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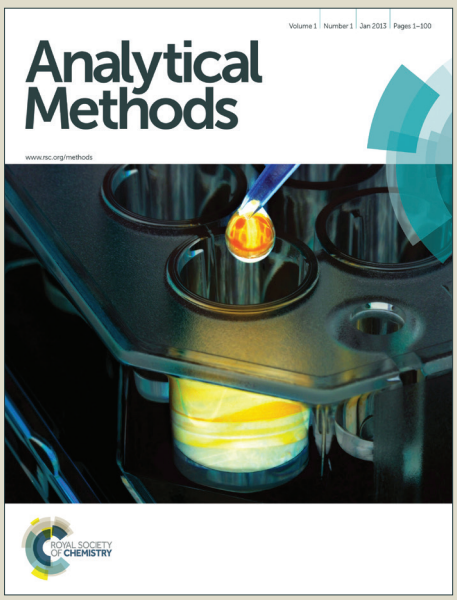

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Determination of Tizoxanide, the Active Metabolite of Nitazoxanide, by Micellar Liquid Chromatography Using Monolithic Column. Application to Pharmacokinetic Studies

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Abstract:

In this study, a micellar liquid chromatography (MLC) method is proposed for the determination of tizoxanide (TZ), the active metabolite of nitazoxanide (NX), and the use of a micellar mobile phase consisting of 0.1 M sodium dodecyl sulphate, 8% n-propanol and 0.3% triethylamine in 0.02 M phosphoric acid buffered at pH 4 as the mobile phase. The method was successfully applied to the analysis of tizoxanide (TZ), the active metabolite of nitazoxanide (NX), in presence of tinidazole (TIN) as internal standard in pure form, in real human urine and plasma without previous extraction step. Analytical separation was performed in less than 10 min using a RP C18 monolithic column with UV detection at 240 nm. The validation study of the proposed method was successfully carried out in an assay range between 0.05 and 20 µg/ mL with limit of detection (LOD) 0.016 µg/ mL and limit of quatification (LOQ) 0.049 µg/ mL. The method was fully validated in accordance with ICH guidelines. The proposed method was successfully applied to quantitatively determine TZ in spiked human urine and plasma. It was also extended to the pharmacokinetic studies of TZ in real human urine and plasma samples.

Keywords: Micellar Liquid Chromatography, Tizoxanide, pharmacokinetics**,** real human urine and plasma

Introduction:

Nitazoxanide (NX) is a novel broad-spectrum antiparasitic agent that is effective against a wide variety of protozoal infections, including *Giardia lamblia, Trichomonas vaginalis, Entamoeba histolytica and Clostridium perfrigens,* helminthes and gram negative organisms (1, 2). NX is a synthetic nitrothiazole derivative. Its chemical structure is 2-acetyloxyl-N-(5-nitro-2-thiazolyl) benzamide, Figure (1). The antiprotozoal activity of NX is believed to be due to interference with the pyruvate–ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reaction, which is essential for anaerobic energy metabolism of the parasites (3).

Following oral administration in humans, NX is immediately and completely metabolized to an active metabolite, tizoxanide (desacetyl-nitazoxanide) (TZ). TZ is the only product identified in feces (two-thirds of the dose) and in urine (one-third of the dose). The parent NX is not detected in plasma. TZ glucuronide has been identified as the main metabolite in plasma, urine and bile (4).

The literature survey has revealed that several methods were reported for the determination of TZ. A liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS-MS) method was developed for the identification of NX metabolites in goat plasma, urine (5) and feces (6). Also, the NX metabolite (TZ) has been determined in human plasma using LC (7) and high performance thin layer chromatography (8).

Nowadays, chromatographic methods are widely applied in contemporary chemistry, e.g. HPLC, HPLC–MS, etc. However, organic solvents are required here, sometimes even in large quantities, including toxic acetonitrile, methanol, etc. Hence, chemical methods with less or no use of organic solvents, the so-called green chemistry methods are attracting great interest.

The proposed method has several advantages over the published methods, it can be applied in laboratories lacking sophisticated instruments such as LC–MS-MS (5, 6), does not require a cation-exchange solid-phase extraction (SPE) step before analysis (8).

Throughout the literature, there is no reported method for determination of the NX metabolite (TZ) in human urine and plasma without previous extraction step. Therefore, it was necessary to develop a simple miceller HPLC method for the determination of TZ in plasma and urine without previous extraction step.

Micellar liquid chromatography (MLC) allows complex matrices to be analyzed without the aid of extraction and with direct injection of samples (9). Micelles tend to bind proteins competitively, thereby releasing protein-bound drugs and proteins, rather than precipitating into the column. Proteins are solubilized and washed harmlessly away, eluting with the solvent front. This means that costs and analysis times are cut considerably (10). Micellar mobile phases usually need less quantity of organic modifier and generate less amount of toxic waste in comparison to aqueous-organic solvents, so that they are less toxic, non-flammable, biodegradable and relatively inexpensive (11). Because of these advantages, MLC is considered an interesting technique for “green” chemistry that copes with current concern about the environment (12).

Nowadays, the most challenging trend in liquid chromatography is the development of new sorbents, which are able to separate complicated substances efficiently. Such sorbents should be able to work in a wide pH range and should perform analysis as fast as possible while sufficient separation, method sensitivity and selectivity remain unaffected. One of these novel types of sorbents is monolithic silica. They have a different structure compared to conventional silica (13). While the

typically used columns are filled with small silica spherical particles, monolithic columns contain a special silica (or another material), which is not formed by particles. They are made by sol–gel technology, which enables formation of highly porous material, containing macropores and mesopores in its structure. Such an LC column consists of a single rod of silica based material with two kinds of pores. The large pores (typically 2 μm) are responsible for a low flow resistance and therefore allow the application of high eluent flow-rates, while the small pores (about 12 nm) ensure sufficient surface area for separation efficiency (13). Due to these facts, higher flow rates can be used while the resolution of the silica rod column is much less affected in comparison to particulate materials after increasing the flow-rate and column back-pressure is still low. Another practical advantage is a short time needed for column equilibration when a mobile phase gradient is used (14). There are a few works that deal with the practical applications of monolithic columns in LC (15).

Experimental

Apparatus

Chromatographic analyses were carried out using a Shimadzu Prominence HPLC system (Shimadzu Corporation, Japan) with a LC-20 AD pump, DGU-20 A5 degasser, CBM-20A interface, and SPD-20A UV-VIS detector with 20 μL injection loop. The columns used were reversed-phase Chromolith® Performance (RP-18e, 100 mm × 4.6 mm i.d.) column obtained from Merck (Darmstadt, Germany) and Nucleodur MN-C18 column (150 mm × 4.6 mm i.d., 5 μm particle size), Macherey-

Nagel, Düren, Germany. Centrifugation was carried out using TDL-60B Centrifuge (Anke, Taiwan). BHA-180T Sonicator (Abbotta Corporation, USA) was used.

Reagents and Materials

All reagents and solvents used were of HPLC grade. Pharmaceutical grade NX and tinidazole (TIN) were used and certified to contain 99.9 and 99.8%, respectively. Methanol, *1*-propanol, acetonitrile, dimethyl suphoxide and sodium dodecyl sulphate (SDS) were from Sigma-Aldrich (Seelze, Germany). Triethylamine and phosphoric acid were from Riedel-deHaën (Seelze, Germany). Regenerated cellulose membrane filters and syringe filters (Minisart RC25) with pore size 0.45 µm were from Sartorius-Stedim (Goettingen, Germany). Commercial Nit Clean® tablets (Batch No. 12162) are labeled to contain 500 mg of NX manufactured by Western Pharmaceutical Industries Co., El-Obour City, Egypt.

Preparation of tizoxanide:

Tizoxanide (TZ) was prepared by acidic hydrolysis of NX (100 mg of NX was refluxed with 100 mL 0f 1M hydrochloric acid at 100 $^{\circ}$ C for 3 h). Complete degradation was followed using plates of silica gel $60F_{254}$ and chloroform- methanol-ammonia solution- glacial acetic acid (95:5: 1:1 by volume, pH=5.8) as a developing system. Filter the formed precipitate, wash with distilled water (4x 3 mL), transfer to a bottom dish and allow drying at room temperature. Structural elucidation of the reaction product was conducted by IR and mass spectroscopy.

HPLC conditions

The HPLC separation and quantitation were performed on a reversed-phase Chromolith® Performance (RP-18e, 100 mm × 4.6 mm i.d.) column. The mobile phase was prepared by mixing 0.1 M SDS, 8% n- proparnol, o- phosphric acid and triethyl amine in the ratio of 91.5: 8 :0.3: 0.2 (v/v/v/v) and adjusted to the apparent pH of 4.0 using phosphoric acid. The flow rate was 1.0 mL/min. All determinations were performed at an ambient temperature of 25℃. The injection volume was 20 µL. The detector was set at 240 nm.

Standard solutions

Stock standard solutions were prepared by dissolving TZ in the least volume of dimethylsulphoxide then completed to the volume with methanol and TIN in methanol to obtain a concentration of 200 µg/ mL. Further dilutions for TZ with methanol were carried out to obtain concentration ranges of 0.05–20 µg/mL. These stock solutions were stored at 4℃ in the dark for one week.

Construction of Calibration Curves

Working solutions containing 0.05–20 µg/mL of TZ were prepared by serial dilutions of aliquots of the stock solutions. All solutions contained 20 µg/ mL TIN as internal standard (IS). 20 µL aliquots were injected (triplicate) and eluted with the mobile phase under the described chromatographic conditions. The peak area ratios (peak area of TZ/peak area of TIN) were determined for each concentration and plotted against the corresponding concentration of drug in µg/ mL. Alternatively, the corresponding regression equation was derived.

Plasma and urine sample preparation (spiking procedure):

To 1 mL of blank plasma or urine samples, add increasing volumes of TZ standard solutions in 10- mL volumetric flasks, diluted to the volume with the mobile phase to obtain a final concentration range of 0.2–10 µg/ mL. All solutions contained 20 µg /mL of TIN as internal standard (IS). After through mixing by sonication for 5 min, the solutions were filtered through 0.45-µm disposable membrane filters. Triplicate 20-µL aliquots were injected for each sample of TZ and chromatographed under the previously conditions described. The peak area ratios (peak area of TZ/peak area of TIN) were determined in case of plasma or urine and plotted against the final concentration to obtain the calibration graph. Alternatively, the corresponding regression equations were derived.

Plasma and urine sample preparation (in vivo procedure)

This investigation conforms to the Egyptian Community guidelines for the use of humans in experiments. The Human Ethics Committee of the Faculty of Pharmacy, Mansoura University, approved the study (code no. 2013-49 on 8/9/2013). The informed written consent was obtained from the volunteers prior to the experiment.

An excretion study of TZ was carried out on three normal, healthy (normal liver, kidney functions and electrocardiogram), female, informed adult volunteers (around 35 years old), with no past history of allergic reaction to NX. Each volunteer was instructed to abstain from all medications for two weeks before administration and also during the study. Also, each volunteer was instructed to be sure of evacuating his bladder as thoroughly as possible exactly before the administration of one tablet of NX (500 mg) with food.

Urine sample

This method was used to investigate the pattern of urinary excretion of TZ. The zero hr. urine sample was collected as a blank. Urine samples were collected at 1 hr. intervals for up to 24 h. The volume of urine specimen was measured and recorded after each collection; 20 mL aliquots were stored at –20℃ until determination.

A suitable volume of the urine specimen from each sampling point was diluted to 10 mL with the mobile phase to reach calibration range. Each sample solution contained 20 µg/ mL TIN as internal standard. The solution was filtered through a 0.45-µm membrane filter. A 20 µL aliquots were injected into the HPLC in triplicate for each solution and chromatographed under the previously conditions described. The peak area ratios were determined. The nominal concentration in urine was obtained from the previously derived regression equation.

Plasma sample

Blood samples were withdrawn at different time intervals at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 6.0 hr into tubes containing 0.5 ml of 2% EDTA solution to prevent blood coagulation. The blood was processed to plasma by centrifugation at 4000 rpm and 15 min. the supernatant plasma samples were transferred into test tubes. A suitable volume of the plasma specimen from each sampling point was diluted to 10 mL with the mobile phase to reach calibration range. Each sample solution contained 20 µg/ mL TIN as internal standard. The solution was filtered through a 0.45-µm membrane filter. A 20 µL aliquot was injected into the HPLC in triplicate for each solution and chromatographed under the conditions described previously. The peak area ratios were determined. The nominal concentration of TZ in plasma was obtained using the corresponding regression equation.

Results and Discussion

Nitazoxanide represents the parent compound of a novel class of thiazolides, which are broad-spectrum anti-parasitic drugs. A single oral dose of NX was generally well tolerated. In particular, no gastrointestinal complaints were recorded.

Complex biological matrices such as urine, plasma are considered to be a powerful challenge for any analyst. High proteins, fats and carbohydrates are the primary components of these matrices, which produce high interferences with the drugs to be determined and affect the method performance. Therefore, it was necessary to develop a simple miceller HPLC method for the determination of TZ, the active metabolite of ZX, in plasma and urine without previous extraction step and clean-up procedure.

The proposed method permits the quantitation of TZ, (pKa = 6.7), in plasma and urine. Figure 2 shows a typical chromatogram indicating good resolution of TZ (t_R = 4.7 min) and TIN (t_R= 1.8 min) as internal standerd.

Selection and Optimization of Chromatographic Conditions

To achieve the best chromatographic conditions, the mobile phase composition was optimized to provide sufficient selectivity and sensitivity in a short separation time. The different chromatographic conditions affecting the separation and resolution of TZ were carefully studied and optimized.

Choice of Column

Two different columns were tested for performance investigations, including: reversed-phase Chromolith® Performance C18 column, and Nucleodur MN-C18 column.

The experimental studies revealed that the first column was more suitable, since it produced well-resolved peaks with a very high sensitivity within a reasonable analytical run time.

Mobile Phase Composition

Several modifications in the micellar mobile phase composition were performed in order to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change of the concentration and type of organic modifier, the surfactant concentration and the pH**.** The mobile phase was prepared using 0.3% triethylamine and 0.02M phosphoric acid. The effect of changing the type of organic modifier on the selectivity and retention time of TZ was investigated using mobile phases containing 10% methanol, *n*-propanol or acetonitrile. *n*-Propanol was the best, giving well-resolved peaks and the highest number of theoretical plates. The effect of changing the concentration of organic modifier on the selectivity and retention times of TZ was investigated using mobile phases containing concentrations of 6–12% *n*-propanol and containing 0.15 M SDS and buffered at pH 4. *n*-Propanol (8%) was the best, giving well-resolved peaks and the highest number of theoretical plates. The effect of changing the concentration of surfactant on the selectivity and retention times of TZ was investigated using mobile phases containing SDS concentrations in the range of 0.07–0.15 M and containing 8% *n*-propanol and buffered at pH 4. SDS (0.1 M) was the best, giving well-resolved peaks and highest number of theoretical plates. The effect of changing the pH of the mobile phase on the selectivity and retention time of TZ was investigated using mobile phases of pH values ranging from 3.0 – 6.0 with 0.1 M SDS concentration and 8% *n*-propanol. pH of 4.0 was the most appropriate, giving well-resolved peaks and

the highest number of theoretical plates. Values of pH higher than 6.0 resulted in very low number of theoretical plates.

System Suitability Test Parameters

To ascertain the reproducibility of the MLC method, system suitability tests were performed using the working standard solutions of TZ. Resolution (Rs), theoretical plates number (N) and tailing factor (T) were measured as the criteria for system suitability testing. These results are satisfactory compared to the minimum values necessary for an acceptable method.

Method Validation

The validity of the proposed MLC method was tested in terms of linearity, ranges, limits of detection, limits of quantification, accuracy and precision.

Linearity and Range

Under the above-described experimental conditions, linear relationships were established by plotting peak areas ratio against TZ concentrations. The concentration ranges were found to be 0.05–20 µg /mL. Linear regression analysis of the data gave the following equation:

$$P = 0.0017 + 0.169\ C \qquad r = 0.9999$$

Where P is the peak area ratio and C is the concentration of drug in µg/ mL and r is the correlation coefficient.

The high values of the correlation coefficients (r-values >0.999) indicate good linearity of the calibration graphs. Statistical analysis of the data gave small values of the % relative error, (% Er) 0.145 % for TZ (16).

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The limit of quantitation (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH Q2B recommendations (16) below which the calibration graph is non linear and it was found to be 0.049 µg / mL. The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected (S/N=3); it was found to be 0.016 µg / mL. The results of LOD and LOQ of TZ are abridged in Table 1.

Accuracy

The accuracy of analytical method is defined as the agreement of the results obtained by this method with the true values. To test the validity of the proposed method, it was applied to the determination of pure samples of TZ over the range of 0.05–20 µg /mL. The results obtained were in good agreement with those obtained using the comparison HPLC method (4). Using Student's t-test and the variance ratio F-test revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively (Table 2) (17).

Precision

The intra-day precision was evaluated through replicate analysis of different concentrations of the drug pure form within the specific working concentration ranges. Each sample was analyzed three successive times. Similarly, the inter-day

precision was evaluated through replicate analysis of the three different concentrations on three successive days. The results obtained are summarized in Table 3. The data presented in Table 3 indicate high precision of the developed method. Good values of the average percentage recoveries and the small values of standard deviations indicate the high accuracy and precision, respectively.

Freeze–thaw stability

The stability of the analytes after three freeze and thaw cycles was determined at low and high concentrations samples. The samples were stored at -20℃ for 24 h and thawed unassisted at room temperature. After completely thawing, the samples were refrozen for 12–24 h. After three freeze–thaw cycles, the concentration of the samples were analyzed. The freeze thaw stability results showed that tizoxanide was stable for at least three freeze thaw cycles. Stability results indicated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. The results were illustrated in table 4.

Applications

Application of the proposed method to spiked human plasma and urine samples

The high sensitivity of the proposed method allowed the determination of TZ, the active metabolite of nitazoxanide, in biological fluids by miceller HPLC method. The plasma and urine samples were spiked with TZ in concentration ranges of 0.2 - 10.0 µg/ mL. Under the above-described experimental conditions, linear relationships were established by plotting peak areas ratio against TZ concentrations. Linear regression analysis of the data gave the following equations:

For plasma, $P = 0.055 + 0.115C$ $(r = 0.9999)$

For urine, $P = - 0.018 + 0.342C$ $(r = 0.9999)$

Where P is the peak area ratio and C is the concentration of drug in µg/ mL and r is the correlation coefficient. The high values of the correlation coefficients (r-values >0.999) indicate good linearity of the calibration graphs. Statistical analysis of the data gave small values of the % relative error, (% Er) 0.0.687 % and 0.618 for plasma and urine respectively (16). The results were shown in table 5.

Application of the proposed method in real human plasma and urine

The proposed method was applied to study pharmacokinetics of TZ in real human plasma and urine. The performance of the proposed method was demonstrated by its application to human urine samples taken from three females volunteers who received Nit Clean® tablet. Figure (3) shows a typical HPLC chromatogram of a real human urine sample taken 4 h after receiving the drug, the concentration of TZ in the urine sample was found to be 7.95 µg/ mL. Figure (4) illustrates cumulative execretion pattern of TZ in Urine after oral administration of Nit Clean® tablets (one tablet contain 500mg NX). The finding is in agreement with the reference method (4).

The performance of the proposed method was demonstrated by its application to human plasma samples taken from three females volunteers who received Nit Clean® tablet. Figure (5) shows a typical HPLC chromatogram of a real human plasma sample taken 2 h after receiving the drug. The maximum concentration of TZ in the plasma sample was found to be 1.98 µg/ mL after 2 h. Figure (6) shows plasma concentration – time profiles of TZ after oral administration of Nit Clean® tablets (one tablet contain 500mg NX). The finding is in agreement with the reference method (4).

Conclusion

In this paper, a green MLC method is proposed. Here, instead of using toxic acetonitrile or flammable methanol, SDS with a small amount of n-propanol is proposed as a media for the analysis of TZ, the main active metabolite of NX, in pure form and in biological samples without any extraction procedure. In comparison with the conventional HPLC method, this method is advantageous not only in it being "green" and less-toxic to living things and the environment, but also yielding a more rapid and sensitive separation than the conventional HPLC method. Moreover, the study is extended to the pharmacokinetic studies of TZ in real human urine and plasma.

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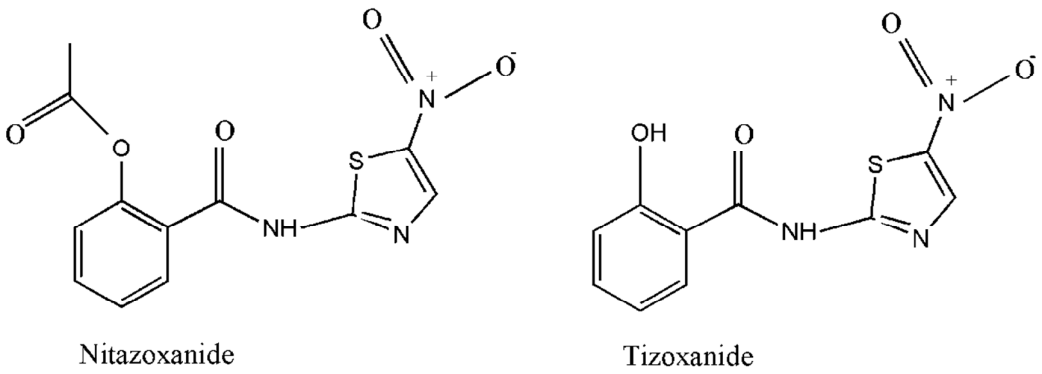

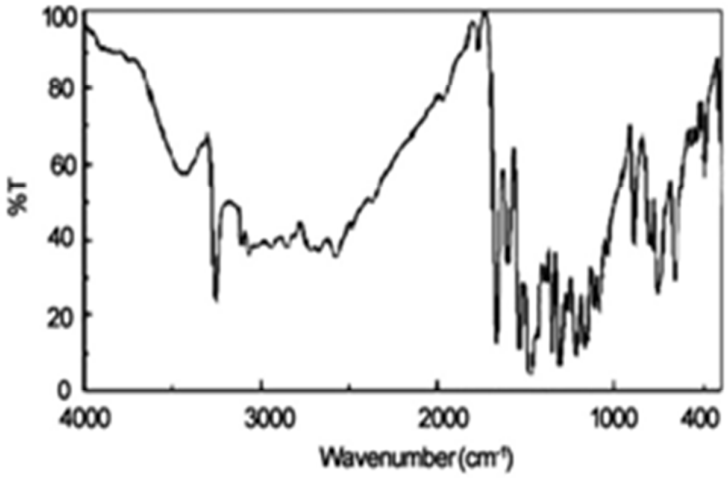

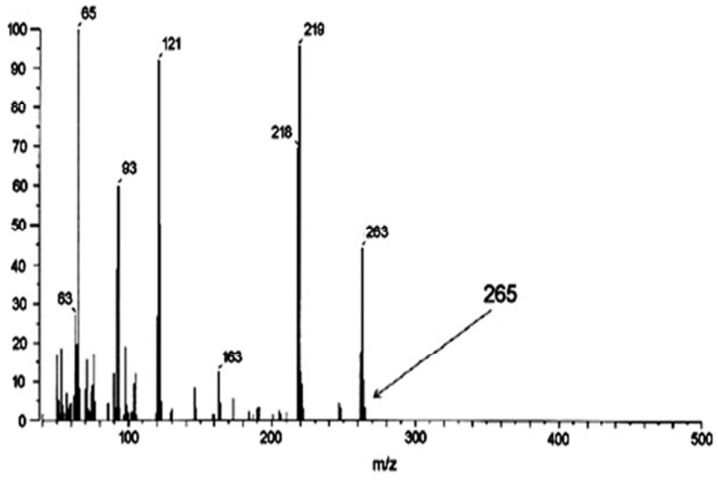

Figure1: Strutcural formula of the Nitazoxanide and Tizoxanide and the IR and mass spectrum of tizoxanide

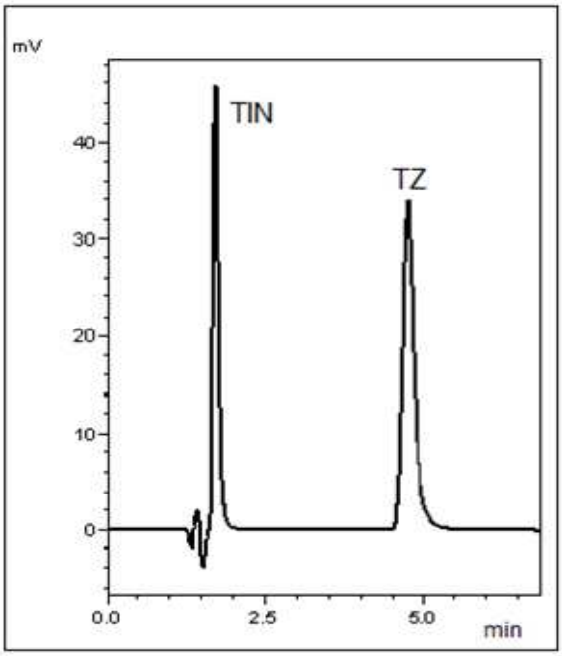

Figure 2: Typical chromatogram of TZ (10 µg/mL) and TIN as IS (20 µg/mL) in pure form under described chromatographic conditions.

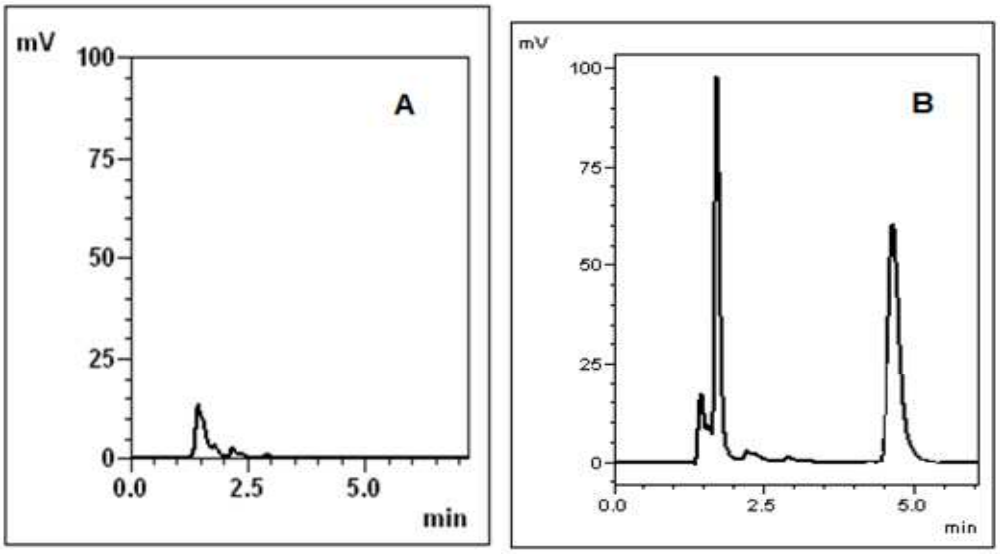

Figure (3): A- Blank urine

B- Urine sample obtained 4.0 hr after oral administration of Nit Clean® tablet under the chromatographic conditions.

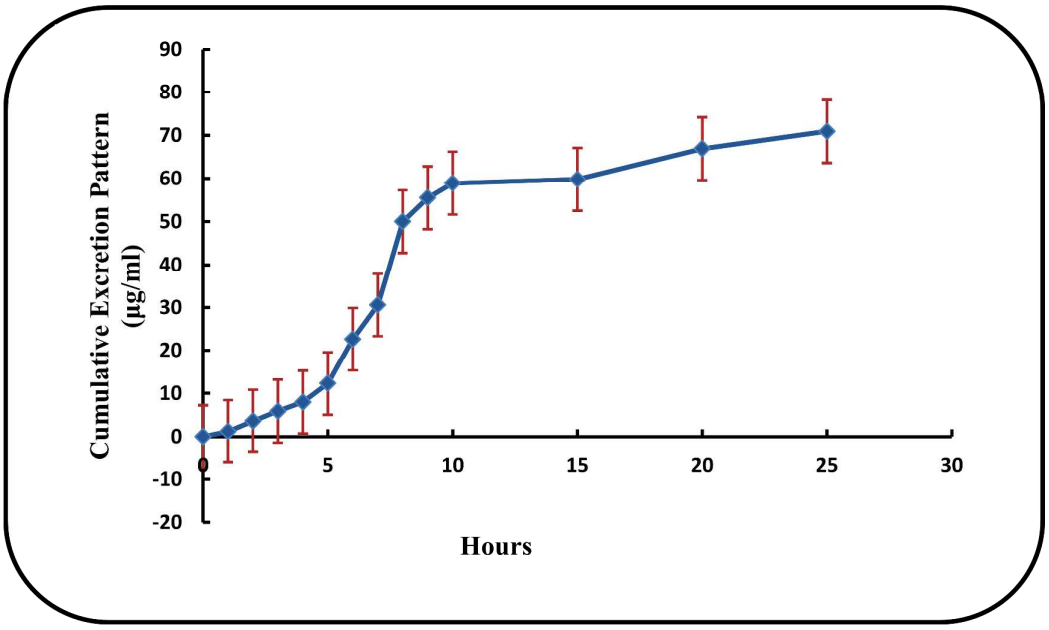

Figure (4): *Cumulative execretion pattern of TZ in Urine after oral administration of Nit Clean® tablets (one tablet contain 500mg NX)

-*The graph is the average of three separate determinations (volunteers).

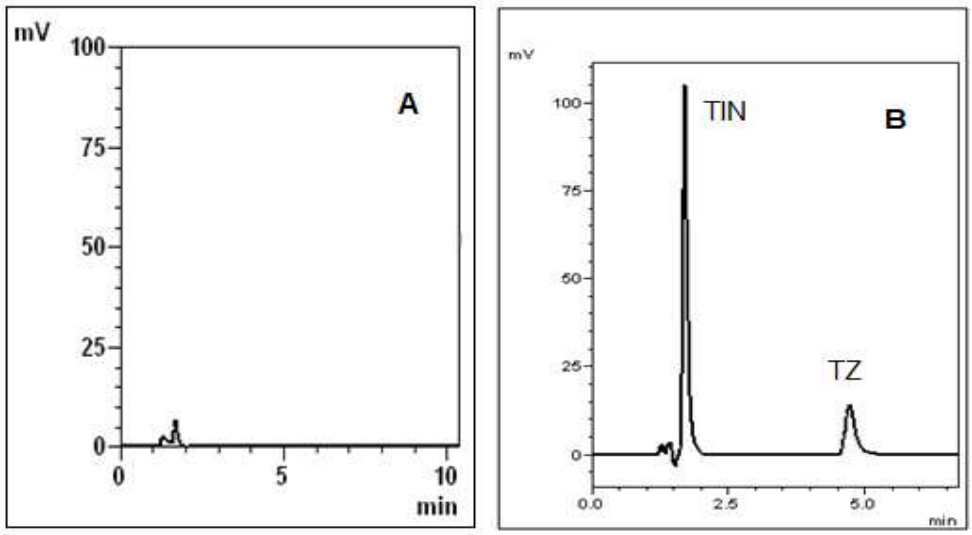

Figure (5): A- Blank plasma

B- Plasma sample obtained 2.0 hr after oral administration of Nit Clean® tablet under the chromatographic conditions.

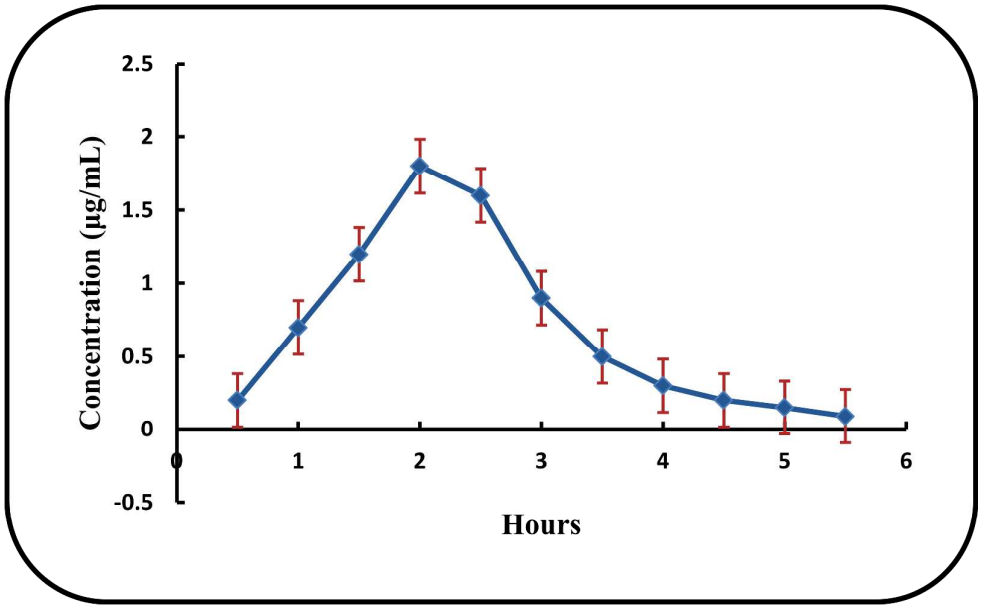

Figure (6): *Plasma concentration – time profiles of TZ after oral administration of Nit Clean® tablets (one tablet contain 500mg NX)

-*The graph is the average of three separate determinations (volunteers).

Table 1 : Effect of experimental parameters on the number of theoretical plates of tizoxanide.

parameter	No. of theoretical plates	Tailing factor
Type of organic modifier		
Methanol	1170	1.102
n-Propanol	3250	1.104
Acetonitrile	2965	1.201
Concentration of n- propanol		
6%	2384	1.302
8%	3264	1.103
10%	2908	1.211
12%	2183	1.101
Concentration of SDS		
0.07	1632	1.112
0.10	3294	1.142
0.12	2690	1.201
0.15	2405	1.154
pH		
3.0	2563	1.312
3.5	2106	1.207
4.0	3310	1.212
5.0	2180	1.176
6.0	1026	1.301

Table 2. Performance data of the proposed chromatographic method

Parameter	Value
Concentration range (µg/ml)	0.05-20
Limit of detection (LOD) (µg/ml)	0.016
Limit of quantification (LOQ) (µg/ml)	0.049
Correlation coefficient (r)	0.9999
Slope	0.169
Intercept	0.0017
standard deviation of the residuals ($S_{y/x}$)	0.002
standard deviation of the intercept (S_a)	0.0008
standard deviation of the slope(S_b)	$9.6x10^{-5}$
%RSD	0.464
% Error	0.145

Table 3. Application of the proposed method to the determination of the studied drug in the pure form

Concentration taken (µg/ml)	Concentration found (µg/ml)	% Found	Reference method (4)
0.05	0.049	99.29	100.46
0.10	0.098	98.71	99.53
0.20	0.198	99.49	100.15
0.50	0.49	98.73	
1.00	0.99	99.42	
2.00	1.997	99.89	
5.00	5.01	100.16	
10.00	10.03	100.29	
15.00	14.98	99.90	
20.00	19.85	99.97	
X`		99.25	100.04
± S.D.		0.461	0.473
t-test	1.76 (2.201)		
F-value	1.0527 (4.260)		

Figures between parenthesis are the tabulated t and F values, respectively at p= 0.05 (15).

Table 4.Validation of the proposed method for determination of TZ in pure form

Concentration added (µg/ml)	% recovery	% RSD	% Error
Intra-day			
5.0	101.00 ±1.6	1.58	0.91
10.0	101.20 ±0.624	0.61	0.35
20.0	99.77 ±0.320	0.32	0.18
Inter-day			
5.0	99.10 ± 0.9	0.90	0.52
10.0	100.06 ±1.10	1.09	0.63
20.0	99.96 ± 0.35	0.35	0.20

Table 5. Freeze – thaw stability of TZ in plasma samples

Concentration added (µg/ml)	Mean± SD	% RSD	% Error
0.2	0.198 ±0.012	6.06	3.498
2.0	1.880 ±0.135	7.20	4.156

Each result is average of three separate determinations

Table 6. The application of the proposed method to the spiked human plasma and urine

Concentration taken(µg/ml)	Concentration found (µg/ml)	% Recovery	Comparison method[4]
Spiked human plasma			
0.2	0.2004	100.22	98.98
0.5	0.4960	99.20	101.14
1.0	1.0300	103.47	99.62
2.0	1.9900	99.085	
5.0	4.9300	98.64	
10.0	10.0300	100.32	
X`		100.28	99.88
± S.D.		1.684	1.145
%RSD		1.680	
%Error		0.687	
Student's t-test	0.365 (2.635)		
Variance ratio F -ratio	2.162 (5.79)		
Spiked human urine			
0.2	0.1946	97.32	100.65
0.5	0.5041	100.82	99.37
1.0	1.0132	101.32	100.21
2.0	1.9714	98.57	
5.0	5.023	100.46	
10.0	9.993	99.93	
X`		99.73	100.07
± S.D.		1.514	0.651
%RSD		1.518	
%Error		0.618	
Student's t-test	0.362 (2.365)		
Variance ratio F -ratio	5.418 (5.79)		