Spectrophotometric and Reverse Flow Injection Method Determination of Nitrazepam in Pharmaceuticals Using O-Coumaric Acid as a New Chromogenic Reagent

Sadeem Subhi Abed

Abstract:

A spectrophotometric- reverse flow injection analysis (rFIA) method has been proposed for the determination of Nitrazepam (NIT) in pure and pharmaceutical preparations. The method is based upon the coupling reaction of NIT with a new reagent O-Coumaric acid (OCA) in the presence of sodium periodate in an aqueous solution. The blue color product was measured at 632 nm. The variation (chemical and physical parameters) related with reverse flow system were estimated. The linearity was over the range 15 - 450 μg/mL of NIT with detection limits and limit of quantification of 3.425 and 11.417 μg mL^{-1} NIT,respectively. The sample throughput of 28 samples per hour was achieved. Suggested method was successfully applied for the determination of NIT in its dosage forms.

Key words: Chromogenic reagent, Nitrazepam, O- Coumaric acid, Reverse flow injection method

Introduction:

Nitrazepam (NIT) (7-nitro-5-phenyl-1H-benzo[e][1,4]diazin-2(3H)-one, M. wt**.** =281.3 g/mol**)**, is a hypotic agent that belongs to the benzodiapine class. NIT is used for healing the sedative effects of insomnia (1), as well as anxiolytic, amnestic, anticovulsant and skeletal muscle relaxant properties. In addition, it has been used in the treatment of stress associated with disorders (2,3). Nitrazepam chemical structure contains an important nitro group (Fig.1). Nitro group plays an important function in the action of these drugs, and the nitro group or even other electron attracting functional is necessary for specific desired biological effect.

Figure 1. Nitrazepam chemical structure ($C_{15}H_{11}N_3O_3$)

Department of Chemistry, College of Science, University of Baghdad, Baghdad, Iraq
E-mail: sadeem.s@sc.uobaghdad.edu.iq
[*]ORCID ID:0000-0001-9606-1972

Different methods have been reported for NIT determination in various samples, including silver nanoparticle enhanced chemiluminescence method (4), liquid-liquid micro-extraction followed by HPLC (5), RP-HPLC(6), UFLC- mass spectrometry (7), capillary electrophoresis(8), multi wavelength spectrophotometry (9), micellar electrokinetic capillary chromatography (10), thin layer chromatography-densitometry(11), gas chromatography-mass spectrometry(12), and HPLC-voltammetry(13). Also various spectrophotometric methods have been reported for determination of NIT, after reduction by Zn/HCl, such as coupling with different reagents namely, N-(1-napthyl)-ethylene diamine dihydrochloride(14), m-aminophenol(15), acetyl acetone(16), vanillin (17), Metol (18), and phloroglucinol(19). Among the different techniques, the spectrophotometric and flow injection analysis methods are considered the most popular and simple methods existing for quick and trace analysis of different pharmaceutical forms (20-22). One of the flow injection analysis modes is reverse flow injection analysis (rFIA), in which the reagent is injected into the sample stream which is reversed to the usual FIA when the sample is injected. The main advantages of rFIA mode are the minimization of reagent consumption and waste disposal, improving the analytical sensitivity by enhancing mixing efficiency, and the possibility of making several different determination on the same

sample solution stream by injection of different reagents (23,24).

The present investigation method offers an improvement of the simple spectrophotometric-rFI technique for the estimation of NIT in both pure and pharmaceutical forms as tablet depending on the oxidative coupling reaction between NIT and O-Coumaric acid as a new chromogenic agent in the presence of sodium periodate in an aqueous medium to produce a blue-colored product.

Materials and Methods:
Tools
• A Shimadzu UV–VIS 260 double beam spectrophotometer (Shimadzu, Kyoto-Japan) had been used for absorbance measurements in FIA procedure. The absorbance measurements were carried out using 1 cm path length of quartz flow matched cells (Cecil, 50 µL internal volume).
• A peristaltic pump of six channels (SHENCHEN, CHINA) was used for impelling the reagents and sample solutions using flexible polyvinyl chloride tubes of (0.8 mm i.d.).
• The injection valve of 6 - ways (KNAUER, Germany) with various sample loops was used to injected sample solutions.
• Flexible Teflon tubes of (0.5 mm i.d.) were used for reaction coils with different lengths. And T - links were used to mix streams of reagents.

For rFIA a two-channel manifold was used (Fig. 2), first channel used to transported the solution of (NIT). And the second channel was used to flow sodium periodate solution, while the reagent O-Coumaric acid (OCA) was injected through the injected valve then it was mixed with the stream of drug and oxidant and the mixing completed in the reaction coil. The blue colored was measured at 632 nm.
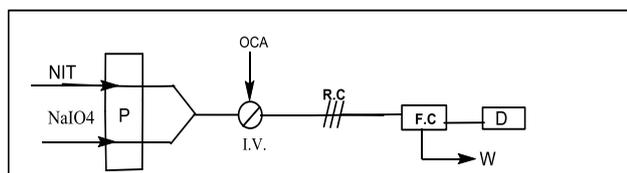
Figure 2. The manifold used for rFIA method for determination of NIT by reaction with OCA in presence of NaIO4, where: IV, injection velve; RC, reaction coil; P, peristaltic pump; FC, flow cell; D, detector; W, waste.

Chemicals and Reagents
All chemicals used were analytical grade reagents, and distilled water was used for the work. NIT Pharmaceutical grade was supplied from SDI, Samara-Iraq. Pharmaceutical dosage forms

containing NIT (Zipex® 5 mg, Aburaihan Pharmaceutical Co., Tehran, Iran) were obtained from local commercial sources.

NIT Solution (500 µg mL^{-1})
The reduction solution of NIT was prepared by dissolving 0.0500 g of NIT in 50 mL of ethanol. Then it was transferred into a beaker and 20 mL of distilled water, 20 mL of 11.65 N hydrochloric acid, and 3.0 g of zinc powder were added. The mixture was allowed to stand for about 15 min at room temperature (25°C). The solution was filtered into a 100 mL volumetric flask, and the residues were washed and diluted to the mark with D.W. Simple dilution was used daily to prepare further diluted solutions.

OCA Solutions (0.05 M)
The solution was prepared daily by diluting 0.8220g of OCA with 100 mL of D.W and then transferred into brown bottle for keeping it out from light. The diluting solutions were prepared with distilled water.

Sodium Periodate NaIO$_4$ (0.03 M)
This solution was prepared by diluting 0.6416 g of NaIO$_4$ with100 mL D.W. Working solutions was prepared by suitable dilution by using the same solvent.

Pharmaceutical Solutions
10 tablets were accurately weighed and powdered. Then, an amount of powder equivalent to 50 mg of NIT was dissolved in approximately 30 mL of ethanol. This solution was filtered into a 50 mL volumetric flask and then washed and diluted to the mark with ethanol to obtain 500 µg mL^{-1} of NIT. The resultant solution was transferred into 125 mL beaker and was reduced as described above. The appropriately diluted solutions of the medicinal formulations were made with distilled water.

Results and Discussion:
Preliminary studies
The absorption spectra of the product and the blank were obtained. In a 10-mL volumetric flask a 25 µg mL^{-1} concentration of reduced NIT, 1 mL of 0.05 M of OCA, and 2 mL of 0.03M NaIO$_4$ were mixed and the blue -colored product was formed immediately. The flasks were made up to the mark with distilled water. The absorption spectra were recorded between 380 and 1000 nm. The maximum value of absorption was dignified at 632 nm versus the reagent blank (Fig. 3).
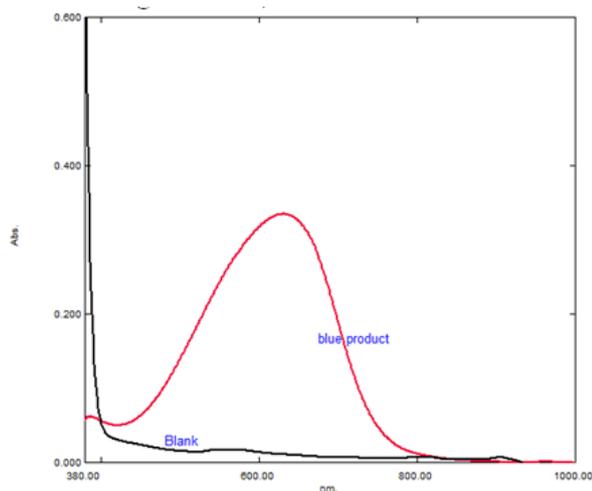
Figure 3. The absorbance spectrum of blue colored product of 25 µg mL^{-1} of NIT measured versus reagent blank, and the blank versus D.W

After studying the reaction stoichiometry of reaction in a batch method using an equimolar

concentration of NIT and OCA (0.00177M for both) and applying the mole ratio method and jobs method, it was found that a 1:1 NIT: OCA ratio was obtained. The mechanism of the reaction was summarized by reduction the nitro group of NIT compound in first step (using zinc powder with concentrated HCl) followed by oxidative coupling between reduced drug and OCA in the presence of NaIO$_4$ (25) as oxidizing agent as shown in Scheme1.The conditional stability constant of the blue colored product was 2914.092M^{-1}. The Gibbs free energy (ΔG)(26) of the reaction was also determinate to indicate the spontaneously of the reaction ΔG was calculated using the equation (ΔG= -2.303RT log K), where T is the absolute temperature (273+25°C), R is the universal gas constant (19.767 J/mol k). The negative value of ΔG refers to the spontaneously of the current reaction.

Shceme1. The probable reaction mechanism

Optimization of rFIA System (Chemical and Physical Parameters)

In order to enhance the conditions of the method, preliminary studies were directed toward optimization of the experimental conditions, so as to select the most favorable parameters for the

reaction, the absorbance was measured at a wavelength of 632 nm against reagent blank. The preliminary chemical and physical parameters are listed in Table1; the reagent was injected three times for each experiment.

Table 1. The preliminary experimental conditions for proposed rFIA

Parameters	Value
Conc. of NIT	100 μg mL^{-1}
Conc. of OCA	0.003 M
Conc. of NaIO$_4$	0.01 M
Total flow rate	2.0 mL/ min
Sample loop	100 μL
Reaction coil	50 cm

Effect of Chemical Parameters

Different concentrations of the reagent (OCA) was injected (from 0.0005 to 0.15M) to choose the best concentration of the reagent. The results indicated that the concentration of 0.005 M of OCA offered the highest absorbance (Fig. 4). Different concentration of sodium periodate was also studied and optimized using the concentration range between (0.005 to 0.06 M), the concentration of 0.03 M showed the higher absorbance and therefore, it was used in all subsequent experiments (Fig. 5).
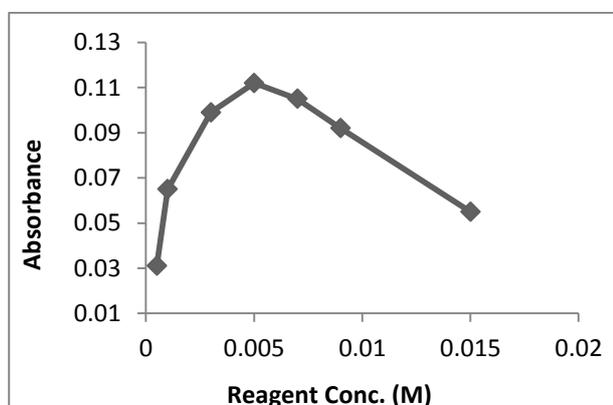
Figure 4. Effect of the concentration of reagent
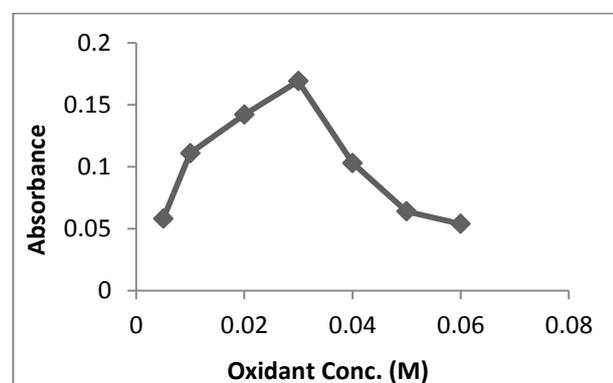
Figure 5. Effect of the concentration of oxidant

Effect of Physical Parameters

The reaction coil lengths effect was examined using variable reaction coils lengths ranged from 0(i.e. without reaction coil) to 250 cm and as shown in Fig. 6, the maximum absorbance and good repeatability were obtained when reaction coil

length of 150 cm used. The higher length of reaction coils caused an increase in the dispersion.

The flow rate between 0.4 and 4.1 mL/min was also studied. As the flow rate increased, the absorbance also increased up to 1.8 mL/min (Fig. 7). Increasing the flow rate leads to the absorbance decrease, and this can be attributed to the dispersion. As a result, 1.8 mL/min was selected as the optimum rate.

The injected reagent volume was examined by varying the sample loop in the range from 75 – 250 μL. Above 100 μL, the absorbance decreased due to the high reagent to sample volume ratio which leads to insufficient ratio and the sample loop of 100 μL was the optimum loop in this work and it was used in further experiments (Fig. 8).

The sampling rate was calculated by recording the period from the injected instant to the appearance of maximum absorbance. The sampling frequency under the optimum conditions (Table 2) was 28 sample per hour (128 second for each sample).
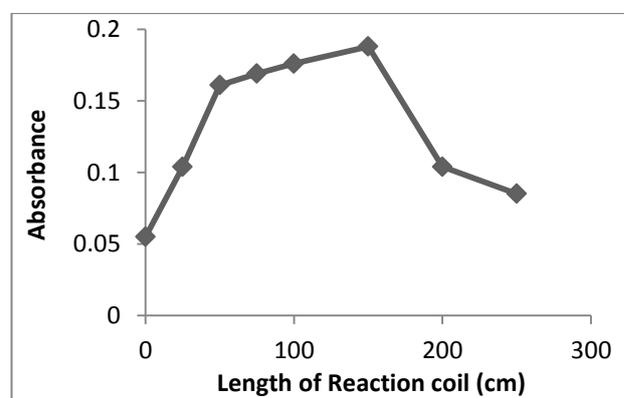
Figure 6. Effect of the reaction coil length
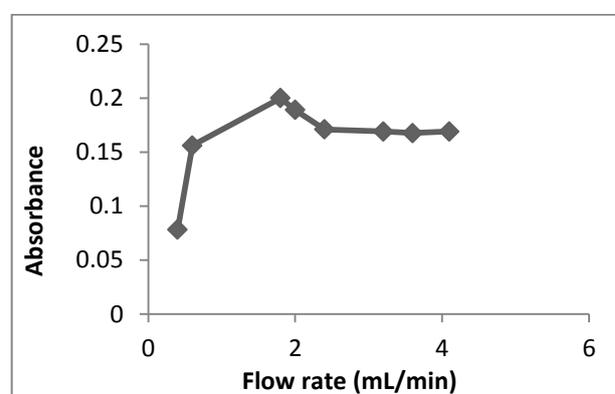
Figure 7. Effect of the total flow rate
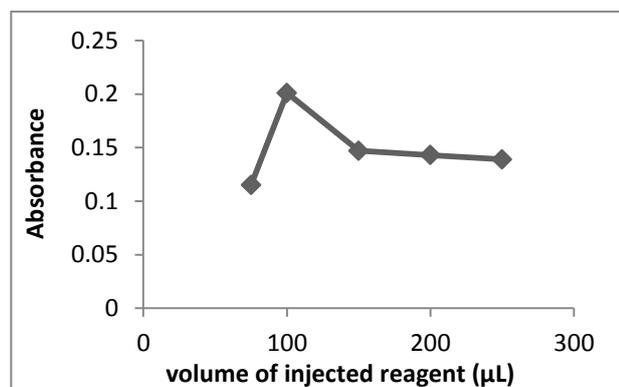
Figure 8. Effect of the volume of injected reagent

Table 2. The optimum experimental conditions for the proposed rFIA system

Parameters	Value
Conc. of OCA	0.005 M
Conc. of $NaIO_4$	0.03 M
Total flow rate	1.8 mL/ min
Sample loop	100 μL
Reaction coil	150 cm

Calibration Curve, Limit of Detection , Accuracy and Precision

A linear calibration curve was achieved over the range 15-450 μg mL^{-1} of NIT with a limit of detection (LOD) of 3.425μg mL^{-1} and limit of quantification (LOQ) of 11.417μg mL^{-1} under the optimal conditions. Table 3 summarizes the statistical analytical treatments for the curve and analytical values, the value of correlation coefficient indicated the high linearity of the suggested method.

To establish the accuracy and precision of the suggested rFIA technique, three different concentrations (five replicates) of NIT were analyzed. The small values of RSD% and Erel% indicated that the method provided good accuracy and repeatability (Table 4).

Table 3. Statistical analysis and analytical data of the calibration curve of NIT.

Parameter	Value
Regression equation	y= 0.0015x- 0.0501
Slope, b (μg mL^{-1})	0.0015
Intercept, a	0.050115
Correlation coefficient, r	0.9996
Linearity percentage, r^2%	99.92
Linearity range (μg mL^{-1})	15- 450
S_b, standard deviation of the slope	0.0000125
S_a, standard deviation of the intercept	0.00291337
$S_{y/x}$, standard deviation of the residuals	0.005966
Limit of quantification, (μg mL^{-1})	11.417
Limit of detection, μg mL^{-1}), $\hat{y}=y_B+3SD_B$	0.0215
Limit of detection, (μg mL^{-1})	3.425
Sandell's sensitivity, S (μg cm^{-2})	0.6666
Sample frequency (sample/hour)	28

Table 4: the precision and accuracy of the rFIA suggested method.

Conc. of NIT μg mL^{-1}		Erel %	Rec. %	RSD %
Present	Found*			
50	50.6000	+ 1.200	101.200	1.2548
100	101.2667	- 1.2667	101.266	1.8850
250	247.2667	- 1.0933	98.906	0.5311

*Average of five determinations

To assess the efficiency of the method and avoid the restrictions from interference by tablet additives, the determinate of NIT was accomplished by using the standard addition method at two different concentration and the results in Table 5 indicated the high accuracy and precision of the method.

Table 5. The accuracy and precision using standard addition method

Pharmaceutical form	Conc. of NIT μg mL^{-1}		Erel %	Rec. %	RSD %
	Present	Found*			
Zipex® tablets (5 mg NIT	100	101.400	+ 1.400	99.400	2.467
	250	245.555	- 1.778	98.222	0.998

*Average of five determinations

Analytical Applications

The rFIA method was effectively applied for the determination of NIT in commercial pharmaceuticals by the analysis of two different concentrations directly, and the results are given in Table 6. The recoveries of NIT were between 100.693to101.137% which revealed that the current method is accurate and the relative standard deviation was between 0.636to 1.504% which revealed that the current method is repeatable for the determination of NIT in commercial pharmaceuticals. The results obtained from the suggested method were in comparison with those obtained by applying the standard method (UV

method) (1).The results of the recovery test were compared with those resulting from using classical UV-method (1) using "Student's *t*-test and (variance ratio) F- test" at 95% confidence level. The

calculated *t* and F - values were less than the theoretical values (Table 7) which indicated that there is no significant difference between both methods.

Table 6. Direct application of the suggested method for the determination of NIT in pharmaceutical forms.

Pharmaceutical form	Conc. of NIT μg mL^{-1}		Erel %	Rec. %	RSD %
	Present	Found*			
Zipex® tablets (5 mg	100	101.1374	+1.137	101.137	1.504
NIT)	250	251.7321	+ 0.693	100.693	0.636

*Average of five determinations

Table 7. the evaluation of the official UV method with the proposed method.

Preparation form	Proposed method	official UV method
	Recovery % (average)	
NIT(pure)	100.4578	100.5417
NIT capsule 5mg	100.9151	101.2417
S^2	0.10456	0.2450
t* (4.303)	0.4909	
F*(161.4)	2.3431	

*theoretical value, t has degree of freedom = $n_1 + n_2 - 2 = 2$, F has degree of freedom = $n_1 - 1 = 1$.

Conclusion:

The proposed rFIA method found to be sensitive, simple, low cost, and offers the advantages of the rapidity with wide linearity range and high sampling rate. OCA is an excellent choice as reagent due to its readily available and sensitivity In addition, the proposed method is applicable for determinate NIT in pharmaceutical formulations in microgram concentration level. Moreover, the method did not need extraction or heating steps and FIA instruments are available in most laboratories.

Conflicts of Interest: None.

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طريقة الحقن الجرياني العكوس والتقدير الطيفي للنايترازيبام في المستحضرات الصيدلانية باستخدام اورثو حامض الكوماريك ككاشف كروموجيني جديد

سديم صبحي عبد

قسم الكيمياء ، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة:

تم اقتراح طريقة طيفية-والتحليل بالحقن الجرياني العكوس لتقدير النايترازيبام في المستحضرات النقية والصيدلانية . تعتمد الطريقة على تفاعل الازدواج للنايترازيبام مع كاشف جديد اورثو حامض الكوماريك بوجود بيرايودات الصوديوم في المحلول المائي. تم قياس الناتج الذائب ذو اللون الازرق عند 632نم. تم الاخذ بنظر الاعتبار المتطلبات الكيميائية والفيزيائية المتعلقة بمنظومة الجريان العكوس ،وعند الظروف المثلى كانت الخطية ضمن المدى(450-15) مايكروغرام لكل مل من النايترازيبام وكانت قيم كل من حد الكشف وحد الكمية عند 3.425 و 11.417 مايكروغرام لكل مل نايترازيبام، وكان معدل النمذجة 28نموذج بالساعة. طبقت الطريقة بنجاح لتقدير النايترازيبام في المستحضرات.

الكلمات المفتاحية: كاشف لوني، نايترازيبام، اورثو- حامض كوماريك، طريقة الحقن الجرياني العكوس

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