



ORIGINAL ARTICLE

Validated stability indicating methods for determination of nitazoxanide in presence of its degradation products[☆]

Nouruddin W. Ali^a, Samah Sayed Abbas^b, Hala El-Sayed Zaazaa^b,
Maha Mohamed Abdelrahman^{a,*}, Mohamed Abdelkawy^b

^aAnalytical Chemistry Department, Faculty of Pharmacy, Beni-Suef University, Hussin El-Shafey Street, Beni-Suef City, Egypt

^bAnalytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, 11562, Cairo, Egypt

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Abstract Three sensitive, selective and reproducible stability-indicating methods are presented for determination of nitazoxanide (NTZ), a new anti-protozoal drug, in presence of its degradation products. Method A utilizes the first derivative of ratio spectra spectrophotometry by measurement of the amplitude at 364.4 nm using one of the degradation products as a divisor. Method B is a chemometric-assisted spectrophotometry, where principal component regression (PCR) and partial least squares (PLS) were applied. These two approaches were successfully applied to quantify NTZ in presence of degradation products using the information included in the absorption spectra in the range 260–360 nm. Method C is based on the separation of NTZ from its degradation products followed by densitometric measurement of the bands at 254 nm. The separation was carried out on silica gel 60F₂₅₄, using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH = 5.80) as a developing system. These methods are suitable as stability-indicating methods for the determination of NTZ in presence of its degradation products either in bulk powder or in pharmaceutical formulations. Statistical analysis of the results has been carried out revealing high accuracy and good precision.

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[☆]This work has been done at Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt.

*Corresponding author. Tel.: +20 141418206; fax: +20 82 2317950.

E-mail address: maha_m_abdelrahman@yahoo.com
(M.M. Abdelrahman)

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1. Introduction

Nitazoxanide (NTZ), as shown in Fig. 1, is a nitrothiazole benzamide compound that has a wide range of antimicrobial activity against parasites and bacterial pathogens [1]. It is chemically designated as N-(5-nitro-2-thiazolyl) salicylamide acetate [2]. It is used for the treatment of cryptosporidiosis and giardiasis in immuno-competent patients. It is absorbed from the gastrointestinal tract following oral administration and is rapidly hydrolyzed to an active desacetyl metabolite (tizoxanide) [3–6].

Nitazoxanide is not official in any Pharmacopoeia. Few methods have been reported for the quantitative estimation of NTZ, including spectrophotometric either alone or in



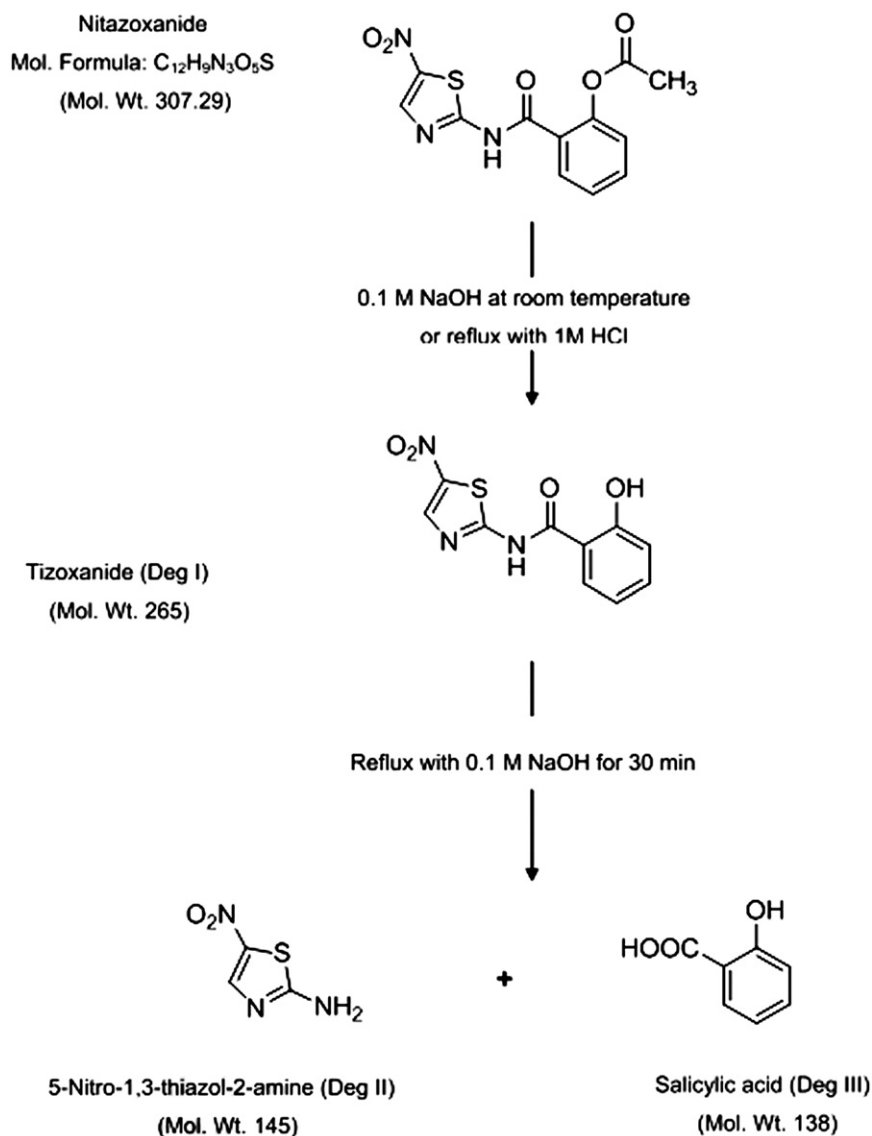


Figure 1 Degradation pathway of nitazoxanide.

combination [7–11], colorimetric [12–14] and liquid chromatographic [15–23] methods.

Literature survey revealed different stability indicating methods performed on NTZ, but up to our knowledge, no data on the structure of degradation products were reported.

An exhaustive study on the stability of NTZ is demanding as the current ICH guidelines requires that stability study should be done using developed and validated stability-indicating assay methods after stress testing on the drug under a variety of conditions [24].

The aim of this work was to develop a comparative study of recent, simple, sensitive, validated stability indicating methods that are of lower cost than the reported HPLC method. These methods include spectrophotometric, chemometric and TLC-densitometric ones for the determination of NTZ in presence of its three degradation products in pure form, laboratory prepared mixtures and in its pharmaceutical formulations.

2. Experimental

2.1. Instruments

× A double beam UV/VIS spectrophotometer (Shimadzu, Japan) model UV-1601 PC with a quartz cell of 1 cm path length. The spectral band width is 2 nm and the wavelength-scanning speed is 2800 nm/min. All data analysis was performed using PLS-Toolbox 2.0 running under MATLAB[®], version 6.5 [25].

× TLC scanner 3 densitometer (Camag, Muttenz, Switzerland).

The following requirements are taken into consideration:

- Slit dimensions: 5 × 0.2 mm
- Scanning speed: 20 mm/s
- Spraying rate: 10 s/μL
- Data resolution: 100 μm/step
- Band width: 6 mm
- Result output: Chromatogram and integrated peak area

- × A sample applicator for TLC Linomat IV with 100 μ L syringe (Camag, Muttenz, Switzerland).
- × An IR spectrophotometer (Shimadzu 435, Kyoto, Japan); sampling was undertaken as potassium bromide disks.
- × A mass spectrophotometer: MS-QB 1000 EX, Finnigan Nat (USA).
- × TLC aluminum plates (20 \times 20 cm) coated with 0.25 mm silica gel 60F₂₅₄ (Merck, Germany).
- × UV lamp with short wavelength 254 nm (USA).

2.2. Samples

2.2.1. Pure standard

NTZ was kindly supplied from Copad Pharma Co., Cairo, Egypt. Its purity was found to be 99.90%, according to a reported HPLC method [23]. Salicylic acid was obtained from El-Nasr Pharm. Co., Cairo, Egypt.

2.2.2. Pharmaceutical formulations

- × Nit Clean[®] tablets (Batch No. 10005) are labeled to contain 500 mg of NTZ manufactured by Western Pharmaceutical Industries Co., El-Obour City, Egypt.
- × Nitazod[®] powder for oral suspension (Batch No. 92456) is labeled to contain 100 mg/5 mL NTZ manufactured by Sigma Pharmaceutical Industries Co. for Al Andalous Medical Co., Cairo, Egypt.
- × Cryptonaz[®] powder for oral suspension (Batch No. 0906189) is labeled to contain 100 mg/5 mL NTZ manufactured by ADWIA Co. for Copad Pharma Co., Cairo, Egypt.

2.2.3. Degraded sample

2.2.3.1. Preparation of degradation product in acid-stress condition. The degradation product under acid stress condition was laboratory prepared by dissolving 100 mg of pure NTZ in the least amount of methanol, re using with 100 mL of 1 M HCl solution for 3 h. Follow complete degradation of NTZ by TLC plates of silica gel 60F₂₅₄ using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system where the spot of NTZ disappeared and one new spot appeared. Filter the formed precipitate (Deg I), wash with distilled water (4 \times 30 mL), transfer to a at bottom dish and allow drying at room temperature. Structure elucidation was conducted by IR and mass spectroscopy.

2.2.3.2. Preparation of degradation products in alkaline-stress condition. Re ux 100 mg of pure NTZ with 100 mL of 0.1 M NaOH solution for 30 min. Follow degradation by TLC using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system. At the beginning, a degradation product similar to that obtained under acid stress condition appeared and then rapidly disappeared followed by the formation of two new spots of the final degradation products. After complete degradation; extract the solution several times (4 \times 30 mL) with ether (to obtain Deg II) and then neutralize the solution with 0.1 M HCl solution and extract several times with ether (4 \times 30 mL) (to obtain Deg III). Evaporate the basic and acidic extracts. The

separated degradation products were subjected to IR and mass spectral analyses for subsequent identification.

2.3. Chemicals and reagents

All chemicals used throughout this work were of analytical grade and the solvents were of spectroscopic grade.

- × Methanol (E.Merck, Germany).
- × Sodium hydroxide (0.1 M aqueous solution) and hydrochloric acid (0.1 M and 1 M aqueous solutions) (El-Nasr Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt).
- × Methanol, chloroform, ammonia solution (Specific gravity 0.91, 33%) and glacial acetic acid (El-Nasr Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt).

2.4. Standard solutions

2.4.1. Stock standard solutions of nitazoxanide and its degradation products (1 mg/mL)

Weigh accurately 0.1 g of each of NTZ, Deg I, Deg II and Deg III into four separate 100-mL volumetric asks, dissolve in and dilute to the volume with methanol.

2.4.2. Working standard solutions of nitazoxanide and its degradation products (100 μ g/mL)

Transfer accurately 10 mL from NTZ, Deg I, Deg II and Deg III stock solutions into four separate 100-mL volumetric asks, complete to the mark with methanol to obtain 100 μ g/mL working standard solutions.

2.5. Laboratory prepared mixtures

Transfer different aliquots of NTZ from its working standard solution (100 μ g/mL) and then add aliquots of its degradation products, Deg I, Deg II and Deg III, from their respective working standard solutions (100 μ g/mL) to prepare mixtures of different ratios of NTZ and its degradation products containing 10%–90% of the degradation products.

2.6. Procedure

2.6.1. Spectral characteristics of nitazoxanide and its degradation products

Transfer accurately and separately, aliquots equivalent to 10, 10, 5 and 5 μ g from NTZ, Deg I, Deg II and Deg III working solutions (100 μ g/mL in methanol), respectively, into 10-mL volumetric asks. Complete to the volume with methanol. Record the absorption spectrum of each solution over the range of 200–500 nm using methanol as a blank.

2.6.2. Construction of calibration graph for first derivative of ratio spectra spectrophotometric method (DD^1)

Transfer accurately aliquots equivalent to 20–240 μ g of NTZ from its working standard solution (100 μ g/mL) into a series of 10-mL volumetric asks, complete to the volume with methanol. Record the absorption spectra of NTZ concentrations against methanol as a blank. Divide NTZ series by the chosen divisor (5 μ g/mL of Deg I). Record the first derivative of the obtained ratio spectra using scaling factor=10 and

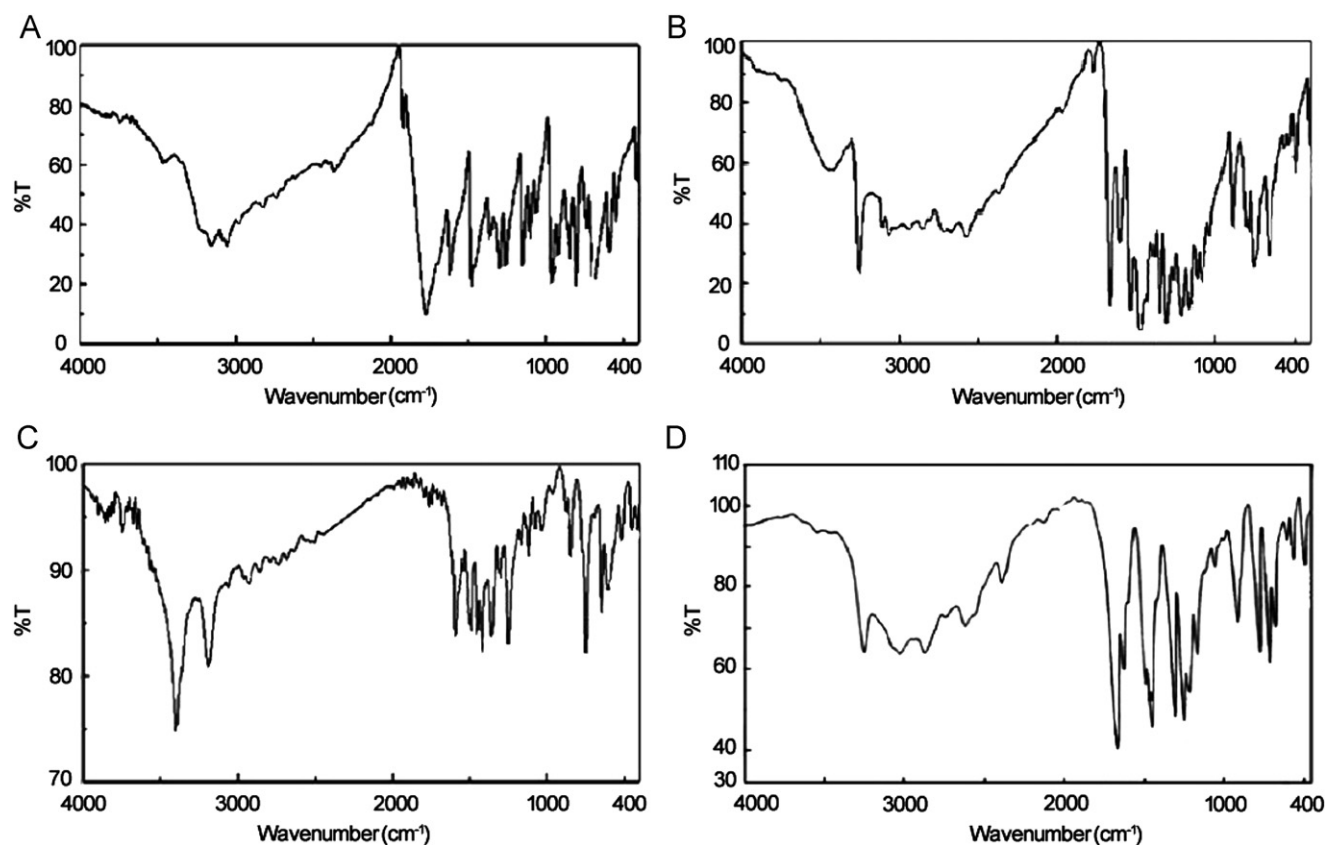


Figure 2 IR spectra of nitazoxanide (A), Deg I (tizoxanide) (B), 5-nitro-1,3-thiazol-2-amine (Deg II) (C) and salicylic acid (Deg III) (D).

$\Delta\lambda=4$ nm. Record the peak amplitude at 364.4 nm and plot against the corresponding concentrations of NTZ and then compute the regression equation.

2.6.3. Chemometric methods

2.6.3.1. Construction of the training set. Prepare different mixtures of NTZ, Deg I, Deg II and Deg III into a series of 10-mL volumetric asks. Transfer accurately aliquots equivalent to 20–100, 10–50, 10–50 and 10–50 μg from Deg I, Deg II and Deg III working solutions (100 $\mu\text{g}/\text{mL}$). Complete to the volume with methanol. Record the absorbance of these mixtures between 200–500 nm at 1 nm interval with respect to a blank of methanol.

The composition of the samples was randomly designed according to five level calibration design [26] in order to obtain non-correlated concentration profiles and this calibration design prepared to obey Beer's law.

Initial developed models were found to have high spectral residuals in the region below 260 and above 360 nm. As a result this region was rejected.

2.6.3.2. Construction of the models. To build the PCR and PLS models, feed the computer with the absorbance and concentration matrices for the training set, use the training set absorbance and concentration matrices together with PLS-Toolbox 2.0 software for the calculations.

2.6.3.3. Selection of the optimum number of factors to build the PCR and PLS models. To select the number of factors for PLS and PCR methods, a cross-validation method, using

leave one out, was used [27]. Given a set of 21 calibration samples, the PCR and PLS calibrations were performed on 20 samples, then the concentration of the sample left out during calibration was predicted. This process was repeated 21 times until each calibration sample had been left-out once. The predicted concentrations were then compared with the known concentrations and the root mean square error of cross validation (RMSECV) was calculated. The RMSECV was calculated in the same manner each time, a new factor was added to the model. The maximum number of factors used to calculate the optimum RMSECV was selected to be 11 (half the number of samples+1) [28]. Visual inspection was used for selecting the optimum number of factors. Upon building the models mean center the data gave better results for both PCR and PLS.

2.6.3.4. Construction of the validation set. Prepare different twelve mixtures of NTZ, Deg I, Deg II and Deg III by transferring different volumes of their working standard solutions (100 $\mu\text{g}/\text{mL}$) into a series of 10-mL volumetric asks and then complete to the volume with methanol. Apply the developed models to predict the concentration of NTZ, Deg I, Deg II and Deg III in each mixture.

2.6.4. Construction of calibration graph for TLC-densitometric method

Into a set of 10-mL volumetric asks, transfer accurately aliquots equivalent to 0.4–2.0 mg of NTZ from its standard stock solution (1 mg/mL), then complete the volume with methanol. Apply 10 μL of each solution to TLC plates as

bands of 6 mm width using a Camag Linomat IV applicator. Space the bands 5 mm from each other and 15 mm apart from the bottom edge of the plate. Pre-saturate the chromatographic chamber with the developing system for one hour and develop the plate by ascending chromatography using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system to a distance of 8 cm. Record the integrated peak areas using scanning wavelength at 254 nm and construct the calibration curve by plotting the integrated peak area versus the corresponding concentration of NTZ and compute the regression equation.

2.6.5. Application to pharmaceutical formulations

- i. *Nit clean*[®] tablets: Weigh, powder finely ten tablets of *Nit clean*[®] and mix well. Transfer an accurately weighed amount of the powder equivalent to 100 mg of NTZ into 250-mL beaker. Add 75 mL methanol, sonicate for 30 min, filter into 100-mL volumetric flask, then wash the residual powder with methanol and finally complete the volume with methanol to obtain 1 mg/mL stock solution. Make the appropriate dilution to obtain 100 µg/mL working solution using methanol. Proceed according to the calibration procedure of each method mentioned before and then calculate NTZ concentration using the corresponding regression equation.
- ii. *Nitazod*[®] and *Cryptonaz*[®] oral suspensions: Transfer 5 mL of each suspension into two separate 250-mL beakers. Then proceed as mentioned above to determine the concentration of NTZ in each solution.
- iii. *Standard addition technique*: Add different known concentrations of pure NTZ to *Nit clean*[®] tablets powder and *Nitazod*[®] and *Cryptonaz*[®] oral suspensions, then proceed as mentioned before to calculate the concentrations of NTZ using the proposed methods.

3. Results and discussion

The ICH guideline Q2(R1) on Stability Testing of New Drug Substances suggests that the testing of those features, which are susceptible to change during storage and are likely to influence quality, safety and efficacy, must be done by validated stability indicating methods. Stress testing should be carried out on a drug to establish its inherent stability characteristics and to support the suitability of the proposed analytical method. Up to our knowledge, the literature survey revealed that none of the most recognized pharmacopoeias or any journals includes this information regarding the stability of nitazoxanide.

Nitazoxanide was subjected to acid, alkaline hydrolysis and photo-degradation. Being ester, in acidic condition the drug undergoes hydrolysis into its corresponding acid (Deg I, active metabolite). The same product is formed first under alkaline condition, which then undergoes further degradation due to cleavage of amide linkage giving two degradation products, namely, 5-nitro-1,3-thiazol-2-amine (Deg II) and salicylic acid (Deg III). Results of photo-degradation of the drug coincide with acid degradation process.

Salicylic acid, one of the degradation products of NTZ, is a mild irritant and its application to the skin may cause dermatitis. It is readily absorbed through the skin and

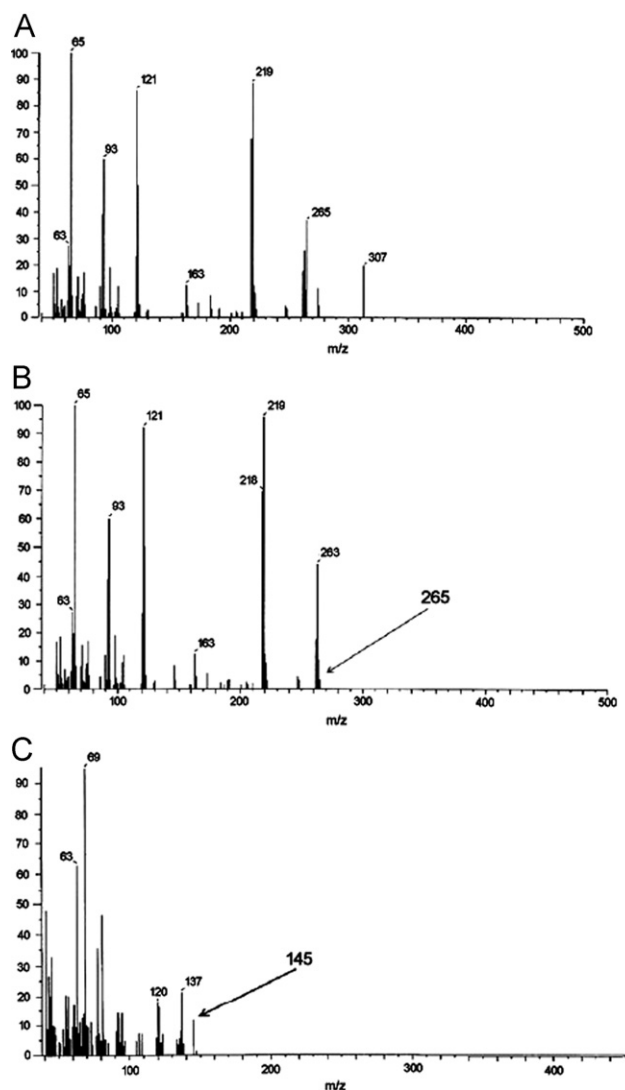


Figure 3 Mass spectra of nitazoxanide (A), Deg I (tizoxanide) (B) and 5-nitro-1,3-thiazol-2-amine (Deg II) (C).

symptoms of acute systemic salicylate poisoning have been reported after excessive use; deaths have occurred, mainly in children [1]. So, bad storage of NTZ may lead to the presence of salicylic acid, as a result of degradation process, which exposes the patient to toxicity. So it is essential to develop analytical procedure, which will serve a reliable, accurate, sensitive and stability indicating method for the determination of NTZ in presence of its degradation products.

3.1. Elucidation of nitazoxanide degradation products

Nitazoxanide was subjected to hydrolysis under acid-stress condition by reacting with 1 M HCl solution for 3 h giving one degradation product (due to cleavage of ester bond). On the other hand, when subjected to hydrolysis under alkaline-stress condition with 0.1 M NaOH solution at room temperature, the same product obtained under acid stress condition in addition to two new degradation products were obtained, so that the solution was heated for 30 min for complete degradation, giving the final two degradation products (due to the cleavage of amide

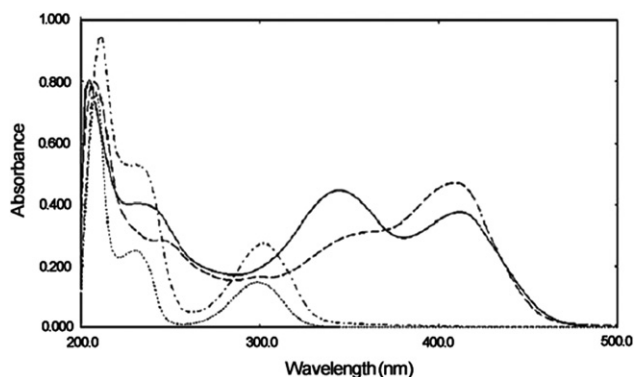


Figure 4 Zero order absorption spectra of 10 µg/mL of NTZ (—), 10 µg/mL of Deg I (- - - - -), 5 µg/mL of Deg II (-.-.-) and 5 µg/mL of Deg III (.....) using methanol as a solvent.

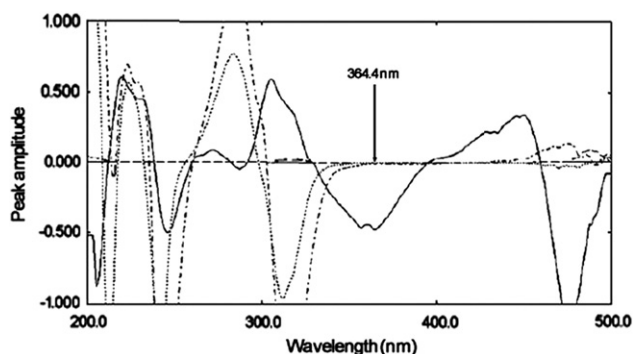


Figure 5 First derivative of ratio spectra of 10 µg/mL of NTZ (—), 10 µg/mL of Deg I (- - - - -), 5 µg/mL of Deg II (-.-.-) and 5 µg/mL of Deg III (.....) using 5 µg/mL of Deg I as a divisor and methanol as a solvent.

bond). Degradation pathway of NTZ is shown in Fig. 1. The degradation products were isolated as mentioned before and characterized by MS- and IR-spectrometry.

3.1.1. Spectral changes

The IR spectrum of intact NTZ shows characteristic bands at 1771 and 1617 cm^{-1} corresponding to carbonyl groups (C=O) of ester and amide linkages. This band of carbonyl group of amide linkage still present in IR spectrum of degradation product results from acid stress condition at 1671 cm^{-1} , while the other carbonyl band of ester linkage that disappeared with appearance of a new broad band at 3256–2578 cm^{-1} indicates presence of (–OH) hydroxyl group, which confirms the suggested degradation product (Deg I). While degradation under alkaline stress condition gives two products that do not have either bands of carbonyl ester group or amide linkage functional group.

The IR spectrum of first degradation product that was extracted from alkaline medium has a forked band at 3396 and 3189 cm^{-1} that indicates presence of (–NH₂) primary amine group, which confirms the expected degradation product (Deg II), while the IR spectrum of second degradation product that was extracted after acidification shows a band at 1662 cm^{-1} that indicates presence of carbonyl group and a

Table 1 Concentration of mixtures of nitazoxanide, Deg I, Deg II and Deg III used in the training and validation sets.

Mixture no.	Concentration (µg/mL)			
	NTZ	Deg I	Deg II	Deg III
1	6	3	3	3
2	10	3	2	2
3	6	2	2	4
4	4	2	4	5
5	4	4	5	4
6	8	5	4	3
7	10	4	3	5
8	8	3	5	5
9	6	5	5	1
10	10	5	1	4
11	10	1	4	1
12	2	4	1	3
13	8	1	3	4
14	2	3	4	4
15	6	4	4	2
16	8	4	2	1
17	8	2	1	2
18	4	1	2	3
19	2	2	3	1
20	4	3	1	1
21	6	1	1	5
22	2	1	5	2
23	2	5	2	5
24	10	2	5	3
25	4	5	3	2
26	10	3	2	5
27	6	2	5	2
28	4	5	2	4
29	10	2	4	4
30	4	4	4	3
31	8	4	3	5
32	8	3	5	4
33	6	5	4	5

The concentrations of mixtures used in the validation set are given in bold font.

broad band at 3236–2597 cm^{-1} indicate presence of (–OH) hydroxyl group (Deg III), as shown in Fig. 2.

3.1.2. Mass spectrometry

In the MS chart of NTZ, the parent peak was identified at m/z 307 (corresponding to the molecular weight of NTZ) while MS chart of degradation product formed under acid stress condition shows parent peak at m/z 265 (corresponding to the molecular weight of Deg I) and that of Deg II shows parent peak at m/z 145, as shown in Fig. 3.

3.1.3. TLC-fractionation

TLC-monitoring of the drug degradation was done on thin layer plates of silica gel F₂₅₄ using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system. The developed plates were visualized under short UV-lamp. Comparing the R_f of the two degradation products that obtained from alkaline stress

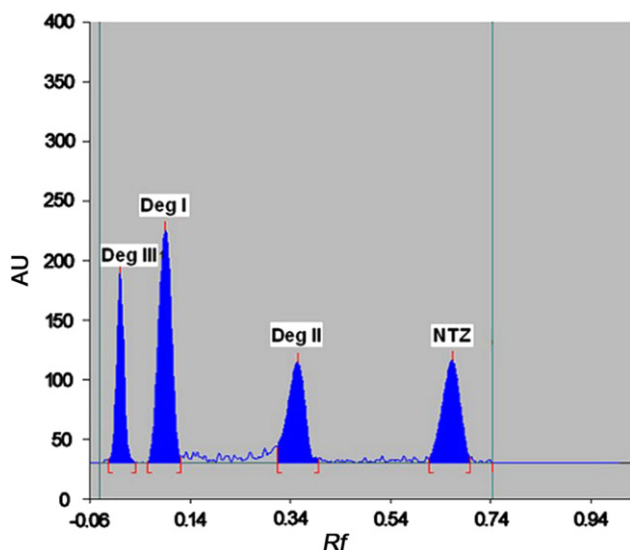


Figure 6 Thin layer chromatogram of separated peaks of Deg III ($R_f=0.03$), Deg I ($R_f=0.13$), Deg II ($R_f=0.37$) and NTZ ($R_f=0.72$) using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system at 254 nm.

condition, shows that the R_f value of Deg III that was obtained after acidification and extraction with ether is equal to the R_f value of standard salicylic acid proving their similarity.

3.2. First derivative of ratio spectra spectrophotometric method (DD^1)

Nitazoxanide and its degradation products (Deg I, Deg II and Deg III) are highly absorbing substances in the UV region of the spectrum, as shown in Fig. 4. On the basis of derivative ratio theory, first derivative of the ratio spectra technique was applied to solve the problem of overlapping spectra of NTZ, Deg I, Deg II and Deg III. The main advantage of the method is that the whole spectrum of interfering substance is canceled. Accordingly, the choice of the wavelength selected for calibration is not critical.

Selection of the divisor and its concentration is of great importance, so Deg I, Deg II and Deg III in different concentrations (5, 10, 15 and 20 $\mu\text{g/mL}$) were tried as divisors. The best results in terms of signal to noise ratio, sensitivity and selectivity followed using 5 $\mu\text{g/mL}$ of Deg I as a divisor.

The absorption spectra of NTZ, Deg I, Deg II and Deg III were divided by the spectrum of 5 $\mu\text{g/mL}$ of Deg I (as a divisor), and DD^1 spectra were obtained, where NTZ could be determined at 364.4 nm (at which no contribution from Deg I, Deg II and Deg III). Different smoothing factor ($\Delta\lambda$) values were tried where a smoothing factor=4 gave the best resolution, different scaling factor values were tested where scaling factor=10 was suitable to enlarge the signal of NTZ to facilitate its measurement and to diminish error in reading signal.

Dividing the absorption spectra of NTZ in the range of 2–24 $\mu\text{g/mL}$ by the absorption spectrum of 5 $\mu\text{g/mL}$ of Deg I, the obtained ratio spectra were differentiated with respect to wavelength ($\Delta\lambda=4$, scaling factor=10) and the interference was eliminated, as shown in Fig. 5.

Table 2 Parameters of system suitability of the developed TLC-densitometric method for the determination of nitazoxanide in presence of its degradation products.

Parameters	Deg III	Deg I	Deg II	NTZ
Symmetry factor	1.01	0.98	1.05	1.02
Resolution (R_s)	1.21		2.57	4.55
Capacity factor (K')	1.20	2.64	5.12	7.35
Selectivity (α)	2.04		2.46	4.77

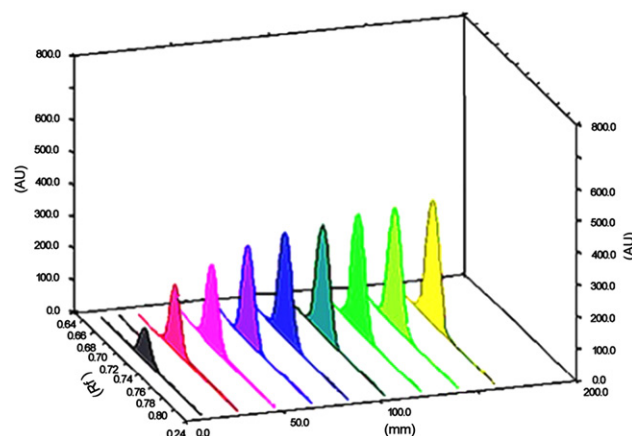


Figure 7 Thin layer chromatogram of nitazoxanide in the concentration range 0.4–2.0 $\mu\text{g/band}$ ($R_f=0.72$) using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system.

DD^1 values showed good linearity at 364.4 nm in the concentration range of 2–24 $\mu\text{g/mL}$. The linear regression equation was found to be

$$A = 0.0480C + 0.0010 \quad r = 0.9999$$

where A is the peak amplitude at 364.4 nm, C is the concentration of NTZ in $\mu\text{g/mL}$ and r is the correlation coefficient.

3.3. Chemometric methods

This method is based on UV-spectrophotometry, and the resulting heavily overlapping responses are processed by chemometrics. In this method, different chemometric approaches were applied for simultaneous determination of NTZ and its degradation products, including PCR and PLS methods. These multivariate calibrations were useful in spectral analysis because the simultaneous inclusion of many spectral wavelengths instead of single wavelength greatly improved the precision and predictive ability [29].

The first step in the simultaneous determination of the components by multivariate calibration methods involves constructing the calibration matrix for quaternary mixture. The calibration set was obtained using the absorption spectra of a set of 21 mixtures of NTZ, Deg I, Deg II and Deg III with different ratios of each component as given in Table 1. Better results were obtained upon rejecting the spectral region above 360 nm and below 260 nm.

Table 3 Determination of nitazoxanide in laboratory prepared mixtures by first derivative of ratio spectra spectrophotometric method and TLC-densitometric method.

% Degradation products	Concentration (NTZ: Deg I: Deg II: Deg III)		Recovery of NTZ ^a (%)	
	DD ¹ (µg/mL)	TLC (µg/band)	DD ¹	TLC
10	18:1:0.5:0.5	1.8:0.1:0.05:0.05	100.56	100.44
25	15:2.5:1.3:1.2	1.5:0.3:0.1:0.1	99.00	99.13
35	6.5:1.5:1:1	1.3:0.3:0.02:0.02	100.43	100.83
45	11:4.5:2.5:2	1.1:0.45:0.25:0.2	101.17	100.89
55	9:6:2.6:2.4	0.9:0.5:0.3:0.3	98.00	97.60
70	6:6.7:3.8:3.5	0.6:0.65:0.4:0.35	101.00	101.50
90	—	0.2:0.9:0.5:0.4	—	100.26
Mean ± SD			100.03 ± 1.25	100.09 ± 1.32

Percent of the degradation products were calculated according to their molecular weight.

^aAverage of 3 determinations.

Table 4 Results of determination of nitazoxanide, Deg I, Deg II and Deg III in the validation set using the proposed multivariate calibration methods.

Mixture no.	Recovery ^a (%)							
	NTZ		Deg I		Deg II		Deg III	
	PCR	PLS	PCR	PLS	PCR	PLS	PCR	PLS
1	100.14	100.11	101.34	101.33	98.32	98.50	102.43	102.05
2	101.43	101.30	102.25	102.20	96.71	96.83	100.78	100.71
3	99.54	99.65	100.78	100.73	98.38	98.43	99.64	99.75
4	97.87	97.91	101.41	101.40	102.34	101.88	101.63	101.6
5	100.63	100.61	100.69	100.67	100.86	100.82	102.59	102.33
6	98.93	98.95	98.35	98.36	99.46	99.46	103.15	102.88
7	100.53	100.50	99.52	99.52	98.61	98.65	96.80	97.25
8	99.10	99.16	101.06	101.05	103.10	103.04	98.47	98.47
9	98.23	98.25	102.17	102.11	102.77	102.80	99.31	99.35
10	97.79	97.82	100.78	100.77	101.31	101.30	98.32	98.33
11	100.58	100.50	97.75	97.75	97.58	97.75	97.40	97.47
12	101.34	101.26	96.78	96.91	103.23	102.86	102.88	102.75
Mean ± SD	99.68 ± 1.29	99.67 ± 1.24	100.24 ± 1.76	100.23 ± 1.72	100.22 ± 2.32	100.19 ± 2.19	100.28 ± 2.25	100.25 ± 2.08

^aAverage of 3 determinations.

In this study, the “leave one out” cross validation method was used and the RMSECV values of different developed models were compared. Five factors were found suitable for both PCR and PLS models. Table 1 shows different concentrations of NTZ, Deg I, Deg II and Deg III used in the validation set.

For evaluation of the predictive abilities of the developed models, several diagnostic tools were used: predictive versus actual concentration plot (model and sample diagnostic); concentration residuals versus actual concentration plot (model and sample diagnostic) and root mean square error of prediction (RMSEP) (model diagnostic), the predicted concentrations of the validation samples were calculated.

3.4. TLC-densitometric method

Chromatographic techniques overcome the problem of overlapping absorption spectra of mixture of drugs or in presence

of impurities or degradation products by separation of these components on TLC plates or chromatographic columns and determining each ingredient by scanning the corresponding chromatogram [30]. It has many applications in the field of pharmaceutical studies, which include the following: stability, impurities, synthetic drugs, pharmacokinetic, enantiomeric purity and drug monitoring in biological fluids [31,32].

To improve separation of bands, it was necessary to investigate the effect of different parameters. These parameters were studied and optimized to achieve maximum separation as follows.

3.4.1. Mobile phase

Initial experiments for the separation of NTZ and its degradation products were performed using various proportions of different solvent systems, which resulted in varying retention factors, tailing of the peaks, larger run times, and moreover improper resolution of the four components. Since

Table 5 Determination of nitazoxanide in pharmaceutical formulations by the proposed methods and application of standard addition technique.

Pharmaceutical formulation	DD ¹ method					Chemometric methods								TLC-densitometric method				
	Taken (µg/mL)	Found ^a ±SD (%)	Pure added (µg/mL)	Recovery ^b (%)	Mean ±SD	Taken (µg/mL)	Found ^a ±SD (%)		Pure added (µg/mL)	Recovery ^b (%)				Taken (µg/mL)	Found ^a ±SD (%)	Pure added (µg/mL)	Recovery ^b (%)	Mean ±SD
							PCR	PLS		PCR	Mean ±SD	PLS	Mean ±SD					
Nit clean [®] tablets	8.00	100.78 ±0.96	6.00	99.00	100.50 ±1.02	4.00	101.12	101.15	3.00	100.67	100.28	100.67	100.27	0.800	101.05 ±0.89	0.600	100.33	100.37 ±0.80
			8.00	101.25			4.00	101.25	101.00	0.800	100.88							
			10.00	101.00			5.00	98.20	98.40	1.000	101.00							
			12.00	101.00			6.00	101.00	101.00	1.200	99.25							
Nitazod [®] oral suspension	8.00	98.58 ±1.07	6.00	101.33	100.08 ±1.41	4.00	98.68	98.70	3.00	99.33	99.90	99.33	99.93	0.800	98.94 ±0.91	0.600	101.67	99.72 ±1.34
			8.00	98.63			4.00	100.75	100.75	0.800	98.63							
			10.00	99.10			5.00	101.00	100.80	1.000	99.30							
			12.00	101.25			6.00	98.50	98.83	1.200	99.25							
Cryptonaz [®] oral suspension	8.00	98.76 ±1.01	6.00	101.67	100.56 ±0.99	4.00	99.43	99.50	3.00	100.67	100.46	100.67	100.43	0.800	97.58 ±1.05	0.600	101.33	100.54 ±1.12
			8.00	100.63			4.00	100.50	100.50	0.800	98.88							
			10.00	100.70			5.00	99.00	99.20	1.000	101.10							
			12.00	99.25			6.00	101.67	101.33	1.200	100.83							

^aAverage of 6 determinations.^bAverage of 3 determinations.

Table 6 Assay parameters and method validation obtained by applying the proposed methods for the determination of Nitazoxanide.

Parameters	DD ¹ spectrophotometric method	Chemometric methods		TLC-densitometric method
		PCR	PLS	
Range	2–24 µg/mL	2–10 µg/mL		0.4–2.0 µg/band
<i>Linearity</i>				
Slope	0.0480	0.9972	0.9973	2.2575
Intercept	0.0010	0.0017	0.0108	0.1262
Correlation coefficient (<i>r</i>)	0.9999	0.9996	0.9997	0.9998
Accuracy (Mean ± SD)	99.85 ± 0.75	99.68 ± 1.29	99.67 ± 1.24	100.07 ± 1.15
Specificity and selectivity	99.88 ± 1.21	–	–	100.07 ± 1.44
<i>Precision (%RSD)</i>				
Repeatability ^a	0.83	0.75	0.68	0.81
Intermediate precision ^b	0.96	0.77	0.84	1.12
LOD ^c	0.95 µg/mL	0.78 µg/mL	0.83 µg/mL	0.10 µg/band

^aThe intraday ($n=3$), average of three different concentrations repeated three times within day.

^bThe interday ($n=3$), average of three different concentrations repeated three times in three successive days.

^cLimit of detection is determined experimentally for TLC-densitometric method and via calculations for the other methods.

the components under investigation are weakly basic (NTZ and Deg II) and weakly acidic (Deg I and Deg III) in nature, addition of glacial acetic acid and ammonia solution in the solvent mixture decreased tailing of the peaks and improved the resolution of the components. Following several attempts in order to develop an appropriate separation method, the peaks ensued following the chromatographic scan were sharp, symmetrical, reproducible and the base line resolution was better with no disturbance upon using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system, as shown in Fig. 6.

3.4.2. Band dimensions

While applying samples as bands, it should be taken into consideration the slight spread of the developed bands due to ordinary diffusion so the band width and the interspaces between bands should be chosen carefully to avoid spread of bands outside the scanning tracks and interference between adjacent bands. Moreover, narrow band width should be avoided to prevent overloading of silica especially if higher volumes are applied, which lead finally to tailing and inaccurate results. Accordingly, the optimum band width chosen was 6 mm and the interspace between bands was 5 mm.

3.4.3. Scanning wavelength

Different scanning wavelengths were tried, like 240, 254, 300 and 365 nm, where the wavelength 254 nm was the best wavelength for detection of all components with good sensitivity of NTZ in the same scan and peaks were sharp and symmetrical and minimum noise was obtained.

3.4.4. Slit dimensions of scanning light beam

The slit dimensions of the scanning light beam should ensure complete coverage of band dimensions on the scanned track without interference of adjacent bands. Different slit dimensions were tried, where 5 mm × 0.2 mm proved to be the slit

dimension of choice, which provides highest sensitivity for NTZ.

3.4.5. System suitability

USP [33] states that system suitability tests are an integral part of liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. Parameters including resolution (R_s), peak symmetry, capacity factor (K') and selectivity factor (α) were calculated using 1.0 µg/band of NTZ. The resolution is always above two, the selectivity more than one and an accepted value for symmetry factor was obtained for NTZ, as shown in Table 2.

This method offers high sensitivity and selectivity for analysis of NTZ in presence of its degradation products using chloroform–methanol–ammonia solution–glacial acetic acid (95:5:1:1 by volume, pH=5.80) as a developing system, where the good separation is obtained by the difference in the retention factor (R_f) values of Deg III ($R_f=0.03$), Deg I ($R_f=0.13$), Deg II ($R_f=0.37$) and NTZ ($R_f=0.72$) as shown in Fig. 6.

The calibration curve was constructed relating the integrated peak area and the corresponding concentrations of NTZ in the range of 0.4–2.0 µg/band, as shown in Fig. 7. The concentration of NTZ was calculated from the following regression equation:

$$Y = 2.2575C + 0.1262 \quad r = 0.9998$$

where Y is the integrated peak area ($\times 10^3$), C is the concentration in µg/band and r is the correlation coefficient.

3.5. Stability-indication

To assess the stability-indicating efficiency of the proposed methods, they were applied to laboratory prepared mixtures containing different ratios of NTZ, Deg I, Deg II and Deg III.

Table 7 Statistical comparison of the results obtained by the proposed methods and the reported method for the determination of pure nitazoxanide.

Parameters	DD ¹ spectrophotometric method	Chemometric methods		TLC-densitometric method	Reported method ^a [23]
		PCR	PLS		
Mean	99.85	99.68	99.67	100.07	99.90
SD	0.75	1.29	1.24	1.15	1.18
% RSD	0.75	1.29	1.24	1.15	1.18
<i>n</i>	6	12	12	6	8
Variance	0.56	1.66	1.54	1.32	1.39
Student's <i>t</i> -test	0.09 (2.18)	0.31 (2.10)	0.34 (2.10)	0.23 (2.18)	
<i>F</i> -value	2.48 (4.88)	1.19 (3.60)	1.11 (3.60)	1.05 (4.88)	

Figures between parenthesis represent the corresponding tabulated values of *t* and *F* at *P*=0.05.

^aRP-HPLC using 0.1% phosphoric acid–acetonitrile (44:45, v/v) at pH=6 with UV detection at 240 nm and flow rate 1 mL/min.

Results given in Table 3 indicate that the proposed DD¹ method is valid for determination of intact NTZ in presence of up to 70% of its degradation products and TLC-densitometric method is selective and valid for determination of NTZ in presence of up to 90% of its degradation products.

To validate the prediction ability of the suggested PCR and PLS models, they were used to predict the concentration of NTZ and its degradation products in laboratory prepared mixtures containing their different ratios; satisfactory results were obtained as shown in Table 4. Also, the chemometric methods allow the determination of the degradation products' concentrations.

As the suggested methods could effectively determine the drug in presence of its degradation products, they can be employed as stability indicating ones.

3.6. Application of the proposed methods to the pharmaceutical formulations

The suggested methods were successfully applied for the determination of NTZ in its pharmaceutical formulations (Nit Clean[®] tablets and Nitazod[®] and Cryptonaz[®] oral suspensions), showing good percentage recoveries as given in Table 5. The validity of the suggested methods was further assessed by applying the standard addition technique as shown in Table 5.

3.7. Method validation

Method validation was performed according to ICH [24] guidelines for the proposed methods. Table 6 shows the results of accuracy, repeatability and intermediate precision of the methods. Other regression equation parameters are shown in Table 6, which shows good linear relationship for the methods as revealed by the correlation coefficient. Results of assay validation parameters of the proposed chemometric methods are demonstrated in Table 6, where satisfactory correlation coefficient (*r*) value was obtained for NTZ in the validation set by PCR and PLS optimized models indicating good predictive abilities of the models.

3.8. Statistical analysis

Results obtained by the suggested methods for determination of NTZ were statistically compared with those obtained by applying the reported HPLC method [23]. The calculated

t- and *F*-values [34] were found to be less than the theoretical ones, confirming accuracy and precision at 95% confidence level, as given in Table 7. The test ascertains that the proposed methods are as precise and accurate as the reported HPLC method [23] and are comparable to one another. The results obtained indicate that the introduced methods can be classified amongst the highly selective and sensitive procedures. These merits suggest the use of the proposed methods in routine and quality control analysis without interference of commonly encountered pharmaceutical formulation additives.

4. Conclusion

In the presented work, three different, simple, accurate and economical stability indicating methods were suggested for determination of NTZ. Moreover, the proposed methods were found to be more selective than the reported HPLC method, which showed severe overlapping between the drug and its degradation products.

First derivative of ratio spectra spectrophotometric method is well established technique that are able to enhance the resolution of overlapping bands. This method is simple, more convenient, less time consuming and economic stability indicating method for determination of NTZ compared to other published LC methods.

The advantage of multicomponent analysis using multivariate calibration is the speed of the proposed procedures, as a separation step avoids. No data about the application of chemometric methods have been found in the literature. It offers a distinct possibility of assaying NTZ in its pharmaceutical formulations without interference due to the excipients or the degradation products and the method is capable of determination of the degradation products concentration.

The advantage of TLC-densitometric method is that several samples can be run simultaneously using a small quantity of mobile phase, thus lowering analysis time and cost per analysis and provides high sensitivity and selectivity.

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