

*Review Article*

**Nitazoxanide: A Review of Analytical Methods.**

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**ABSTRACT**

Nitazoxanide is an antiprotozoal and anthelmintic agent, which is mostly used in the treatment and prevention of wide variety of protozoa, helminthes and gram negative organisms. This article studies published analytical techniques that are reported so far for the determination of nitazoxanide in bulk, pharmaceutical formulation and biological samples. They include various techniques like spectrophotometry, electrochemical methods, capillary electrophoresis, high performance liquid chromatography, high performance thin layer chromatography, and liquid chromatography-mass spectrophotometry.

**KEYWORDS**

Nitazoxanide, Analytical methods, antiprotozoal, anthelmintic.

## 1. INTRODUCTION

Nitazoxanide (NTZ) is a new antiparasitic and antiprotozoal agent having broad spectrum of activity. It is a nitrothiazole derivative and its chemical name is 2-acetyloxyl-N-(5-nitro-2-thiazolyl) benzamide (Fig. 1) (1). It was initially developed as a veterinary anthelmintic with activity against intestinal nematodes, cestodes and trematodes. NTZ was approved by the US Food and Drug Administration (FDA) in 2002 for use in human beings (2). It is used for treating both intestinal protozoal infections and helminthiasis (3). It is also used for treating diarrhea caused by *Giardia lamblia* as well as for cryptosporidiosis in immune-compromised patients, including those with AIDS or HIV infection (4, 5, 6, 7, 8). The antiprotozoal activity of NTZ is believed to be due to interference with the pyruvate ferredoxin oxidoreductase (PFOR) enzyme dependent electron transfer reaction which is essential to anaerobic energy metabolism. Studies have shown that the PFOR enzyme from *Giardia lamblia* directly reduces nitazoxanide by transfer of electrons in the absence of ferredoxin. The DNA-derived PFOR protein sequence of *Cryptosporidium parvum* appears to be similar to that of *Giardia lamblia*. Interference with the PFOR enzyme-dependent electron transfer reaction may not be the only pathway by which nitazoxanide exhibits antiprotozoal activity. NTZ is a light yellow/ pink crystalline powder which is insoluble in water and poorly soluble in ethanol. It has a molecular mass of 307.283 g/mole and molecular formula of  $C_{12}H_9N_3O_5S$  (9). After ingestion, it is converted to the active metabolites tizoxanide and tizoxanide glucuronide. In plasma, more than 99% of NTZ is bound to proteins. It is available in the market as tablets and oral suspension.

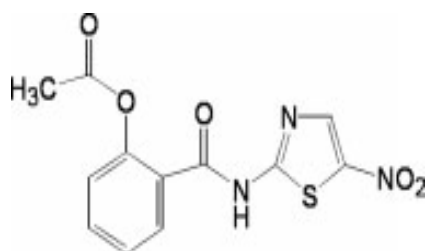


Fig. 1: Structure of Nitazoxanide.

## 2. MATERIALS AND METHODS

### 2.1. Solubility

According to Biopharmaceutical Classification System (BCS), nitazoxanide is a class IV drug (low solubility and low permeability). It is slightly soluble in acetone, chloroform and very slightly soluble in methanol and practically insoluble in water. The melting point of nitazoxanide is 202°C. (10)

### 2.2. Sample preparation strategies

Sample preparation is the integrated part of analytical methodology, and it was reported that approximately about 30% errors contributed from sample analysis was due to sample preparation. Various diluents used for the analysis of nitazoxanide include acetonitrile:0.2M potassium dihydrogen

phosphate 70:30 (pH 3.0 adjusted with o-phosphoric acid), acetonitrile; 0.3M potassium dihydrogen phosphate: methanol 70:10:20 (pH 3.5 adjusted with o-phosphoric acid), 1N hydrochloric acid, methanol, ethanol, sodium hydroxide, ammonia acetate, glacial acetic acid, acetonitrile, chloroform, ammonia solution. Solvent used are 1, 4-dioxane, dimethyl formamide (DMF), acetonitrile, ethanol. The sample preparation technique for the extraction of nitazoxanide from the biological matrices like plasma, serum, urine, liver, kidney and brain was by deproteination with acetonitrile, ethanol and followed by centrifugation.

*2.3. Analytical Methods*

*2.3.1. Spectrophotometry*

In the literature, 9 methods were reported for the estimation of NTZ using spectrophotometry, of which 7 methods are for determining NTZ alone, whereas the remaining are for quantifying NTZ in combination with other drugs substance.

Table 1 shows the summary of the reported spectroscopic methods indicating the basic principle,  $\lambda$  max, solvent, limit of detection (LOD) and limit of quantification (LOQ).

**Table 1.**

<b>Compounds</b>	<b>Method</b>	<b><math>\lambda</math> max (nm)</b>	<b>Solvent</b>	<b>LOD (<math>\mu</math>g/ml)</b>	<b>LOQ (<math>\mu</math>g/ml)</b>	<b>Ref.</b>
<b>NTZ</b>	Hyposchromic shift based method	343.5	Methanol:0.1M citric acid (80:20)	0.12	0.39	11
<b>NTZ in dosage form</b>	Spectrophotometry	238.3	Acetonitrile:water(9:1)	-	-	12
<b>NTZ</b>	Spectrophotometry	732	1ml ferric chloride (1%) and 2ml MBTH (0.1%)	0.1147	0.3824	12
<b>NTZ</b>	Simultaneous Equations Method	218.5	Ferric chloride	0.7653	0.8796	13
<b>NTZ</b>	First derivative spectroscopy	277	Ferric chloride	1.2374	1.1134	
<b>NTZ</b>	Second derivative spectroscopy	260,314	Ferric chloride	1.6543	1.2467	
<b>NTZ</b>	spectrophotometry	344	ethanol	0.907	0.299	14
<b>NTZ, Ofloxacin</b>	Q-analysis method	346.36	1NHCl in methanol	-	-	15
<b>NTZ, Ofloxacin</b>	Vierodts method	346.3,296.49	1N HCl in methanol	-	-	15
<b>NTZ, Ofloxacin</b>	Dual wavelength method	333.6, 359.2, 302.4, 289.2	1N HCl in methanol	-	-	15

*2.3.2. Electrochemical methods*

The determination of electrochemical behavior of NTZ was studied using voltammetry. The authors used hanging mercury electrode as sensor for the NTZ in Britton-Robinson universal buffer of pH values 2 to 11. Methods used for voltammetric determination are cyclic and square-wave voltammetry, Cyclic voltammetric Square wave cathodic adsorptive stripping voltammetry (SW-CAdSV) and Differential pulse cathodic adsorptive stripping voltammetry (DP-CAdSV), linear sweep cathodic adsorptive stripping voltammetry (LS-CAdSV), differential pulse polarography (DPP).

**Table 2.**

<b>Compounds</b>	<b>Method</b>	<b>Linear response</b>	<b>Correlation coefficient</b>	<b>LOD (µg/ml)</b>	<b>LOQ (µg/ml)</b>	<b>Ref.</b>
<b>NTZ in pharmaceutical formulation</b>	cyclic and square-wave voltammetry	20–140 mg/mL	0.9938	5.23 µg/mL	17.45 µg/mL	16
<b>NTZ in human serum</b>	LS-CAdSV DP-CAdSV SW-CAdSV	3×10 <sup>-9</sup> to 5×10 <sup>-9</sup> mol L <sup>-1</sup> , 1×10 <sup>-7</sup> mol L <sup>-1</sup> , 1×10 <sup>-9</sup> to 1×10 <sup>-7</sup> mol L <sup>-1</sup>	0.985, 0.990, 0.999	9x10 <sup>-10</sup> , 1.5×10 <sup>-9</sup> , 3×10 <sup>-10</sup> mol L <sup>-1</sup>	3×10 <sup>-9</sup> , 5×10 <sup>-9</sup> , 1×10 <sup>-9</sup> mol L <sup>-1</sup>	17
<b>NTZ in human urine</b>	DP-CAdSV, SW-CAdSV	1×10 <sup>-9</sup> to 1×10 <sup>-8</sup> mol L <sup>-1</sup>	0.9965, 0.9985	2.078×10 <sup>-10</sup> , 1.365×10 <sup>-10</sup> mol L <sup>-1</sup>	4.551×10 <sup>-10</sup> , 6.926×10 <sup>-10</sup> mol L <sup>-1</sup>	18
<b>NTZ in human breast milk</b>	DP-CAdSV, SW-CAdSV	1×10 <sup>-8</sup> to 1×10 <sup>-9</sup> mol L <sup>-1</sup>	0.9999, 0.9993	0.601×10 <sup>-10</sup> mol L <sup>-1</sup> , 0.718×10 <sup>-10</sup> mol L <sup>-1</sup>	2.00×10 <sup>-10</sup> mol L <sup>-1</sup> , 2.393×10 <sup>-10</sup> mol L <sup>-1</sup>	18
<b>NTZ in bulk form</b>	DP-CAdSV, SW-CAdSV	1x10 <sup>-9</sup> to 1x10 <sup>-8</sup> mol L <sup>-1</sup>	0.9961, 0.999	1.878x10 <sup>-10</sup> mol L <sup>-1</sup> , 1.078x10 <sup>-10</sup> mol L <sup>-1</sup>	of 6.262 x10 <sup>-10</sup> mol L <sup>-1</sup> , 3.595x10 <sup>-10</sup> mol L <sup>-1</sup>	18

Shital Gandhi *et al* (19) developed a simple, sensitive and highly selective electrochemical method for the simultaneous determination of NTZ and ofloxacin in aqueous media (Britton-Robinson buffer, pH-8.36) on a hanging mercury drop electrode (HMDE) using differential pulse polarography (DPP). Using DPP a separation of about 936 mV between the peak oxidation potentials of nitazoxanide and ofloxacin present in binary mixtures was obtained. The quantification limits for the simultaneous determination of NTZ and ofloxacin were 0.083 µg/ml and 0.208 µg/ml.

## 2.4. Chromatography

### 2.4.1. HPLC

#### 2.4.1.1. Biological samples

A high-performance liquid chromatographic method was optimized and validated for the determination of desacetyl NTZ (tizoxanide), the main active metabolite of NTZ in human plasma, urine and breast milk by Ghada M.*et al* (20). 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. Human urine sample was prepared by alkalizing with 0.1M sodium hydroxide sonicated for 15 min and neutralized with 0.1M hydrochloric acid. The LOD was found to be 3.56 and LOQ was found to be 11.87. Human breast milk sample was prepared by homogenizing and mixing with nifuroxazide and orthophosphoric acid. The LOD was found to be 4.47 and LOQ was found to be 14.9. Human plasma was prepared by mixing with acetonitrile, NF 0.1M sodium hydroxide. It is sonicated for 15 min., neutralized by 0.1M hydrochloric acid. The LOD 4.8 was found to be and LOQ was found to be 16.24.

#### 2.4.1.2. Pharmaceutical samples

Analytical methods for the determination of isoniazid in pharmaceutical dosage forms using HPLC

**Table 3.**

Mobile phase	Column	Detection	$\lambda$ max (nm)	Flow rate	LOD	LOQ	Ref.
<b>Study aim: Quantification of NTZ in presence of its alkaline degradation product</b>							
<b>acetonitrile: 50 mM ammonium acetate buffer (50:50, v/v, pH 5.0 adjusted with acetic acid)</b>	Inertsil C8-3 column (150 × 4.6 mm i.d.)	UV	298 nm	1 mL/min.	0.0410	0.1242 µg/mL	21
<b>Study aim: Isocratic reverse phase high pressure liquid chromatographic Method for the simultaneous determination of NTZ and Ofloxacin from combined dosage form</b>							
<b>2.0gm</b>	Phenomen	UV	305 nm	1.5	0.174µg/	0.034µ	22

**sodium dihydrogen phosphate and 5M triethylamine are mixed into 500mL Milli Q water and pH was adjusted to 4.5 by orthophosphoric acid**

ex Luna mL/min ml and g/ml  
 C18 0.21µg/ and  
 reversed- ml 0.08µg/  
 phase ml  
 column

**Study aim: Simultaneous determination of NTZ and ofloxacin**

**Tetrabutyl ammonium hydrogen sulphate: methanol: acetonitrile (20:20:60)**

RP C-18 UV 282nm, 0.5ml/m 0.00013 0.0001 23  
 column 278 nm in 06, 306,  
 (Shimadzu liquid 6.183 6.183  
 chromatog  
 raph LC-  
 10ATVP)

**Study aim: Simultaneous determination of NTZ and ofloxacin in pharmaceutical preparation.**

**acetonitrile: 0.2M potassium dihydrogen phosphate in ratio 70:30 (pH 3.0 adjusted with orthophosphoric acid)**

Hypersil UV 319nm 1.0ml/m - - 24  
 BDS C8 in  
 column  
 (5µ  
 particles  
 size) (250  
 mm X 4.6  
 mm)

**Study aim: Determination of NTZ in oral suspension dosage form**

**mixture of acetonitrile: ammonium dihydrogen phosphate buffer (0.075 M) in the ratio 45:55 (% v/v) adjusted to pH 3.0 with orthophosphoric acid**

Qualisil UV 240 nm 1.5 0.25 0.77 25  
 BDS C18 mL/min  
 (4.6 ×  
 250mm,  
 5µ)

**Study aim: Simultaneous Estimation of NTZ and Ofloxacin from Tablet Dosage**

<b>acetonitrile:</b>	Princeton	UV	265nm	1.0ml/m	26
<b>potassium</b>	SPHER			in	
<b>dihydrogen ortho</b>	C18				
<b>phosphate (pH</b>	column				
<b>4.5,</b>	(250mm×/				
<b>10mM) (60:40</b>	4.6 mm				
<b>v/v)</b>	i.d.)				

T. Sakamoto *et al* (27) developed a simple and rapid determination method for NTZ using reverse-phase HPLC and Ultra Performance Liquid Chromatography (UPLC).

Mobile phase consisted of a mixture of phosphate buffer (pH 6.0) and acetonitrile HPLC System-HPLC Shimadzu Class-VP HPLC system. A Waters symmetry C18 Column (150 mm\_4.6 mm I.D., 5 mm particle size, Waters Co., Milford, MA, USA),

The UPLC SYSTEM- A waters ACQUITY UPLC system. C18 (50 mm\_2.1 mm I.D., 1.7 mm particle size, Waters Co., MA, USA)

The tailing factor of the NTZ peak was 2.0 for HPLC and 1.2 for UPLC, respectively. The retention times of IS (nifuroxazide) and NTZ were 22.1 and 24.8 min for HPLC, and 3.2 and 3.5 min for UPLC, respectively. The correlation coefficients were 0.9988 (HPLC) and 0.9963 (UPLC). The RSDs of quantitative values of sample solution were calculated to be 4.06% to 4.64% for HPLC and 0.15% to 0.36% for UPLC.

#### 2.4.2. GC

The residual solvents in NTZ was developed by Jiang Shanet *al* (28) were separated by a DM-WAX column (30 m×0.25 mm,0.5µm) with an FID detector. The injector temperature and the detector temperature was set at 200°Cand 250°C, respectively. The containers of head-space injector were in equilibrium at 80°Cfor 30 min. N,N-Dimethyl formamide was used as the solvent. The detected solvents were separated completely. A good linearity of the two solvents was obtained within the range ol 250-750µg/ml( $r=0.9991$ ) and 30-90µg/ml ( $r=0.9991$ ), respectively. The average recovery of acetone and dichloromethane was 99.15%and 99.18%with RSD of 2.17%and 2.97% (n=9), respectively.

#### 2.4.3. LC-MS

LC-MS method was developed by Zhanzhong Zhao et.al (29) developed a sensitive and specific method for the identification of NTZ metabolites in goat feces by liquid chromatography–electrospray ionization tandem mass spectrometry with negative ion mode was developed. After extraction procedure the pretreated samples were injected on an XTerra MS C8 column with mobile phase (0.2 mL min<sup>-1</sup>) of acetonitrile and 10 mM ammonium acetate (adjusted to pH 2.5 with formic acid) followed by a linear gradient elution, and detected by MS–MS. Identification and structural elucidation of the metabolites were performed by comparing their retention times ( $R_t$ ), full scan, product ion scan, precursor ion scan and neutral loss scan MS–MS spectra to those of the parent drug or other available standard. The parent drug (NTZ) and its deacetyl metabolite (tizoxanide) were found in goat feces after the administration of a single oral dose of 200 mg kg<sup>-1</sup> of NTZ. Tizoxanide was detected in goat feces for up to 96 h after ingestion of NTZ.

Huang Xet al (30) utilized a hybrid linear ion trap/Orbitrap mass spectrometer providing a high mass resolution and accuracy was used to investigate the metabolism of NTZ in rats, pigs, and chickens. The results revealed that acetylation and glucuronidation were the main metabolic pathways in rats and pigs, whereas acetylation and sulfation were the major metabolic pathways in chickens, which indicated interspecies variations in drug metabolism and elimination. With the accurate mass data and the characteristic MS(n) product ions, we identified six metabolites in which tizoxanide and hydroxylated tizoxanide were phase I metabolites and tizoxanide glucuronide, tizoxanide glucose, tizoxanide sulfate and hydroxyl tizoxanide sulfate were phase II metabolites. Hydroxylated tizoxanide and tizoxanide glucose were identified for the first time. All the comprehensive data were provided to make out the metabolism of NTZ in rats, pigs and chickens more clearly.

The photodegradation of NTZ was studied by M.D. Malesuik *et al* (31) in order to investigate the degradation kinetics of this drug. The analyses of the degraded samples were performed by a stability-indicating liquid chromatographic method. The column utilized was a Phenomenex (Torrance, CA) Synergi Fusion C18 column (250mm, 4.6 mm, i.d., 4 $\mu$ m particle size) coupled to a C18 guardcolumn (4.0mm-3.0mm, i.d., 4  $\mu$ m). a mobile phase of ophosphoric acid 0.1% (v/v) (pH 6.0 adjusted by addition of triethylamine)– acetonitrile (45:55, v/v) run at a flow rate of 1.0mL/min and using PDA detection at 240 nm. The light source was an UVC – 254 nm30Wlamp (Philips, Amsterdam, Holland) fixed to a chamber in a horizontal position. Degradation Rate Constant (k), Half-life ( $t_{1/2}$ ), and  $t_{90}$  for NTZ in Pharmaceutical Formulations Solutions Submitted to Photodegradation and Determined by LC Method.

**Table 4.**

<b>Dosage forms</b>	<b>k/min</b>	<b><math>t_{1/2}</math> (min)</b>	<b><math>t_{90}</math> (min)</b>
<b>Tablets</b>	4.81,10 <sup>-2</sup>	201. 56	40.30
<b>Powder for oral suspension</b>	5.38, 10 <sup>-2</sup>	183.83	36.75

#### 2.4.4. HPTLC:

A new simple, rapid, and selective high-performance thin-layer chromatographic (HPTLC) method with metronidazole as the internal standard has been developed by Salvador Namuret al (32) for analysis of tizoxanide (a metabolite of nitazoxanide) in human plasma. The analyte was extracted from human plasma by cation-exchange solid-phase extraction (SPE). In HPTLC the stationary phase was silica gel 60F<sub>254</sub> and the mobile phase was toluene-ethyl acetate-acetic acid 6.2:13.4:0.4 ( v/v ). UV detection and quantification were performed at 313 nm for the internal standard and 410 nm for tizoxanide. Data were fitted to a quadratic mathematical function by polynomial regression. The working range was 400–16000 ng mL<sup>-1</sup>. The method was validated for accuracy and precision. The average recovery was 85.5%. A validated stability indicating HPTLC was developed by CL Gopuet al(33) for determination of nitazoxanide in bulk and in formulation. They carried out separation in TLC alumina plates precoated with silica gel 60F<sub>254</sub> using mixture of ethyl acetate-toluene-methanol (3.9:6.1:1 and 4.1:5.9:1 v/v/v) as mobile phase.



The detection of spot was carried out by using UV detector at 350 nm. The linearity of calibration curve was found to be between 400-1600ng per spot.

### **3. CONCLUSION**

A large number of techniques are available for the estimation of nitazoxanide in pharmaceutical formulations and biological samples. The survey of analytical data revealed that HPLC methods are predominant for the estimation of drug alone or in combination with other drugs in various formulation types. So for the precise and accurate separation of nitazoxanide in various formulations recommended method of analysis includes HPLC with UV detector as it provides faster analysis time and has more separation selectivity than most other available techniques. This review carried out an overview of the state-of-art analytical methods for the determination of nitazoxanide in different formulations using various analytical techniques.

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