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Method development and validation of anti-protozoal agent- Nitazoxanide by RP-HPLC and it's stability related impurities studies

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ABSTRACT

A simple and new Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method was developed for the simultaneous estimation of Nitazoxanide in Pure form and Marketed Pharmaceutical Dosage form. The separation involved an isocratic elution of this component on Symmetry C18 (4.6 x 150mm, 5µm, Make: XTerra) using a mobile phase composition Methanol: Phosphate buffer = 60:40. The flow rate was 1.0 ml/min and the analyte monitored at 242nm. The performance of the method was validated according to the present ICH guidelines for specificity, linearity, accuracy, precision and robustness. Retention times of Nitazoxanide were found to be 2.96 min respectively. The percentage recoveries for Nitazoxanide were found to be within the limits i.e. 97-102% respectively. Calibration curves were linear with coefficient correlation between 0.990 to 0.999. Typically the regression equation for the calibration curve was found to be y = 26326x + 38649 (R^2=0.995) Nitazoxanide respectively. The developed method can be successfully employed for routine quality control analysis of Nitazoxanide marketed formulation.

Keywords: Nitazoxanide, Method development, Method Validation, Retention time.

INTRODUCTION [1 2 3]

Reversed Phase Chromatography**:** Since 1960's chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography. A large number of chemically bonded stationary phases based on silica are available commercially. Silica based stationary phases are still most popular in reversed phase chromatography however other absorbents based on polymer (styrene-di-vinyl benzene copolymer) are slowly gaining ground. The retention decreases in the following order: aliphatic > induced dipoles (i.e. CCl_4) > permanent dipoles (e.g.CHC_{l3}) > weak Lewis bases (ethers, aldehyde, and ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases. As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers. In reversed phase systems the strong attractive forces between water molecules arising from the 3-imentional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase. Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution

rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase. The importance of Chromatography is increasing rapidly in pharmaceutical analysis. The exact differentiation, selective identification and quantitative determination of structurally closely related compounds are possible with chromatography. Another important field of application of chromatographic methods is the purity testing of final products and intermediates (detection of decomposition products and by-products). As a consequence of the above points, chromatographic methods are occupying an ever-expanding position in the latest editions of the pharmacopoeias and other testing standards. The various components of a HPLC system are herewith described.[3,4]

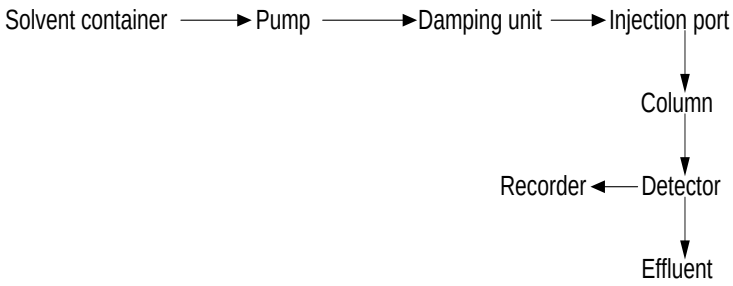


Fig 1: Various Components of HPLC

METHOD DEVELOPMENT

Analytical method is a detailed description of different steps necessary to perform analytical tests which may include preparation of samples, reagents, use of apparatus, generation of calibration curve and use of formulae for calculations. Analytical method development is required to analyze Herbal Products, New process & reactions, New molecules, Active ingredients(Macro analysis), Residues(Micro analysis), Impurity profiling etc.

These are the minimum requirements of the specifications of the method for the intended purpose. The steps of method development consist of the following steps which are common to most types of projects: Method development plan definition, Background information gathering, Laboratory method development, Generation of test procedure

A method should be developed with the goal to rapidly test preclinical samples, formulation prototypes and commercial samples.

Method Development by HPLC**:** According to ICH guidelines this following procedure is applicable to the development of new analytical methods by HPLC.

Method of validation: It is not always essential to validate every analytical performance parameter are: Accuracy, Precision, Specificity, Limit of detection, Limit of quantization, Linearity and range, Ruggedness, Robustness.

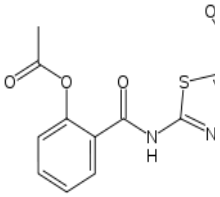
The parameters, as defined by the ICH and by other organizations and authors, are summarized in below and are described in brief in the following paragraphs. Specificity(1,2), Selectivity, Precision(1,2), Repeatability (1)Intermediate precision, Reproducibility,(3)Trueness, Bias, Linearity (1,2), Range (1,2),Limit of detection (1,2), Limit of quantization (1,2)Robustness (2,3), Ruggedness (2)The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates each).

DRUG PROFILE

Name: Nitazoxanide

Description: Nitazoxanide, also known by the brand name Alinia, is a synthetic nitrothiazolyl-salicylamide derivative and an anti-protozoal agent. It is approved for treatment of infectious diarrhea caused by Cryptosporidium parvum and Giardia lamblia in patients 1 year of age and older.

Structure:



Chemical formula: $C_{12}H_9N_3O_5S$, Molecular weight: 307.283g/mol,

Category: Anti-parasitic agent,

Solubility: Freely soluble in dimethyl formamide, sparingly soluble in acetone, practically insoluble in water,

Uses: Nitazoxanide is a first-line choice for the treatment of illness caused by Cryptosporidium parvum or Giardia lamblia infection in immunocompetent adults and children.

MATERIALS AND METHODS:

S.No	Instruments/Equipments/Apparatus
1.	Hitachi L2130 with D Elite 6000 Software with Isocratic with UV-Visible Detector (L-2100),
2	ELICO SL-159 UV-Vis spectrophotometer
2	Electronic Balance
3.	Ultra Sonicator (Wensar wuc-2L)
4.	Thermal Oven
5.	Shimadzu- ODS (C_8) RP Column, 150 mm x 4.6 mm.
6.	P^HAnalyzer- (ELICO)

Chemicals and Reagents

S.N.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	HPLC grade water	----	----	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	A.R.	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9	L.R.	Sd fine-Chem ltd; Mumbai
6.	Ortho phosphoric acid	99.9	L.R.	Sd fine-Chem ltd; Mumbai

HPLC Instrumentation & Conditions

The HPLC system employed was HITACHI L2130 with D Elite 2000 Software with Isocratic with UV-Visible Detector (L-2400), Standard & sample preparation for UV-spectrophotometer analysis:25 mg of Nitazoxanide standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase.Further dilution was done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Optimized Chromatographic Conditions

Column : Waters C_{18}, 5µm, 25cmx4.6mm i.d.
Mobile Phase : Buffer: Methanol (40:60)
Flow Rate : 1.0ml/minute
Wave length : 242 nm
Injection volume : 20 µl
Run time : 10 minutes
Column temperature : Ambient

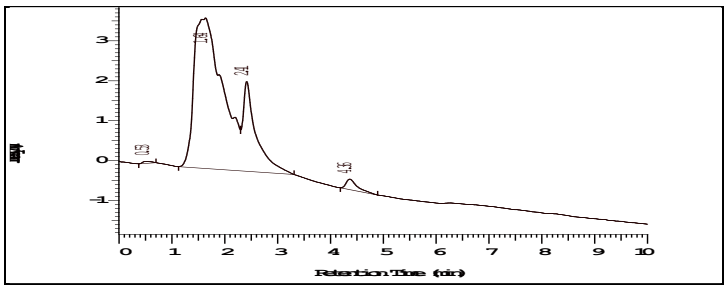


Fig 2: Chromatogram for Blank

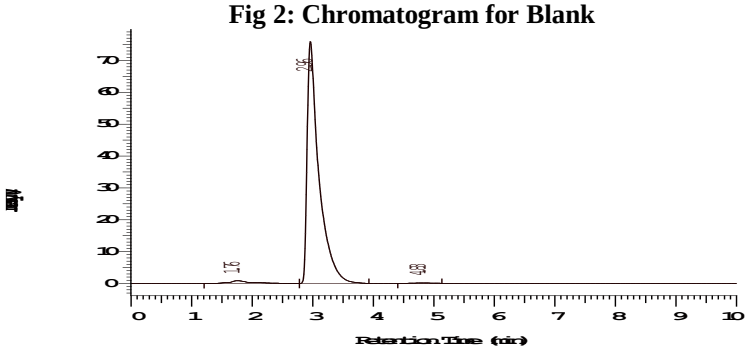


Fig 3: Chromatogram for Nitazoxanide (Rt 2.96 min)

Table 1: Results of Optimized Condition

S.NO	RT(min)	PEAK AREA	PEAK CONCENTRATION
1	2.96	1246755	99.89

Table2: Results of force degradation studies of Nitazoxanide API.

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	43.75	54.61	98.36
Basic Hydrolysis (0.I M NaOH)	24Hrs.	43.32	55.02	98.32
Thermal Degradation (50 ^{0}C)	24Hrs.	97.39	----------	97.39
3 % Hydrogen peroxide	24Hrs.	59.75	30.28	100.03

METHOD VALIDATION

Accuracy

Table 3: Results of accuracy-80%-1

Drug Name	RT	Peak Area	Tailing Factor	Plate count
NITAZOXANIDE	2.96	832564	0.84	2245

Table 4: Results of accuracy-100%-1

Drug Name	RT	Peak Area	Tailing Factor	Plate count
NITAZOXANIDE	2.96	1103706	1.02	2925

Table 5: Results of accuracy-120%-1

Drug Name	RT	Peak Area	Tailing Factor	Plate count
NITAZOXANIDE	2.99	1332456	1.05	2845

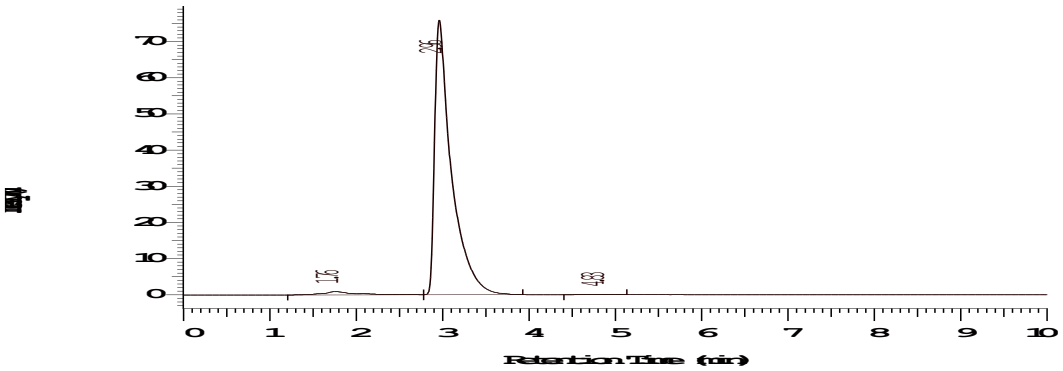


Fig 4: Chromatogram for Repeatability- 1

Drug Name	RT	Peak Area	Tailing Factor	Plate count
NITAZOXANIDE	2.95	1202543	0.98	2441

INTRA-ASSAY & INTER-ASSAY

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Nitazoxanide revealed that the proposed method is precise

Table 6: Results of intra-assay & inter-assay

Conc. Of Nitazoxanide (API) (µg/ml)	Observed Conc. Of Nitazoxanide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
20	20.01	0.86	20.03	0.87
40	40.02	0.30	40.03	0.32
60	59.97	0.13	59.95	0.11

Linearity & Range

The calibration curve showed good linearity in the range of 0-100 µg/ml, for Nitazoxanide (API) with correlation coefficient (r^2) of 0.995 (Fig-42). A typical calibration curve has the regression equation of y = 26326x + 38649 for Nitazoxanide.

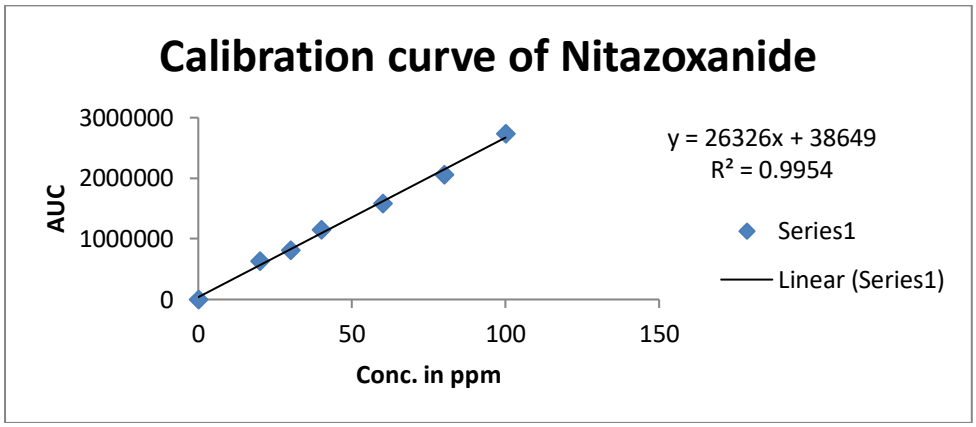


Fig 5: Calibration curve of Nitazoxanide (API).

Table 6: Results of linearity

CONC.	AUC (n=6)
0	0
20	635564
30	804839
40	1144133
60	1581052
80	2055851
100	2736626

Robustness: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & Acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-42, % RSD < 2%) the developed RP-HPLC method for the analysis of Nitazoxanide (API).

Table 7: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.06
Flow (0.9 ml/min)	0.04
Temperature (27^{0}C)	0.08
Temperature (23^{0}C)	0.11
Wavelength of Detection (244.5 nm)	0.03
Wavelength of detection (240.5 nm)	0.02

LOD & LOQ: Limit of detection (LOD) and Limit of quantification (LOQ) for Nitazoxanide were determined by performing various trials at different lowest concentrations. The final LOD and LOQ values at least concentrations were detected by observing signal to noise ratios respectively. The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.03 & 0.09 µg/ml respectively.

Estimation Of Nitazoxanide In Tablet Dosage Form

NITAZOXANIDE500 mg twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 100 mg of drugs were transferred to 100 ml volumetric flask, and 70 ml of Hplc grade methanol was added and solution was sonicated for 15 minutes, there after volume was made up to 100 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with hplc grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (3.5 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

Table8: Assay of Nitazoxanide tablets

Brand name of tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	% PURITY
Aliniav (Romark Laboratories)	500 mg	500.16 (±0.09)	100.16%

RESULTS AND DISCUSSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Nitazoxanide, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Develosil C_{18}, 5µm, 150 x 4.6 mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good. Mobile phase & diluents for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1NHCl).

The drug was found to be highly soluble in methanol and slightly soluble in Acetonitrile. Drug was insoluble in water. Using these solvents with appropriate composition newer methods can be developed and validated. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Nitazoxanide it is evident that most of the HPLC work can be accomplished in the wavelength range of 220-280 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20 µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay and which can help in the analysis of Nitazoxanide in different formulations.

CONCLUSION

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Nitazoxanide API. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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