Original Article

Determination of Nitazoxanide in Biological Matrices by LC-MS/MS Technique: Method Development and Validation

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ABSTRACT

Background: Determination of pharmaceuticals in the biological matrices is essential for toxicologic and pharmacokinetic applications. The main objective of the current work was to develop a bioanalytical method for quantifying nitazoxanide in biological samples by LC-MS/MS. **Materials and Methods:** Chromatographic elution of nitazoxanide and linagliptin were achieved on C_{18}-hypersil (5 μ, 50x4.6mm) stationary phase with mobile phase consisting of acetonitrile and 0.1% HCOOH (75:15, v/v) processed at 0.8 ml/min flow rate. Linagliptin was utilized as an internal standard (IS). **Results:** The method was linear in the concentration levels of 0.53–21.2 ng/ml with more than 0.999 r^2 values, consisting of 0.53 ng/ml as the lower limit of quantification (LLOQ). Analytes were subjected to pretreatment by liquid-liquid extraction (LLE) procedure with average extraction recovery findings within 99.93±5.04%. Method accuracy findings were present in between 96.44% to 104.31%, and the assessed percentage relative standard deviation findings for within and between run precision were ≤ 4.68%. **Conclusion:** The developed LC-MS/MS procedure for quantitating nitazoxanide in the biological matrix was suitable for routine analysis of patients' blood samples for pharmacokinetic studies and drug monitoring.

Key words: Nitazoxanide, Antiviral, LC-MS/MS, Validation, Accuracy.

INTRODUCTION

Nitazoxanide (NTZX) is a broad-spectrum antiviral and antiparasitic compound utilized to manage viral, helminthic and protozoal infections. It is also helpful in treating infections caused by Giardia lamblia and Cryptosporidium parvum in immunocompetent patients and influenza.[1-3] The widely recognized mechanism of NTZX is the distraction of energy metabolism in the anaerobic microorganisms by preventing the pyruvate: ferredoxin/flavodoxin oxidoreductase (PFOR) cycle. NTZX in parasitical protozoa produces the lesions in cell membrane and depolarizes the mitochondrial membrane while inhibiting protein disulfide isomerase, nitroreductase-1, and quinone oxidoreductase NQO-1 enzymes. In addition to these actions, NTZX prevents an enzyme that detoxifies (glutathione-S-transferase) and modifies the gene Avr-14, coding for the α-subunit of glutamate-gated chloride ion channel present in nematodes.[4-6] In *E. coli* the drug will block pyruvate dehydrogenase, pH homeostasis and alter the membrane potential in Mycobacterium tuberculosis, suppress the CU (chaperone/usher) path of the gram-negative bacterial organism, and increase the macrophage autophagy in tuberculosis host patients.[7]

This drug prevents viral duplication by preventing the viral hemagglutinin maturation and the viral transcription factor IE-2 (immediate early-2), also it activates translation of eukaryotic initiation factor-2α. Finally, it shows an inhibition activity on

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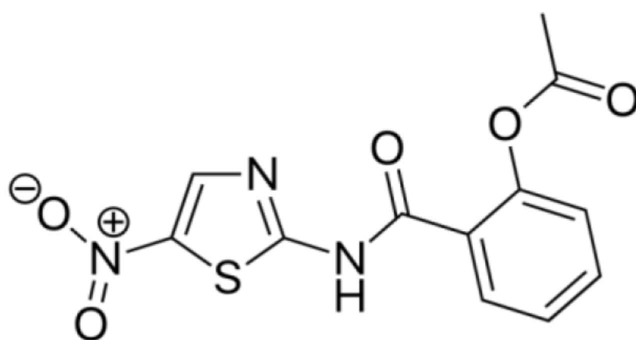

Figure 1: Nitazoxanide chemical structure.

tumor cell development by varying NTZX detoxification (glutathione-S-transferase P1), anti-cytokines activity, autophagy, c-Myc inhibition and unfolded protein response. NTZX chemically designated as [2 - [(5- Nitro-1,3-thiazol-2-yl) carbamoyl] phenyl] ethanoate (Figure 1) with molecular mass and formula of 307.283 g/mol and $C_{12}H_9N_3O_5S$, respectively.[7-9]

Literature review about NTZX showed few analytical methods reported on UV-spectrophotometer,[10] reverse-phase liquid chromatography,[11-13] UPLC,[14] and Liquid chromatography with tandem mass spectrophotometer[15,16] for its quantification. The present aim of the research is to establish a sensitive and precise LC-MS/MS technique for quantitating NTZX in biological media. An extraction procedure was optimized utilizing the LLE method for attaining a better percentage recovery of NTZX. The established method validation was executed as per the guiding principles of bioanalytical method validation of EMA[17] and FDA.[18]

MATERIALS AND METHODS

Chemicals and reagents

NTZX and IS (linagliptin) were procured from MSN Labs, Hyderabad, India. HPLC grade methanol and acetonitrile (ACN) solvents were bought from A.B enterprises, Mumbai, India. HCOOH and ammonium formate were bought from Merck, India. Water for the analytical technique was processed form the Integral-3 MilliQ purifier equipment, Millipore, USA. Blank human plasma (drug free) was bought from Laxmi Sai Clinicals, Hyderabad, India, and was monitored in a freezer till the method of analysis.

Equipment

The Liquid chromatography with tandem mass spectrometric system was Applied Biosystems/MDS SCIEX API-6500 furnished with electro-spray ionization source. Data acquisition and instrument control were processed by Analyst software-1.5.2 version. The HPLC system was Shimadzu LC-30AD liquid chromatography comprised four pumps, an autosampler (SIL-30AC), and a CTO-20AC prominence column oven compartment. Samples were extracted with Chromabond-C_{18} stationary columns of Germany. Digital micropipettes of different µl capacity were calibrated and utilized during the LLE technique.

Chromatographic elution of nitazoxanide and linagliptin were achieved on C_{18}-hypersil (5 µ, 50x4.6mm) stationary phase with mobile phase consisting of acetonitrile and 0.1% HCOOH (75:15, v/v) processed at 0.8 ml/min flow rate. Linagliptin was utilized as an internal standard (IS).

LC-MS/MS parameters

NTZX and IS were eluted in a chromatographic system encompassing C_{18}-hypersil (5 µ, 50x4.6mm) stationary phase with mobile phase consisting of acetonitrile and 0.1% formic acid (75:15, v/v) processed at 0.80 ml/min flow rate. The analyte infusion volume was maintained at 5.0 µl. The elution time for the separation of drug and IS was found to be 3.0 min. The detection in MS-system was processed at +ve ionization approach utilizing ESI as source. MS conditions were: N_2 gas was employed as collision/drying gas, 10.0 ml/min flow of gas, 250°C gaseous temperature, 4500 V capillary voltage and 20.0 psi nebulizer pressure. Quantitation of drug and IS were processed by utilizing MRM (multiple reaction monitoring) mode. The examined molecular to product ion molecular weight transitions for NTZX was 308.12→96.0 and for the IS was at 472.54/456.21.

Protocol for standard stock solutions

Stock solution of NTZX was processed by solubilizing 10.0 mg of NTZX in 10ml of ACN to produce 1.0 mg/ml primary stock. The resulting stock was monitored at −20.0°C till further method of analysis. The working stock of 50 µg/ml drug solution was processed by serial dilution method with ACN as diluent. Primary and working stock solution of IS were processed in same manner with diluent and it was further diluted to get 5.0 ng/ml solution.[16, 19]

Protocol for sample extraction

Sample solution was prepared by transferring 0.3ml of plasma into 10ml tube along with 100 µl of IS (50.0 ng/ml) and further diluted. The resulting solution vortexed for 1 min and further 5ml of butyl methyl ether and ethyl acetate solvent was mixed and vortexed for 5 min in a centrifuge at 4000 rpm. Resulting solution was evaporated by applying steam of N_2 and water bath. Reconstitute with 250 µl of mobile phase mixture and

shake for 5 min and contained in auto-sampler vial then directly infused to LC system.

Validation

Validation of the developed LC-MS/MS technique was performed as per the EMA[17] and FDA [18] guidelines related to bioanalytical method.

Sensitivity and calibration curve

Solution consisting of NTZX 10 µg/ml was processed from the NTZX working stock solution by serial dilution method using diluent. From the resulting solution eight non-zero calibration standards were processed over 0.53–21.2 ng/ml with diluent. All the calibration standards were subjected to extraction and examined in triplicates to assess the linearity of the analytical method. The lowest concentration as LLOQ (0.53 ng/ml) solution was assessed for the quantification to prove the sensitivity of the analytical method.

Carry-over and specificity

After infusing, the highest concentration of analyte blank was infused and examined to assess the carry-over. The response in the blank should be less than twenty percent of LLOQ of analyte and five percent of the IS response. Six blank samples of human plasma were collected from six different individuals to examine the intervention by variable endogenous compounds to be present in the sample at retaining time of NTZX and IS. This infusion of standard solutions of NTZX and IS was essential to identify the nosiness between each sample.

Precision and accuracy

Four different quality control (QC) standards including LLOQ concentration were processed individually from the calibration solutions.[20] These concentrations were 15.9 ng/ml (HQC), 10.6 ng/ml (MQC), 1.484 ng/ml (LQC) and 0.53 ng/ml (LLOQ). The within and between run precision and accuracy of a developed process was estimated by examining 6 duplicate solutions of each concentration in 3 infusions on 3 alterative days. The percentage recoveries were calculated and the findings should be in the range of ±15.0% of the original value of QC standards and within ±20.0% for LLOQQC. The between and within run %RSD calculated findings should be ≤15.0% for QC standards except for LLOQQC which should be ≤20.0%.

Matrix effect

Matrix effect was assessed by collecting 6 blank human plasma samples from six different lots. The matrix factor (MF) and IS normalized MF were determined for each lot of plasma sample.[21] MF was determined at LQC, and HQC concentration levels, and the %RSD value of the IS normalized MF should be ≤15.0% for 6 different lots of plasma samples. MF and IS normalized MF were calculated form the following formulae:

$$\text{Analyte MF} = \frac{\text{Peak response in presence of matrix ions}}{\text{Average peak response in aqueous samples}}$$

$$\text{IS normalized MF} = \frac{\text{MF of analyte}}{\text{MF of IS}}$$

Recovery

The recovery of the analytical method was determined by comparing the findings of QC extracted samples with blank extracted samples spiked with respective QC standards after the extraction process.[22,23] The degree of percentage recovery should be constant and reproducible.

Stability

The NTZX stability was evaluated after exposing the QC standards to variable storage environments.[17,21] The applied parameters comprises 3 freeze and thaw sequences (kept at −20.0°C for 12.0 hr), long-term stability at a storage temperature of −20.0°C for 30 days, short-term stability at the temperature of room for 8.0 h and the prepared extract sample stability after 24.0 hr at 4°C.

Dilution integrity

NTZX dilution integrity was processed by five times diluting the 6 human plasma aliquots consisting of 21.2 ng/ml of drug. The resulting samples were subjected for extraction and examined.[18] The found concentration levels were equated with the undiluted samples of a human plasma consisting of 1.484 ng/ml to check whether the dilution effect was within the precision and accuracy range or not. The precision and accuracy should be considering the previously stated acceptance limit.

RESULTS

Chromatographic system optimization

The chromatographic conditions of analytical technique were adjusted to produce the separated symmetrical peak responses within the reasonable time and better method sensitivity. acetonitrile and 0.1% formic acid

(75:15, v/v) were selected as a better and more efficient movable phase for the successful elution of drug and IS. Asymmetric NTZX peak response was produced with methanolic movable phase. Mobile phase of ammonium formate with ACN produced very poor resolution and unsymmetrical peaks. On changing the ammonium formate of movable phase with 0.1% HCOOH (5%) improved peak resolution of NTZX and IS. 0.1% HCOOH in the ratio of 15% produced a good symmetrical peak response with high resolution. Zorbax-SB- C8 (4.6mm × 250mm, 5µ) (Agilent, USA), X-bridge–C_{18} (4.6mm×150 mm, 5µm) and C_{18}-Hypersil (50 × 4.6 mm, 5 µ) (Thermo Fisher scientific) stationary columns were subjected for the optimization over the analytical method development. The first two column types separated NTZX drug after 8.0 min. The 3rd type of column was the utmost one which was produced the symmetrical type of peak responses for NTZX and IS, were eluted in the total run time of 3.0 min.

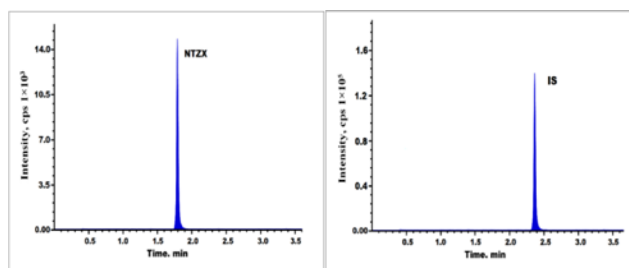

Figure 3: Chromatograms of nitazoxanide at LQC level.

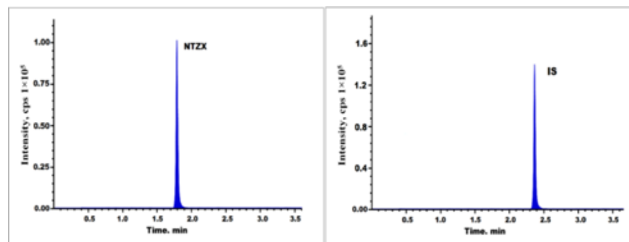

Figure 4: Chromatograms of nitazoxanide at MQC level.

DISCUSSION

Validation

Carry-over and specificity

Figures 2 to 5 represent the blank plasma sample and LLOQQC chromatograms. These chromatograms prove that blank plasma has no interfering components when equating with LLOQ sample. The analyte and IS samples were eluted at 1.8 and 2.38 min having a resolution of > 5.0. The carry-over was processed as per the method and evaluated. The result was <2.5% of LLOQQC for NTZX and < 0.86% for IS.

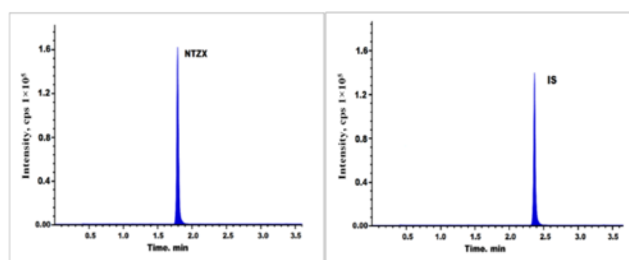

Figure 5: Chromatograms of nitazoxanide at HQC level.

Sensitivity and calibration curve

The linearity graph was plotted by considering the peak response ratio of analyte and IS against the actual concentration of analyte over the concentration of 0.53-21.2 ng/ml and the results were shown in Table 1. The linearity equation was found to be $y = 0.0823x + 0.0413$ and in this equation 'y' value represents peak response ratio of NTZX to IS and 'x' value represents the NTZX concentration level. The regression coefficient (r^2) was ≥ 0.999.

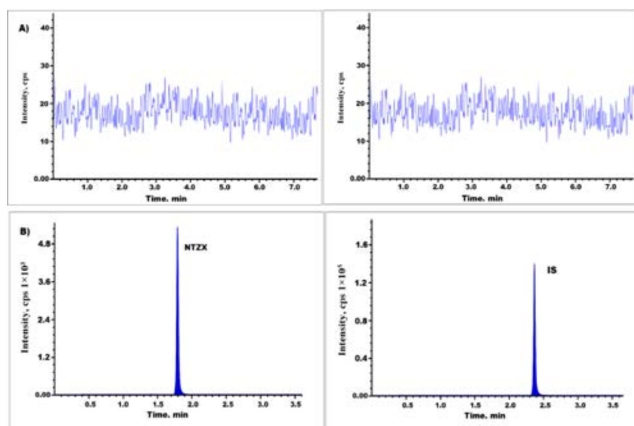

Figure 2: Chromatograms of blank plasma (A) and LLOQ (B) samples.

Precision and accuracy

The precision and accuracy findings of NTZX determinations were produced in Table 2. Within and between run accuracy were estimated and the findings were present in between 95.21-104.10%. The percentage RSD findings for within the run precision values were < 5.24%. The between run precision %RSD findings were <5.31%. The analytical method's findings precision and accuracy showed that the method has a high degree of precision and accuracy.

Table 1: Linearity data for nitazoxanide.										
Actual conc. (ng/mL)	0.53	1.06	2.9	5.7	9.6	13.5	17.55	21.2	Slope	Intercept
1	0.50	0.97	2.87	5.64	9.69	13.47	18.11	20.72	0.0821	0.0432
2	0.51	1.02	2.93	5.69	9.65	13.38	18.46	20.80	0.0828	0.0394
3	0.53	1.04	2.95	5.61	9.48	13.29	17.96	20.56	0.0819	0.0412
Mean	0.51	1.01	2.92	5.65	9.59	13.38	18.17	20.69	0.0823	0.0413
±SD	0.013	0.038	0.039	0.043	0.092	0.089	0.256	0.121	0.0005	0.0019
%CV	2.479	3.73	1.364	0.759	0.962	0.66	1.41	0.587	--	--
% Accuracy	96.93	95.5	100.63	99.10	99.87	99.10	103.56	97.63	--	--

Coefficient of variance: CV; standard deviation: SD.

Table 2: The accuracy and precision data for nitazoxanide.						
Nominal Concentrations in ng/ml	Within-run			Between-run		
	Mean[a] ± SD	Precision (RSD%)	Accuracy (Average%)	Mean[a] ± SD	Precision (RSD%)	Accuracy (Average%)
0.53	0.551±0.08	5.24	104.1	0.522±0.22	5.31	103.15
1.484	1.495±0.13	1.95	97.35	1.483±0.23	4.21	96.98
10.6	10.73±0.82	3.54	98.01	10.62±5.92	3.21	95.21
15.9	15.36±1.13	4.27	102.3	15.34±14.52	4.74	102.45

Standard deviation: SD; a= Six replicates; relative standard deviation: RSD.

Table 3: The stability results for NTZX in human plasma						
Storage condition	LQC		MQC		HQC	
	Accuracy (%Mean)	Precision (%RSD)	Accuracy (%Mean)	Precision (%RSD)	Accuracy (%Mean)	Precision (%RSD)
Room temp., 8 h	103.61	3.56	104.31	1.79	96.75	2.54
30 day at −200C	97.45	2.41	97.41	4.12	93.85	4.68
3 freeze-thaw cycles	96.47	3.62	96.44	3.95	102.63	3.97
Extract, 24 h at 4^0C	102.56	1.95	96.46	3.71	104.12	1.88

RSD- relative standard deviation

Stability

The developed method was assessed for stability at LQC, MQC and HQC levels and the findings were shown in the Table 3. The measured accuracy findings for NTZX estimations were present in between 93.85–104.32% of the original concentration levels and were present in the acceptable limit. It proves that the NTZX has a high degree of stability over variable storage conditions.

Matrix effect

The parameter matrix effect was significant for an LC-MS/MS analysis to find whether the plasma matrix components produced ionic enhancement and suppression effect at the MS detection. The MF was evaluated at HQC and LQC standards and the values were 0.99 ± 0.03 and 0.98 ± 0.02 respectively. The IS normalized MF percentage RSD findings of 6 variable lots were ≤4.85% at LQC and HQC levels. It proves that there was no interference with the matrix components of plasma with the drug and IS response.

Recovery and dilution integrity

NTZX was subjected for the average recovery studies at 1.484 ng/ml, 10.6 ng/ml and 15.9 ng/ml and the findings were 97.56%, 104.21%, and 98.01%, respectively were shown in the Table 4. The overall %RSD was found to be 3.04% and the average recovery of internal standard

Table 4: NTZX extraction recovery results.

Concentration level	X	Y	% Recovery	% Mean recovery	%RSD
LQC (1.484 ng/ml)	15196	14825.2	97.56		
MQC (10.6 ng/ml)	108554	113124	104.21		
HQC (15.9 ng/ml)	162780	159541	98.01		
IS	142116	137668	96.87	99.93	3.04

Average recoveries of extracted samples: Y; Average recoveries of unextracted samples: X

was found to be 96.87 ± 0.53. Dilution integrity of the method was assessed by processing five folds dilution of 6 samples consisting NTZX of 21.2 ng/ml and the percentage accuracy was found to be 97.42 ± 2.46%.

CONCLUSION

An accurate analytical method was developed and validated for the estimation of NTZX in biological matrices of humans by LC-MS/MS utilizing linagliptin as IS. The chromatographic elution of NTZX and linagliptin was attained on a C_{18}-hypersil (50 x 4.6 mm, 5 μ) column with an isocratic mobile phase, made up of acetonitrile and 0.1% formic acid (75:15, v/v) processed at 0.8 ml/min flow rate. Analytes were subjected for pretreatment by LLE method with average extraction recovery findings within 99.93±5.04%. Method accuracy findings were present in between 96.44% to 104.31% and the assessed percentage relative standard deviation findings for within and between run precision were ≤ 4.68%. The validation data of the developed method represents that the proposed method applicable for the pharmacokinetics and bioequivalence studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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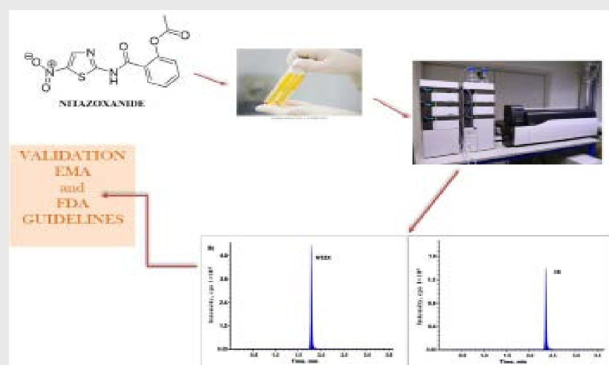
PICTORIAL ABSTRACT



SUMMARY

NTZX is a broad spectrum antiviral and antiparasitic compound utilized in the management of viral, helminthic and protozoal infections. The main objective of the current work was to develop a bioanalytical method for the quantification of NTZX in biological samples by LC-MS/MS. Elution of NTZX and IS were achieved on C_{18}-hypersil (5 μ, 50x4.6mm) stationary phase with mobile phase consisting of acetonitrile and 0.1% HCOOH (75:15, v/v) processed at 0.8 ml/min flow rate. Method was linear over 0.53–21.2 ng/ml concentration level with R^2 value more than 0.999. Analytes were subjected for pretreatment by liquid-liquid extraction (LLE) procedure with average extraction recovery findings within 99.93±5.04%. Method accuracy findings were present in between 96.44% to 104.31% and the assessed percentage relative standard deviation findings for within and between run precision were ≤ 4.68%.

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