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Article

Validated and Optimized High-Performance Liquid Chromatographic Determination of Tizoxanide, the Main Active Metabolite of Nitazoxanide in Human Urine, Plasma and Breast Milk

Ghada M. Hadad[1], Randa A. Abdel Salam[1]* and Samy Emara[2]

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, University of Suez Canal, Ismailia 41522, Egypt, and
[2]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Misr International University, Egypt

*Author to whom correspondence should be addressed. Email: dr_rasalam@yahoo.com

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A high-performance liquid chromatographic method was optimized and validated for the determination of desacetyl nitazoxanide (tizoxanide), the main active metabolite of nitazoxanide in human plasma, urine and breast milk. The proposed method used a CN column with mobile phase consisting of acetonitrile–12mM ammonium acetate–diethylamine in the ratio of 30:70:0.1 (*v*/*v*/*v*) and buffered at pH 4.0 with acetic acid, with a flow rate of 1.5 mL/min. Quantitation was achieved with UV detection at 260 nm using nifuroxazide as internal standard. A simplified direct injection of urine samples without extraction in addition to the urinary excretion pattern were calculated using the proposed method. Also, the effectiveness of protein precipitation and a clean-up procedure were investigated for biological plasma and human breast milk samples. The validation study of the proposed method was successfully carried out in an assay range between 0.2 and 20 μg/mL.

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. 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. TZ undergoes conjugation, primarily by glucuronidation, and passes through urine in the form of TZ-glucuronide (4, 5).

A literature survey has found that several methods are reported. A liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS-MS) method has been developed for the identification of NX metabolites in goat plasma, urine (6) and feces (7). Also, the NX metabolite (TZ) has determined in human plasma using LC (8) and high-performance thin layer chromatography (HPTLC) (9). The proposed method has several advantages over the published methods: it can be applied in laboratories lacking sophisticated instruments such as LC–MS-MS (6, 7), does not require a cation-exchange solid-phase extraction (SPE) step before analysis (9), can be applied for determination of TZ in human urine and breast milk (8) and furthermore, determines TZ in human plasma in a relatively short run time (8).

Throughout the literature, there is no published method for determination of the NX metabolite (TZ) in human urine and breast milk. Therefore, it was necessary to develop a simple reversed-phase isocratic HPLC method for the determination of TZ in plasma, urine and human breast milk. Urine samples were directly injected, while plasma and milk samples were injected after a pretreatment and clean-up procedure to remove the interfering substances found in both matrices, which hinder the chromatographic analysis. The limits of detection for the determination of TZ in human urine, plasma and breast milk were 35.6, 48.7 and 44.7 ng/mL, respectively and limits of quantitation for determination of TZ in human urine, plasma and breast milk were 118.7, 162.4 and 149.0 ng/mL, respectively.

Experimental

Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12A degasser, Rheodyne 7725i injector with a 20 μL loop and SPD-10AVP ultraviolet–visible (UV–Vis) detector.

A Werk Nr type centrifuge (Hettich, Germany) was used in the clean-up procedure of plasma and milk samples.

Materials and reagents

Pharmaceutical grade NX and nifuroxazide (NF) were used and certified to contain 99.9 and 99.8%, respectively.

TZ (>99.9%) was prepared by acidic hydrolysis of NX (200 mg of TZ was refluxed with 100 mL, 1M hydrochloric acid at 100°C for 24 h) and recrystallized four times with chloroform and characterized by proton magnetic resonance, MS, infrared and HPLC–UV. Acetonitrile and methanol used were HPLC grade (BDH, Poole, UK). Sodium hydroxide, ammonium acetate and diethylamine (Sigma-Aldrich, St. Louis, MO), orthophosphoric acid (BDH Laboratory Supplies, Poole, UK)

and acetic acid (Riedel-de Haën Laboratory Chemicals, Germany), *n*-hexane (Sigma-Aldrich), and dimethyl sulfoxide (Sigma-Aldrich) were used.

Commercial Nitazode emulsion (Batch No. 71783) was manufactured by Sigma Pharmaceutical Industries for Alandalous Medical Co., and labeled to contain 100 mg NX / 5 mL.

HPLC conditions

The HPLC separation and quantitation were performed on a 250 × 4.6 mm (i.d.) Luna 5-μm CN column (Phenomenex, Torrance, CA). The mobile phase was prepared by mixing acetonitrile, 12 mM ammonium acetate and diethylamine in a ratio of 30:70:0.1 (v/v/v) and adjusted to the apparent pH 4.0 using acetic acid. The flow rate was 1.5 mL/min. All determinations were performed at an ambient temperature of 25°C. The injection volume was 20 μL. The detector was set at 260 nm. Data acquisition was performed on class-VP software, which is a Shimadzu specification.

Standard solutions

Stock standard solutions were prepared by separately dissolving NX in acetonitrile, TZ in dimethylsulphoxide and NF in methanol to obtain a concentration of 100 μg/mL. Further dilutions for TZ with dimethylsulphoxide were carried out to obtain concentration ranges of 0.2–20 μg/mL. These stock solutions were stored at 4°C in the dark.

Urine sample

Different volumes of TZ stock standard were transferred to a 10-mL volumetric flask, 1 mL of blank urine was added and the solutions were diluted to 10 mL with the mobile phase to obtain a concentration range of 0.2–20 μg/mL. All solutions contained 10 μg/mL NF as internal standard (IS). The solutions were filtered through 0.45-μm disposable membrane filters. Triplicate 20-μL injections were made for each concentration of TZ and chromatographed under the conditions described previously. The peak area ratios (peak area of TZ/peak area of NF) were determined for each concentration and plotted against the corresponding concentration to obtain the calibration graph of TZ.

Standard solutions and extraction procedure for plasma samples

Before the samples were injected into the chromatograph, protein denaturing and precipitation procedures were carried out. Standard solution of TZ was further diluted 10-fold with drug-free human plasma to obtain a concentration range of 0.2–20 μg/mL. The spiked plasma samples were kept in an ice bath the entire time.

Into 2.0-mL Eppendorf tubes, 0.5 mL of acetonitrile and 10 μg/mL NF as IS were added to a spiked plasma sample (0.5 mL), then vortexted for 20 s and centrifuged for 10 min at 3,000 rpm and 4°C. The upper layer was transferred to another tube, filtered through a 0.45-μm Millipore filter, evaporated by nitrogen at ambient temperature and then reconstituted with 500 μL mobile phase; 20 μL were injected on the analytical column for analysis using the same analytical mobile phase. The peak area ratio of each concentration was plotted against the corresponding concentration to obtain the calibration graph of TZ.

Standard solutions and extraction procedure for milk samples

Standard solution of TZ was further diluted 10-fold with drug-free human milk to obtain the concentration range of 0.1–20 μg/mL. Into 2.0-mL Eppendorf tubes, 10 μg/mL NF as IS and concentrated orthophosphoric acid (50 μL) was added to a spiked milk sample (0.5 mL). The mixture was shaken for 5 s, followed by addition of 500 μL of acetonitrile. The mixture was vortexed for approximately 10 s at high speed and then centrifuged for 5 min at 4,000 rpm and 4°C. The supernatant solution was decanted into another 15-mL glass centrifuge tube and 500 μL of *n*-hexane was added. The mixture was vortexed for approximately 10 s at high speed and then centrifuged for 5 min at 4,000 rpm and 4°C. The upper, organic layer was removed using a Pasteur pipette. The aqueous layer was transferred to another tube filtered through a 0.45-μm Millipore filter, evaporated by nitrogen at ambient temperature and then reconstituted with 500 μL mobile phase; 20 μL were injected on the analytical column for analysis using the same analytical mobile phase. The peak area ratio of each concentration was plotted against the corresponding concentration to obtain the calibration graph of TZ.

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 Faculty of Pharmacy, Suez Canal University, approved the study.

An excretion study of TZ was carried out on a normal, healthy (normal liver, kidney functions and electrocardiogram), male, informed adult volunteer (28 years, 85 kg, 178 cm height), with no past history of allergic reaction to NX. The volunteer was instructed to abstain from all medications for two weeks before administration and also during the study. Also, the volunteer was instructed to be sure of evacuating his bladder as thoroughly as possible exactly before the administration of 25 mL NX emulsion (500 mg) with food.

Urine sample

This method was used to investigate the pattern of urinary excretion of TZ. The 0-h urine sample was collected as a blank. Urine samples were collected at 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°C until determination.

A suitable volume of the urine specimen from each sampling point was alkalinized with an equal volume of 0.1M sodium hydroxide and sonicated for 15 min. The mixture was neutralized by the addition of 0.1M hydrochloric acid. Suitable dilution was carried out to 10 mL with the mobile phase to reach calibration range. Each sample solution contained 10 μg/mL NF 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 for TZ.

Plasma samples

Blood samples were collected into tubes containing disodium EDTA. Samples were centrifuged at 4,500 rpm for 10 min. The resultant plasma (0.5 mL) was mixed with 0.5 mL of acetonitrile, 10 μg/mL NF and 0.5 mL of 0.1M sodium hydroxide. This mixture was sonicated for 15 min. The mixture was neutralized by addition of 0.1M hydrochloric acid. The extraction procedure described previously was followed and the concentration of TZ was calculated using regression equation.

Milk samples

The human breast milk samples were obtained from a normal, healthy, breastfeeding, informed female volunteer (30 years, 65 kg, 170 cm height). The volunteer was instructed to abstain from all medications for two weeks before the study. The 0-h breast milk sample was collected as a blank. Breast milk samples were collected and the large pooled sample of human breast milk was homogenized and stored in −20°C until analysis. The breast milk sample (0.5 mL) was mixed with 10 μg/mL NF as IS and concentrated orthophosphoric acid (50 μL), then the extraction procedure described previously was followed and the concentration of TZ was calculated using regression equation.

Results and Discussion

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

Complex biological matrices like urine, plasma and milk are considered to be a powerful challenge for any analyst. High protein, fat and carbohydrates are the primary components of these matrices, which produce high interferences with the drugs to be determined and affect the method performance. Owing to this, a special procedure is required for sample preparation and clean-up procedure, particularly because of the high protein and lipid content found in milk samples.

Deproteinization and extraction

Because TZ is present primarily in the conjugated form in human urine and plasma (5), and because of the lack of a conjugate TZ reference standard, hydrolysis of the conjugate form to the free form is needed for the precise determination of the total TZ found in urine and plasma. Various hydrolysis conditions were investigated, including hydrolysis time and the concentration of acid or alkali. It was found that alkaline hydrolysis was preferable to the acidic hydrolysis, because alkaline hydrolysis is faster. Hydrolysis of the conjugated TZ was completed by using 0.5 mL of 0.1M sodium hydroxide within 15 min at ambient temperature.

Most of the methods reported for determination of drugs in plasma and tissue involve protein precipitation with an organic solvent. Commonly used organic solvents are acetonitrile, ethanol and methanol; acetonitrile is the most often used and gives a higher percent recovery of TZ from plasma protein.

A combination of acetonitrile and sodium hydroxide was found to be ineffective in precipitating proteins from breast milk spiked samples with TZ and gives poor recovery of this drug.

The second approach for precipitation of protein from breast milk spiked samples with TZ was built on the use of a combination of acetonitrile and acid. Either phosphoric acid or hydrochloric acid was found to be effective in precipitating proteins from milk to give a clean supernatant suitable for analysis by HPLC. The use of phosphoric acid was preferable to hydrochloric acid because of the possible adverse effect of hydrochloric acid on the column material. Formic acid was examined for precipitation of milk proteins with acetonitrile and found to give incomplete protein precipitation. After treating the milk sample with acetonitrile and phosphoric acid, *n*-hexane was used for removing milk fats. *n*-Hexane is a better extraction solvent, because it leads to fewer interfering peaks in the chromatograms with good recovery of TZ.

Chromatographic conditions

The development of a liquid chromatographic method requires a careful combination among the polarities of the analyte, stationary phase and mobile phase to obtain good separations in reasonable times.

Furthermore, when performing analysis of biological fluids, especially with complex matrices such as urine, plasma and human breast milk, it is necessary to have an efficient chromatographic separation between endogenous components and compounds of interest.

To optimize the HPLC assay parameters, the effects of acetonitrile concentration and pH of the mobile phase on the capacity factor (K′) were studied.

The influence of acetonitrile concentration of the mobile phase on the separation efficiency between NF, NX and TZ and endogenous compounds from different biological samples was investigated at pH 4. It was found that decreasing the concentration of acetonitrile to less than 20% caused a severe increase in the retention time of the TZ peak that resulted in band broadening and excessive tailing, whereas increasing the concentration of acetonitrile more than 55% caused a decrease in retention time that led to inadequate separation of the three peaks and biological matrices. The best separation was obtained by using 30% acetonitrile.

The influence of pH of the mobile phase on the retention behavior of NF, NX TZ and endogenous compounds of biological samples was investigated. It was found that variation of the pH of the mobile phase resulted in a maximum K′ value at pH 6 with loss of peak symmetry for TZ. At lower pH values (less than 3), poor resolution for TZ, NX, NF and different biological matrices was observed. At pH 3.5–4.5, improved resolutions of the three peaks were observed; however, at pH 4.0, optimum resolution with reasonable retention time was affected.

The effect of the concentration of ammonium acetate in the mobile phase was studied between 12 and 50 mM. The lowest concentration of ammonium acetate (12 mM) was selected because results were similar to those obtained at other concentrations and it was less damaging to the column.

Diethylamine was added as organic amine modifier to the aqueous–organic eluent to improve the sharpness of the TZ peak.

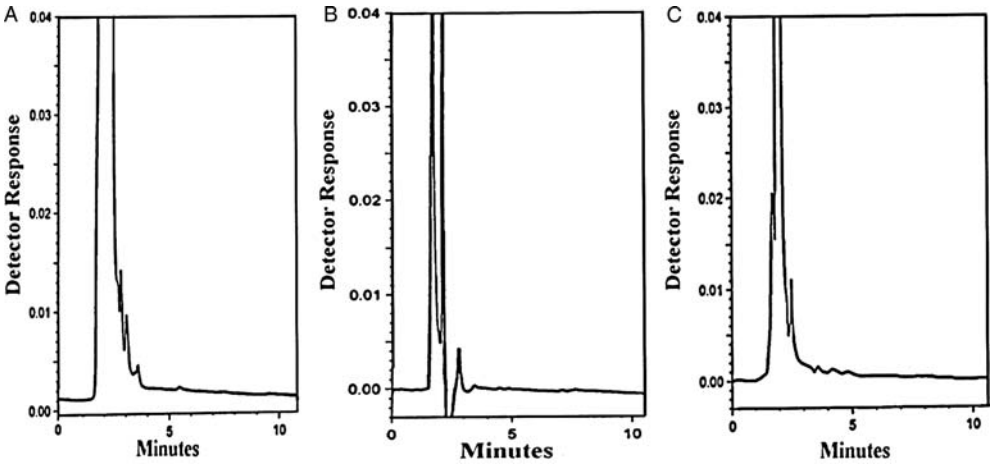

Figure 1. Typical HPLC chromatograms obtained from analysis of: blank human urine (A); blank human plasma (B); blank human breast milk (C).

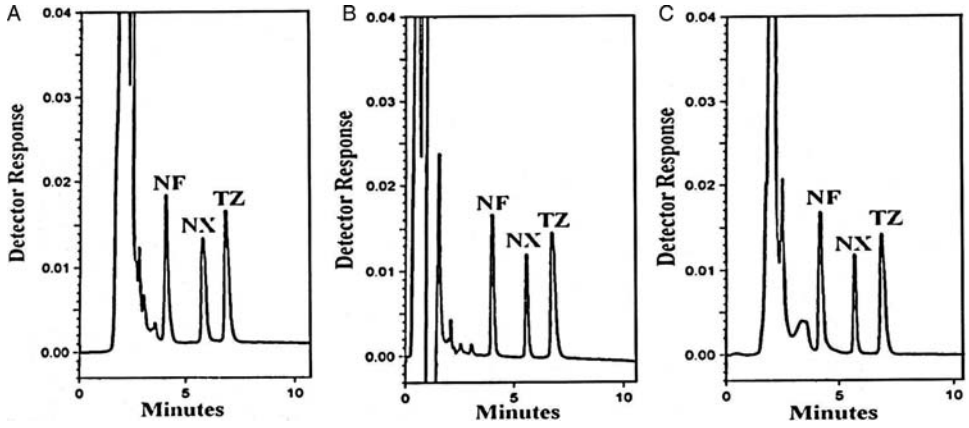

Figure 2. Typical HPLC chromatograms obtained from analysis of 10 μg/mL NF, 10 μg/mL NX and 10 μg/mL TZ spiked to: human urine (A); human plasma (B); human breast milk (C).

Chromatograms of blank human urine, plasma and breast milk (Figures 1A, 1B and 1C, respectively) showed no interfering peaks at the retention times of NF, NX and TZ. Figures 2A, 2B and 2C show typical chromatograms for the samples prepared from human urine, plasma and breast milk containing NF, NX and TZ in which the drugs were well separated. The average retention times ± standard deviation (SD) for NF, NX and TZ were found to be 4.15 ± 0.06, 5.54 ± 0.02 and 7.10 ± 0.04 min, respectively, for seven replicates. The peaks obtained were sharp and had clear baseline separation. The system suitability results are given in Table I.

The performance of the proposed method on real samples was demonstrated by its application to a human urine sample taken from a male volunteer who received Nitazode medication. Figure 3A 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 7.8 μg/mL after 4 h.

The performance of the proposed method on real samples was demonstrated by its application to a human plasma sample obtained from a volunteer patient who received Nitazode medication. The concentration of TZ in the plasma sample was found to be 1.9 μg/mL after 2 h.

Table I
System Suitability Test Results of the Developed Method for Determination of NF, NX and TZ*

Comp.	Retention time (min)	Retention factor (K′)	Selectivity α	Resolution Rs	Tailing factor	RSD (%) of retention time	Plate count
NF	4.15	5.83	1.39 (a_1)	2.94 (b_1)	1.08	1.43	68032
NX	5.54	8.11	1.32 (a_2)	1.56 (b_2)	1.20	0.43	59143
TZ	7.10	10.68	—	—	1.05	0.59	54315

*The retention time of unretained peak is 0.608 min; a_1 and b_1 are α and Rs calculated for NF–NX; a_2 and b_2 are α and Rs calculated for NX–TZ.

Also, the performance of the proposed method on a real human breast milk sample was assessed with the help of a breastfeeding mother who agreed to receive Nitazode medication. The concentration of TZ in the breast milk sample was found to be 1.4 μg/mL after 6 h.

Urinary excretion pattern of TZ

Because NX is rapidly and completely absorbed and metabolized into TZ, which is partially excreted in human urine, the

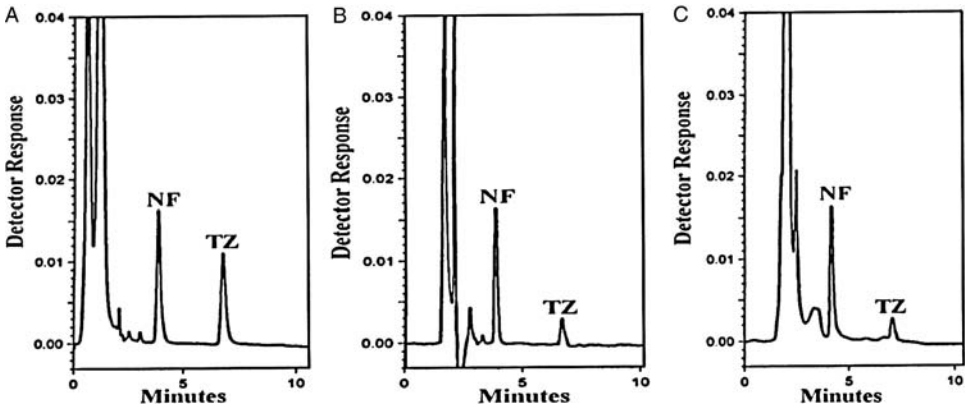

Figure 3. Typical HPLC chromatograms obtained after oral dosing of 500 mg of NX emulsion from analysis of real samples of: human urine (4 h) (A); human plasma (2h) (B); human breast milk (6 h) (C).

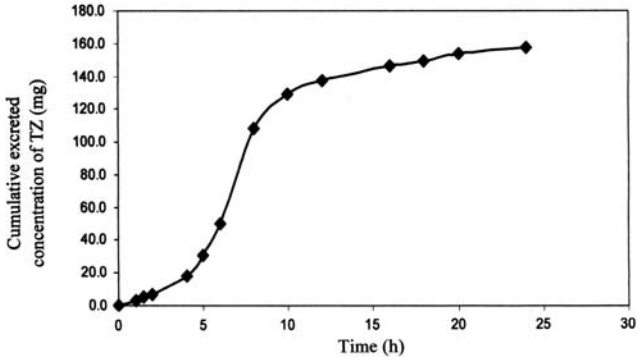

Figure 4. Cumulative excretion of TZ in the urine of a healthy human male volunteer after administration of a single oral dose of 500 mg NX.

clinical applicability of the method for determination of the cumulative amount of TZ excreted in urine after oral administration of 500 mg NX emulsion was investigated, which reflects the bioavailability of NX. The cumulative amount of TZ excreted in urine was found to be 157.6 mg after 24 h following oral administration of a 25-mL Nitazode emulsion containing 500 mg TZ (Figure 4).

Validation

Linearity

The linearity of the proposed method was evaluated by analyzing seven concentrations of TZ ranging between 0.2 and 20 μg/mL in different biological matrices. Each concentration was repeated three times. The assay was performed according to previously established experimental conditions. Calculations of calibration curves were based on the peak area ratios of TZ to the IS, NF. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p = 0.05$) different from zero (Table II).

Characteristic regression data for the method, obtained by least squares treatment of the results, are given in Table II.

Precision

Precision was determined to judge the quality of the method. The precision of the method, expressed as coefficient of variation (CV) (%), was determined by analysis of three different concentrations within the linearity range for TZ in urine, plasma and breast milk samples. Intra-day precision was assessed from the results from five replicate analyses of each sample on the same day. Inter-day precision was determined by analysis of the samples on five consecutive days. The CV (%) for intra-day and inter-day assays for human urine, plasma and breast milk samples was less than 2. The results obtained from the determination of intra-day and inter-day precision are listed in Table III.

Range

The calibration range was established by consideration of the practical range necessary to give accurate, precise and linear results. Calibration ranges for the proposed HPLC method are given in Table II.

Detection and quantitation limits

In accordance with ICH recommendations (10), the approach based on the SD of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were practically assessed and given in Table II.

Selectivity and accuracy

Method selectivity and accuracy were achieved by addition of appropriate volumes of standard solution of TZ (different concentrations, but within the linearity range concentration) to urine, plasma and breast milk samples obtained 2, 4 and 6 h after a single administration of a commercially available NX suspension. After spiking with a fixed concentration of NF IS and analyzing according to the previous procedure, satisfactory results were obtained (Table IV) for TZ, indicating the high selectivity and accuracy of the proposed method. No interference resulted from biological matrices.

Robustness

The effect of percent of organic strength on resolution was studied by varying acetonitrile from 28 to 32%. The effect of

Table II
Characteristic Parameters of the Calibration Equations for the Proposed HPLC Method for the Determination of TZ in Human Urine, Plasma, and Breast Milk

Parameters	TZ in human urine	TZ in human plasma	TZ in human milk
Calibration range (μg/mL)	0.2–20	0.2–20	0.2–20
Detection limit (μg/mL)	3.56×10^{-2}	4.87×10^{-2}	4.47×10^{-2}
Quantitation limit (μg/mL)	11.87×10^{-2}	16.24×10^{-2}	14.90×10^{-2}
Regression equation (Y)* Slope (b)	9.39×10^{-2}	9.32×10^{-2}	8.90×10^{-2}
SD of the slope (S_b)	1.43×10^{-3}	1.94×10^{-3}	0.19×10^{-2}
RSD of the slope (%)	1.52	2.08	2.09
Confidence limit of the slope†	9.26×10^{-2} -9.53×10^{-2}	9.13×10^{-2} -9.51×10^{-2}	8.72×10^{-2} -9.08×10^{-2}
Intercept (a)	1.22×10^{-2}	1.88×10^{-2}	-0.20×10^{-2}
SD of the intercept (S_a)	1.48×10^{-2}	2.01×10^{-2}	1.92×10^{-2}
Confidence limit of the intercept†	$(-0.22 \times 10^{-2}) - (2.65 \times 10^{-2})$	$(-0.71 \times 10^{-3}) - (3.83 \times 10^{-2})$	$(-2.07 \times 10^{-2}) - (1.67 \times 10^{-2})$
Correlation coefficient (r)	0.9998	0.9997	0.9997
Standard error of estimation	1.04×10^{-2}	1.41×10^{-2}	1.35×10^{-2}

*$Y = a + bC$, where Y is the response (peak area ratio), a is the intercept, b is the slope, and C is the concentration of TZ (μg/mL) in human urine, plasma and breast milk.
†95% confidence limit.

Table III
Intra-Day and Inter-Day Precision for the Assay of TZ in Human Urine, Plasma and Breast Milk

Concentration (μg/mL)	Intra-day precision						Inter-day precision					
	Human urine		Human plasma		Human milk		Human urine		Human plasma		Human milk	
	Recovery (%)*	CV (%)	Recovery (%)*	CV (%)	Recovery (%)*	CV (%)	Recovery (%)*	CV (%)	Recovery (%)*	CV (%)	Recovery (%)*	CV (%)
0.2	98.74 ± 0.98	1.00	96.28 ± 0.38	0.40	96.18 ± 1.18	1.22	97.88 ± 1.43	1.46	96.04 ± 1.21	1.26	95.80 ± 1.43	1.50
10.00	98.16 ± 1.32	1.35	101.30 ± 0.61	0.60	95.78 ± 0.94	0.98	98.94 ± 1.37	1.38	95.16 ± 1.73	1.81	95.66 ± 1.54	1.61
20.00	98.82 ± 0.75	0.76	95.32 ± 1.32	1.39	95.96 ± 1.36	1.42	101.20 ± 0.30	0.29	101.90 ± 1.58	1.55	95.12 ± 1.48	1.56

*Mean ± SD from five determinations.

Table IV
Accuracy of the HPLC Method Determined by Recovery of TZ from Real Human Urine, Plasma, and Breast Milk Samples Spiked with TZ

Sample number	Concentration of TZ (μg/mL)			Added concentration (μg/mL)			% Recovery of added		
	Urine	Plasma	Milk	Urine	Plasma	Milk	Urine	Plasma	Milk
1	3.20	1.90	0.21	0.5	0.50	0.50	97.8	96.7	93.2
2	3.10	1.86	0.20	1.00	1.00	1.00	98.6	96.5	94.5
3	7.86	1.10	0.85	5.00	5.00	5.00	96.8	95.8	94.8
4	7.70	1.15	0.81	10.00	10.00	10.00	98.5	96.1	94.6
5	11.90	0.28	1.40	5.00	15.00	15.00	98.9	97.3	95.6
6	12.00	0.32	1.44	6.00	16.00	16.00	97.8	96.9	95.7
Mean							98.07	96.55	94.73
SD							0.76	0.54	0.91
CV (%)							0.78	0.56	0.96

the pH of the mobile phase on resolution was studied by varying the pH from 3.8 to 4.2 and the flow rate of the chromatographic method was changed from 1.3 to 1.7.

In all of the deliberately varied chromatographic conditions (flow rate, percentage organic strength and pH of the mobile phase), the resolution between NF, NX and TZ and other components in the biological matrices was not altered.

Stability

In anticipation of unexpected delays during analysis, it is important to have information about the stability of all solutions. When the stability of TZ and the IS in the mobile phase was tested, it was found the samples were stable for at least 4 h at room temperature. Urine, plasma and breast milk samples spiked with TZ were evaluated for stability after freezing and thawing. The drug was stable through at least three freeze–thaw cycles. The stability of spiked urine, plasma and breast milk samples during storage for 4 weeks at −20°C was also evaluated; no significant change was observed. The spiked urine, plasma and breast milk samples were found to be stable for at least 4 h at room temperature.

Conclusion

An optimized reversed-phase HPLC method was developed for direct quantitative analysis of TZ, the main active metabolite of NX in human urine, without any extraction procedure before the separation. The urinary excretion pattern of the metabolite was easily established. Also, a suitable procedure for preparation of samples with a subsequent analytical method was developed for reliable drug determination in complex and variable biological matrices such as plasma and breast milk. The proposed HPLC method provides simple, accurate, sensitive and robust determination of TZ in different and complex biological matrices.

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References

1. Diaz, E., Montage, J., Ramirez, E., Bernai Am, R.; Nitazoxanide: An important advance in anti-parasitic therapy; *Journal of Tropical Medicine and Hygiene*, (2003); 68: 384–385.
2. Raether, W., Hänel, H.; Nitroheterocyclic drugs with broad spectrum activity; *Parasitology Research*, (2003); 90: S19–S39.
3. Physicians' Desk Reference. Thomson PDR, Montvale, NJ, (2005); pp. 2958–2959.
4. Rossignol, J.F., Stachulski, A.; Syntheses and antibacterial activities of tizoxanide, an *N*-(nitrothiazolyl)salicylamide, and its *O*-aryl glucuronide; *Journal of Chemical Research*, (1999); (S): 44–45.
5. Broekhuysen, J., Stockis, A., Lins, R.L., De Graeve, J., Rossignol, J.F.; Nitazoxanide: Pharmacokinetics and metabolism in man; *International Journal of Clinical Pharmacology and Therapeutics*, (2000); 38: 387–394.
6. Zhao, Z., Zhang, L., Xue, F., Wang, X., Zheng, W., Zhang, T., etc.; Liquid chromatography–tandem mass spectrometry analysis of nitazoxanide and its major metabolites in goat; *Journal of Chromatography B*, (2008); 875: 427–436.
7. Zhao, Z., Zhang, L., Xue, F., Zhang, T.; Metabolic profile of nitazoxanide in goat feces; *Chromatographia*, (2008); 68: 731–738.
8. Agarwal, S., Solomon, W.D., Gowda, K.V., Selvan, P.S., Ghosh, D., Sarkar, A.K., etc.; Bioequivalence study of a fixed dose combination of nitazoxanide and ofloxacin in Indian healthy volunteers; *Arzneimittelforschung*, (2007); 57: 679–683.
9. Namur, S., Cariño, L., González-De La Parra, M.; Development and validation of a high-performance thin-layer chromatographic method, with densitometry, for quantitative analysis of tizoxanide (a metabolite of nitazoxanide) in human plasma; *Journal of Planar Chromatography—Modern TLC*, (2007); 20: 331–334.
10. The European Agency for the Evaluation of Medical Products; ICH Topic Q2B Note for Guidance on Validation of Analytical Procedures: Methodology GPMP/ICH/281/95, (1996).