes a reactor containing PbO_2 immobilized on inert support as an oxidant. On passage through the reactor, the reagent (2,2'-dihydroxybiphenyl) oxidized and then coupling with the sample (reduced drug) to form a blue complex monitored spectrophotometrically at 630 nm at the end of system.

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EXPERIMENTAL

A digital UV-Vis 260 double beam spectrophotometer (Shimadzu) was used in all absorbance measurements. A four channels peristaltic pump (Shenchen, LabV1, China) and 6-way injection valve (Knauer, Germany) provided with variable volumes were used to assemble the flow-injection manifold. The injection valve and the flow cell (50 µL) were connected through a Teflon reaction coil (0.5 mm i.d.). A simple one-line reverse flow manifold was used (Figure. 1). Through the injection valve, 2,2'-dihydroxybiphenyl (DHB) was injected into the stream of reduced NIT solution (using a plastic syringe) which was then oxidized through the PbO_2 solid phase reactor (length: 5.5 cm, particle size: 1 mm) and then mixed in a 100 cm reaction coil. The stream of solution were propelled using peristaltic pump at flow rate of 1.2 mL/min and finally the absorbance of the blue colored product was then monitored at 635 nm at the end of manifold.

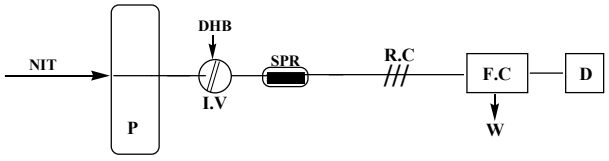


Figure 1. Diagram of the rFIA manifolds: p, peristaltic pump; I.V, injection valve; R.C, reaction coil; F.C, flow cell; D, detector; W, waste; SPR, solid phase reactor.

Materials and reagents

All reagents used were of analytical grade and double-distilled water was used throughout. Nitrazepam (NIT) was obtained from State Company for Drug Industries and Medical Appliances (SDI, Iraq). Commercial NIT tablets from several sources (Zipex ® 5 mg-Aburaihan Pharmaceutical Co., Tehran-Iran, Mogam® 5 mg-Domina Pharmaceuticals, Syria) were purchased from the local market, and were used as test tablets. 2,2'-Dihydroxybiphenyl (British Drug Houses, UK), cellulose acetate (BDH Chemicals Ltd., Poole, England), lead(IV) dioxide (Merck, Chemicals Ltd., Germany), zinc powder (Fluka, Buchs, Switzerland), hydrochloric acid (BDH), N,N-dimethyl formamide (BDH) and acetone (BDH) were used as received.

Preparation of reduced NIT solution. A 500 µg/mL of stock solution of reduced NIT was prepared by dissolving 0.05 g of NIT in 50 mL of ethanol, then transferred into a 150 mL beaker and a 20 mL of double-distilled water, 20 mL of HCl (11.7 N), and 3.0 g of zinc powder were added. The reduction mixture allowed to stand for about 15 min at ambient temperature and then the solution was filtered into a 100 mL volumetric flask. The residues were washed and diluted to the mark with double-distilled water. Simple dilution was used daily for prepared more diluted solutions.

DHB solutions (0.05 M). These solutions were daily prepared by dissolving 0.4655 g of DHB into 50 mL of ethanol and then transferred into brown bottles. Working standard solutions were prepared by serial dilutions of appropriate volumes of the standard stock solution with bidistilled water.

Immobilization of PbO_2 and preparation the reactor. The PbO_2 immobilization was made onto cellulose acetate (inert support) using dimethylformamide and aceton as catalyst, as reported by Zhang and Tang [15]. A 0.5 g of cellulose acetate (CAC) was dissolved in 0.5 mL dimethylformamide and 3 mL of acetone, and after manual homogenization, 4 g of lead dioxide powder was added gradually to the mixture and stirred until viscosity was increased. Then the

homogenous mixture was washed with distilled water, and a rigid solid was obtained and left to dry. After drying, the rigid polyester product was cut by scissors and the particle size was selected by passing the pulverized material through sieves with different mesh sizes. Glass tubes of different lengths (2 mm i.d.) were packed with particles which were hold inside the tubes by inserting small pieces of sponge into the ends of tubes. The solid-phase reactor was inserted in the flow system between injection valve and reaction coil.

Preparation the solution of NIT tablets. For approximately 20 tablets, the material from each tablet was accurately weighed, and estimated the mean weight of the contents of a single tablet. After that, the material was homogenised and an appropriate amount (equivalent to 50 mg of NIT) was sampled, dissolved in ethanol and filtered into a 50 mL volumetric flask. The solution diluted to the mark with ethanol to obtain 1000 µg/mL of NIT. The resultant solution was transferred into a beaker and was reduced as described previously. Working solutions were prepared by sampling a required volume and diluted with distilled water.

RESULTS AND DISCUSSION

Preliminary studies. Solid phase material was examined outside the flow manifold and the absorption spectra of the blue product and the blank were studied manually before automating the proposed reaction. In a 10 mL volumetric flask, 50 µg/mL concentration of reduced NIT, 1 mL of 0.05 M of DHB, and 0.1 g of immobilized PbO_2 were mixed and swirled. The Preliminary investigations proved that the blue product was formed immediately. After diluting the flasks with water and filtration the absorption spectra were recorded and the maximum value of absorption was measured at 635 nm versus the reagent blank (Figure 2).

In order to understand the mechanism of reaction, the stoichiometry of the reaction was studied using an equimolar concentration of NIT and DHP and applying the mole ratio method. The results indicate that 1:1 NIT:DHP ratio was obtained under this reaction. The amino group of NIT (which formed after reduction the nitro group with zinc), have a strong electron donating couples with DHP after oxidized by PbO_2. The proposed mechanism of the reaction is shown in Scheme 1 [16, 17].

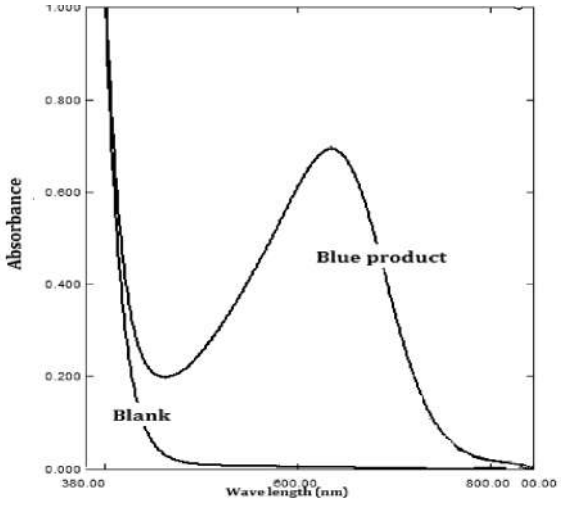
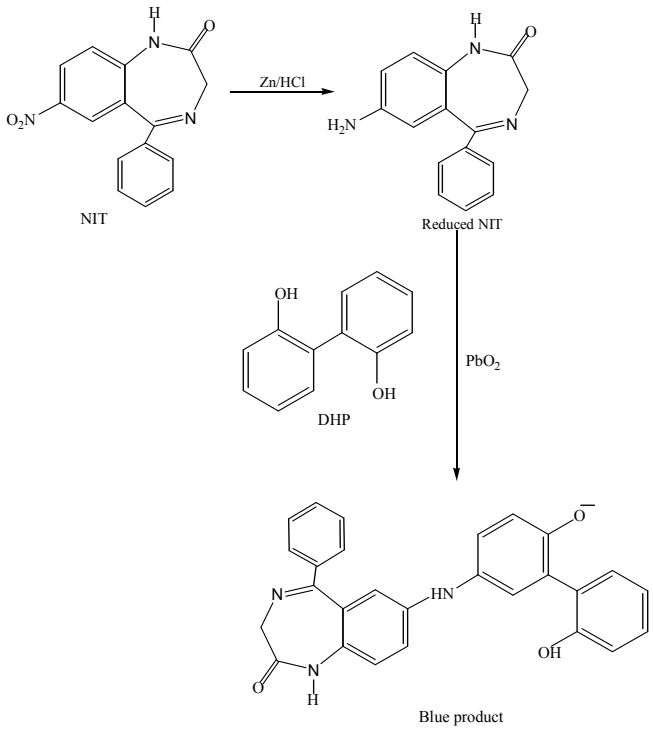


Figure 2. (a) The absorption spectra of 40 µg/mL of NIT measured against the reagent blank, as well as the reagent blank measured against distilled water.



Scheme 1. Proposed mechanism of the reaction between NIT and DHB.

Optimization of experimental variables. All the variables affecting in the performance of the suggested method were studied by the univariant method at a fixed NIT concentration 100 µg/mL. The optimum conditions were selected depending on the sensitivity, reproducibility and sampling rate. To obtain a stable response and decrease the effect of particle compaction in the reactor, the solid phase reactors were conditioned previously by pumping the carrier solution for 10 min before first injection. Reduced NIT was propelled by a peristaltic pump and the reagent (DHB) was injected three times for each experiment.

Selection of flow injection system and manifold. In order to obtain either a long lifetime of the solid phase reactor or a high sensitivity of the suggested method, a reverse flow system was used rather than normal system [14]. Reverse flow system minimizing the consumption of the oxidant (immobilizing PbO_2) as a result of continuous passing of the reagent (DHB) through the reactor. Different flow manifolds (single and double line) were examined to obtain different reaction paths. Several solutions were investigated as carriers such as distilled water and acids. The results indicated that the manifold in Figure 1 gave the best absorbance and minimum dispersion and was thus chosen for further investigation. A single-channel rFIA manifold was adopted. The new reagent (2,2'-dihydroxybiphenyl) was injected inside the stream of the reduced NIT solution using the injection valve, which was then oxidized by SPR-PbO_2 and then mixed in the reaction coil. Finally, the absorbance of blue product was measured at 635 nm through the detector.

Solid-phase reactor parameters. The performance of solid-phase reactors can be affected by several parameters such as the reactor composition, length of reactor, degree of backing and particle size. In order to obtain the best results for the proposed method, these factors were evaluated and studied. The preparation of PbO_2 solid-phase reactors were evaluated for four different ratios of PbO_2/CAC: 4/0.25, 4/0.5, 2/0.25, and 6/0.25 (w/w, g). The results show that the reactor prepared with ratio of 4/0.5 showed the highest sensitivity and was adopted as satisfactory.

Usually a small amount of CAC must be avoided to ensure efficient immobilization of PbO_2 and to reduce the partial solubility of solid particles during the continuous flow of solution. Three particles sizes range of 0.3-1.18 mm, were selected by sieving the solid material. The reactors were filled with different sizes but the same weight of 0.17 g which caused differences in particles packing that may be create an extra spaces over time in the case of small particles. With these spaces usually the dispersion will increased. The best results in terms of sensitivity and reproducibility were obtained with particle size of 1 mm.

The influence of the reactor length was also studied in the range of 3.5-8 cm. Figure 3 shows that absorbance signals increased gradually with the increase of the column length up to 5.5 cm and then the signal decreased. A reactor length of 5.5 cm was selected as it gave high absorbance with good precision, in addition the reactors length greater than 5.5 cm allowed the increase in the hydrodynamic pressure that affected on the flow rate and analytical response.

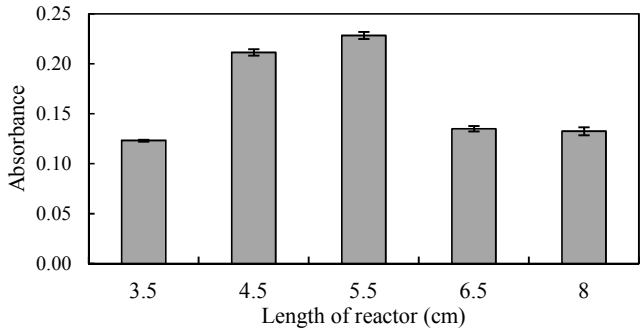


Figure 3. Effect of the length of reactor.

The degree of packing of the reactor affects the column efficiency. Strong packing of PbO_2 in the reactor (> 0.21 g in this case) must be avoided to reduce resistance against the flowing of carrier solution propelled by the pump. For the optimum reactor of 5.5 cm: length and 1 mm: particles size, the degree of packing was evaluated using different amounts of pulverized material. The obtained results show that 0.21 g of the solid-polymer gave the highest absorbance and good reproducibility.

Flow injection parameters. The effect of flow variables such as concentration of DHB, sampling loop, flow rate, and reactor coil length were studied depend on compromise absorbance intensity, repeatability, rate of sampling, and baseline stability.

Effect of DHB concentration. The effect of varying DHB concentration in the range 0.005 to 0.04 M was explored. DHB concentration up to 0.02 M gradually increased the absorbance (analytical signal). Further increase in the DHB concentration resulted in a slight decrease in the signal. Results obtained indicated that the concentration of 0.02 M gave the highest sensitivity and good repeatability and was thus chosen for the next experiments.

Effect of the total flow rate. The effect of total flow rate on analytical signals was examined using different flow rates. The highest absorbance and precision were obtained with total flow of 1.2 mL/min, beyond this value the absorbance was decreased due to the decrease in the contact time between the injected sample and the lead dioxide particles in the reactor. As a result, 1.2 mL/min was selected as the optimum rate.

Effect of the mixing coil length. The influence of the coil lengths from 50-200 cm was tested. The maximum absorbance and good repeatability were obtained with a 100-cm reaction coil length, therefore this length was selected for further experiments. The absorbance of the color product decreased constantly with an increase of coil length, due to the effect of sample dispersion.

Effect of injection volume. The effect of injection volume on the analytical signal was investigated in the range 65-170 µL. A 75 µL volume gave the best response and precision and was used in the following experiments.

Reactor life-time and stability. After optimizing all the manifold variables, the repeatability and long-term stability of the SPR were investigated by measuring the absorbance of the product several times at optimum conditions. It was found that the pulverized material of SPR was still stable more than one month. The prepared reactors gave reproducible results for at least 33 successive injections with a variation less than 2.3, with a life time greater than 38 injections (RSD ≤ 5). In addition the time consumed for appearance of maximum absorbance after each injection was 1.5 min. As a result, the analytical frequency (sampling rate) was 40 samples per hour.

Calibration graph and analytical features. Under the optimum conditions, a linear analytical response (r = 0.9995) within 10-300 µg/mL NIT concentration range was obtained with y = 0.0029x + 0.0118, where Y is the absorbance and X is the NIT concentration in µg/mL. Limit of detection "three times the standard deviation of the blank/slope of the analytical curve" was 4.5 µg/mL. The figure of merits and the statistical treatments are summarized in Table 1.

Table 1. Statistics and analytical values of the calibration graph.

Parameter	Value
Regression equation	y = 0.0029x + 0.0118
Correlation coefficient, r	0.9995
Linearity percentage, r^2%	99.90
Dynamic range (µg/mL)	10-300
Slope, b (mL/µg)	2.86×10^{-3}
Intercept, a	1.18×10^{-2}
Standard deviation of the residuals, $S_{y/x}$	9.68×10^{-3}
Standard deviation of the slope, S_b	3.33×10^{-5}
Standard deviation of the intercept, S_a	5.37×10^{-3}
Sample through-put (h^{-1})	40
LOD (µg/mL)	4.47
LOQ (µg/mL)	14.9

Table 2. Accuracy and precision of the proposed method.

Sample	Conc. (µg/mL) of NIT		RE (%)*	Rec. (%)*	RSD(%)*
	Added	Found			
1	100	99.3	-0.70	99.3	1.7
2	150	147	-2.3	97.7	2.9

*Average of three determinations.

The precision of the rFIA method was established by repeated runs of two concentration of NIT solution (five replicates). The relative standard deviation values (< 2.9%) indicated that the proposed method is highly precise (Table 2).

Interference study. The probable interferences that may affect on the estimation of NIT caused by common excipients used in pharmaceutical tablets were studied. The analytical signal produced by a 100 µg/mL standard solution of NIT is compared with that of a similar concentration of NIT solution with additions of five-fold (talc, poly vinyl pyrrolidone, magnesium stearate, starch) of each one of these excipients. The results show that all experienced interferences have insignificant effect on the determination of NIT and the proposed method has good selectivity.

Applications. The developed SPR-rFIA system was applied for determination of NIT in two types of pharmaceutical tablets commercially available, and results were compared to those obtained by applying standard IP (UV method) method [18]; the results obtained are shown in Table 3. The application of the statistical t- and F-test [19, 20] (95% confidence level) indicated insignificant differences between two methods and within an acceptable range of error, proving the accuracy of the proposed method using SPR.

Table 3. Application of the SPR-rFIA and official methods for determination of NIT in tablets.

Pharmaceutical form	Proposed method					Official method				
	Taken conc. (µg/mL)	Found conc. (µg/mL)	Rec. (%)[a]	Mean Rec. (%)	RSD (%)[a]	Taken conc. (µg/mL)	Found conc. (µg/mL)	Rec. (%)[a]	Mean Rec. (%)	RSD (%)[a]
Zipex® Tablets (5 mg Nitrazepam)	100	98.3	98.3	99.0	1.39	15	15.1	100.7	100.9	2.12
	150	149.5	99.7		1.36	20	20.2	101.0		0.81
Mogam® Tablets (5 mg Nitrazepam)	100	97.8	97.8	98.5	2.29	15	15.4	102.7	101.1	1.15
	150	148.8	99.2		1.29	20	19.9	99.5		2.89
Pure NIT				98.5					99.3	
t (2.78)[b]	1.66	$(n_1 - 1) = 1$, $(n_2 - 1) = 1$, $(n_1 + n_2 - 2) = 2$								
F (19.0)[b]	11.8									

[a]Average of five determinations; [b]Theoretical value; Conc., concentration; RSD, relative standard deviation.

CONCLUSION

The present work involved proposed a new spectrophotometric-reverse flow injection method combined with solid phase reactor for determination of NIT. A new reagent (2,2'-dihydroxybiphenyl) used for the first time in analysis of drugs have been used as coupling reagent. The proposed FIA method shown to be sensitive, as indicated by the detection limit (4.5 µg/mL) of NIT, in addition the method applied effectively in determination of NIT in tablets. The reproducibility of PbO_2 reactor, and the stability of analytical signal with simplicity of the single line manifold, makes it an attractive alternative for estimation of NIT in a flow system.

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