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Research Article

DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF NITAZOXANIDE AND IT'S RELATED SUBSTANCES IN ACTIVE PHARMACEUTICAL INGREDIENTS

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ABSTRACT

Objective: Nitazoxanide (NAT) is a veterinary antibiotic used for the treatment of protozoal infections in livestock and sheep. The estimation of NAT, its process impurities, and degradation products have not been reported till date. In this study, we aimed to develop and validate a chromatographic separation method for the determination of NAT, its process impurities, and related substances as per International Conference on Harmonization (ICH) guidelines.

Methods: Chromatographic separation of process impurities such as 2-amino-5-nitro thiazole also called NAT-related substance A, aspirin, and degradation impurities such as salicylic acid and tizoxanide (TIZ) were separated by reverse-phase high-performance liquid chromatography using gradient elution. The separation of NAT and TIZ was most critical as they are structurally similar. The mobile phase consisted of a gradient elution containing a composition of acetonitrile and 2% orthophosphoric acid solution adjusted to pH 2.5 at a flow rate of 1 mL/min. Separation was achieved on a YMC Pack C8 L7 column with a run time of 40 min. The detection was carried out using a photodiode array detector and quantification was carried out at 210 nm. Forced degradation study was also conducted to confirm the specificity. The robustness and ruggedness of the method were evaluated.

Results: The relative retention times (RRT) for aspirin, salicylic acid, NAT, and TIZ were 6.33, 6.52, 22.43, and 6.45, respectively, indicating good separation. The asymmetry factor for all the peaks is ranged from 1.1 to 1.2 indicating acceptable chromatography. The % recovery from spiked studies ranged from 90% to 110% for all the impurities when spiked in the range of 50–150% of their nominal concentrations. For all the known impurities, the limit of detection ranged from 0.06 to 0.20 parts per million (ppm) and the limit of quantification ranged from 0.19 to 0.61 ppm.

Conclusion: The method was validated as per ICH guidelines and further was successfully applied for the quality evaluation of NAT in bulk active pharmaceutical ingredients.

Keywords: Nitazoxanide, Aspirin, 2-amino-5-nitro thiazole, Tizoxanide, Salicylic acid.

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INTRODUCTION

Nitazoxanide (NAT) was first approved for veterinary use in the late 1980s [1]. In humans, NAT was approved by the U.S. Food and Drug Administration in 2002. It is primarily used to treat a variety of parasitic infections in animals, particularly for controlling protozoal infections in livestock, including cattle, sheep, and goats. In children, it is used to treat giardiasis. In immune-compromised individuals such as those with human immunodeficiency virus/acquired immunodeficiency syndrome, NAT is helpful to treat cryptosporidiosis [2,3]. NAT has antiviral properties [4] and has been investigated for use against a range of viral infections, including influenza, rotavirus [5], and, more recently, coronaviruses such as severe acute respiratory syndrome coronavirus 2 (the virus responsible for COVID-19) [6,7]. However, its use in these contexts is still under study, and it is not widely approved for these indications.

Technically, NAT is [2-[(5-nitro-1,3-thiazol-2-yl) carbamoyl] phenyl] ethanoate (Fig. 1a) and is manufactured by reacting 2-amino-5-nitro thiazole (ANT) (Fig. 1b) and (Fig. 1c) in presence of thionyl chloride [8,9]. The process impurities may potentially include unreacted or excess ANT, unreacted aspirin, and salicylic acid (Fig. 1d) (a by-product formed by the hydrolysis of aspirin). Another potential impurity formed during the process is tizoxanide (TIZ) (Fig. 1e) which is a result of thermal hydrolysis in an acid and/or alkaline medium. TIZ possesses similar efficacy and safety as that of NAT. TIZ is also the *in vivo* metabolite of NAT. Till date, NAT is not official in any pharmacopeia. A specification was developed in-house to achieve consistency in product quality. Accordingly, each of the impurities i.e ANT, aspirin, and salicylic acid shall not exceed 0.1%. The limit of unknown impurities shall not exceed 0.1%. Moreover, TIZ shall not exceed 0.5%. Total impurities shall not exceed 0.5%.

In the current study, we successfully developed and validated a reverse-phase high-performance liquid chromatography (HPLC) method for the determination of process impurities, degradation products, and related substances of NAT. Since NAT is not official in any pharmacopeia, the limits of impurities were narrowed down (not more than [NMT] 0.5% as total impurities) such that the responses of all known and unknown impurities are captured in the chromatogram. The lack of pharmacopoeial specification, four known impurities (ANT, acetylsalicylic acid, salicylic acid, and TIZ), their degradation products formed during exposure to stress studies, the low sensitivity of detection to be achieved, separation of structurally similar TIZ from NAT, transformed the method specificity more complex and challenging.

Impurities in pharmaceuticals can lead to adverse events [10] including life-threatening conditions [11] and product instability [12].

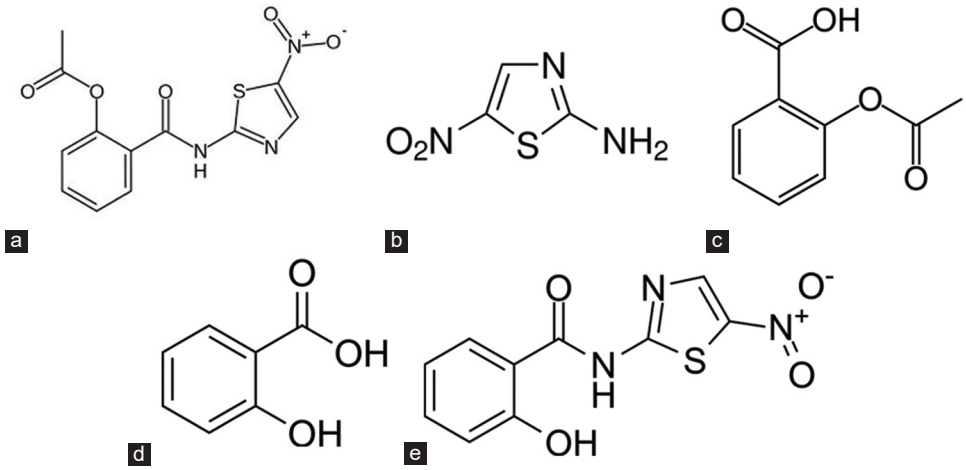

Fig. 1: (a) Nitazoxanide (NAT). (b) 2-amino-5-nitrothiazole. (c) 2-acetoxy benzoic acid (Aspirin). (d) 2-hydroxy benzoic acid (Salicylic acid). (e) Structure of Tizoxanide

Determination of impurities in pharmaceuticals is essential [13-15] to ensure patient safety, maintain drug efficacy, ensure consistent quality, and prevent a product recall [16-18]. Methods of the determination of NAT in the formulation are scarce in the literature [19-21]. At present, there is no published literature describing the estimation of NAT and its impurities and related substances. This manuscript reports a validated HPLC method for the determination of NAT and all its related substances.

METHODS

Instrumentation

A Shimadzu LC 2010 (Shimadzu, Japan) equipped with a column temperature oven, autoinjector, and photodiode array detector (PDA) detector was used for the study. Data acquisition was facilitated using a LC Solutions software synchronized to the Labsolutions Server. YMC Pack C8 Columns (250×4.6 mm i.d; 5 µm) (YMC Co. Ltd, Japan) with L7 packing or equivalent were used. The injection volume is set at 25 µL. pH meter (Model: PICO+, Labindia make), Analytical balance (Model: ME155DU/A, Mettler Toledo; Sensitivity 0.01 mg), and Ultrasonicator (Model: VCX 750, Vibracell Sonics make) were used for the study. Class A glassware (Borosil, India) was used throughout the study.

Standards, chemicals, and reagents

Reference standards (purity>99.98% w/w) of NAT, ANT, salicylic acid, and aspirin were gift samples from a local manufacturing unit in Mumbai. Milli-Q water was used for solution preparation. Solvents such as dimethylformamide and acetonitrile were of HPLC grade and purchased from Merck Ltd. Chemicals for preparation of buffers were of the highest grade and sourced from Merck Ltd. Test substance (batch sample) of NAT containing NMT 0.1% w/w of TIZ was taken for final evaluation of the method.

Preparation of analytical solutions

Preparation of standard solutions

A mixture solution of 70:30:1% v/v/v solution of acetonitrile, water, and dimethyl formamide was prepared, mixed thoroughly, and then sonicated to remove any dissolved gases. This solution was used as the diluent throughout the experiment. Stock solutions of NAT, ANT, aspirin, and salicylic acid were prepared separately using the diluent such that the final concentration was 250 parts per million (ppm). The stock solution of TIZ was prepared such that its concentration was 1250 ppm. A mixture of NAT and its impurities is then prepared such that it contains 2.5 ppm each of NAT, ANT, aspirin, salicylic acid, and 12.5 ppm of TIZ. This solution is treated as the system suitability solution or 100% reference standard solution.

Preparation of sample solutions

Approximately 250 mg of the test substance is weighed and dissolved in 100 mL of diluent. This sample solution is suitably diluted before analysis as such or spiked with impurities solutions for evaluating various validation parameters.

Preparation of buffers and mobile phase

The mobile phase consisted of two separate reservoirs containing acetonitrile and 2% v/v orthophosphoric acid in water adjusted to pH 2.5. Based on the results of the experimental design, the gradient programming for the mobile phase was fixed.

Chromatographic separation and quantitation

The gradient program (time in min/% mobile phase-B) 0.01/30, 25/70, 30/70, and 40/30 was applied on a YMC Pack C8 column (L7 packing) with dimensions of 250×4.6 mm and 5 µm. The flow rate of 1.0 mL/min was best suited for the separation. Peak responses were quantified at 210 nm for NAT and related substances using a PDA.

Method validation

System suitability

The reference standard solution (treated as 100% level) was used for the evaluation of system suitability. The results are treated acceptably if the relative standard deviation (RSD) of six replicate injections for peak area is within 2%, the mean theoretical plates are not less than (NLT) 2000, tailing factor (Tf) NMT 2.0, and resolution is NLT 2.0. A blank sample at the beginning of the injection sequence was analyzed to check interference due to solutions. The sequence also contained a blank sample at the end to ascertain any carryover effect.

Specificity and forced degradation studies

Specificity due to impurities and related substances

The specificity of an analytical method is defined as the absence of interference due to impurities, related substances, and/or degradation products at the retention time of the peaks under study. This interference sometimes can also be in the form of peak response amplification or attenuation caused by co-analytes, impurities, degradation products, or pH buffers in solution. Evaluation of specificity is the prerequisite for method validation. In this study, the effect of forced degradation was also included as a part of specificity.

Individual standard solutions of NAT and its impurities at 100% level and limit of quantification (LOQ) concentrations are analyzed initially. The sample solution was also analyzed separately to check the presence of known and unknown impurities.

Table 1: Results of system suitability

Analyte	Retention time	Tailing factor	Resolution	Number of theoretical plates
NAT	15.95±0.006	1.13±0.01	22.42±0.057	59681±403
ANT	5.55±0.01	1.07±0.012	--	4445±38.1
Aspirin	7.66±0.011	1.07±0.005	6.35±0.005	8599±65.4
Salicylic acid	9.69±0.008	1.11±0.001	6.49±0.012	17030±88
TIZ	17.61±0.008	1.15±0004	6.45±0.013	76884±375

Values of mean±standard deviation indicated above are for replicate measurements of n=6. ANT: 2-amino-5-nitro thiazole, TIZ: Tizoxanide

Table 2: Results of specificity

Specificity	
Analyte	**RRT**
ANT	--
Aspirin	6.33
Salicylic Acid	6.52
NAT	22.43
TIZ	6.45

RRT: Relative retention time, ANT: 2-amino-5-nitro thiazole, TIZ: Tizoxanide

Specificity due to forced degradation products

For forced degradation, the untreated sample solution is used for comparison. Similar quantities as taken for sample solution were separately weighted into volumetric flasks and initially treated separately with 10 mL of HCl (0.1 M, 0.5 M), NaOH (0.1 M, 0.5 M), and H_2O_2 (3% w/w, 5% w/w). The volumetric flasks are then placed in a water bath at 55°C for 1 h to allow degradation. After the exposure time, the samples are neutralized and further made up to volume with a diluent solution and analyzed. To study the effect of thermal stress, the sample is initially placed in a hot air oven at 105°C for 6 h. This sample is then made up to volume. For UV treatment, the sample is exposed to UV light of 254 nm such that the exposure is NLT 1.2 million lux h and 200-watt h/m^2. The results are compared with those of the untreated samples exposed to diffused daylight and also with the sample solution placed in an amber-colored volumetric flask.

To study the effect of humidity and temperature, the sample is placed for 24 h in a 40°C/75% relative humidity stability chamber. This sample is compared with that of the untreated sample. The peak purity is also verified for each of the samples. The mass balance was also determined after the degradation.

For specificity, the results are considered acceptable if none of the impurities, related substances, and/or degradation products interfere with the peak responses of NAT and the known impurities. Furthermore, the response obtained in the untreated sample mixture should be comparable with that of the peak response obtained in individual analytes. The relative retention times (RRTs) in the LOQ and reference solutions are similar to that of the sample solution. The theoretical plates for the peaks of interest are NLT 2000, Tf NMT 2.0, and the resolution is NLT 2.0.

Stability of solutions

Room temperature (25±2°C) stability for 24 h and refrigerated stability (8±2°C) for 1 week was conducted using a reference standard solution. The results are compared with freshly prepared solutions of similar concentration. A correction factor is applied to compensate for weight variations (if any).

Linearity and range

A calibration curve in the range of LOQ-150% level was plotted. The linear calibration standard solutions contained a mixture of NAT and its impurities at LOQ, 50%, 80%, 100%, 120%, and 150% of the standard concentration. LOQ was determined as described in "*Detection and quantification limits*" in the next section. Linearity is treated as acceptable if the regression coefficient (r^2) is NLT 0.99 for each of the analytes. Furthermore, the %RSD for triplicate measurements at each concentration is NMT 2% for NAT and NMT 5% for known impurities.

Precision

System precision was performed using 100% solution. Six replicate injections of the 100% reference solution were injected and the data were tabulated.

For method precision, six separate test substance solutions were prepared and analyzed. To verify the repeatability in response to the known and unknown impurities, spiked studies were also conducted. Twelve test substance solutions were prepared of which six preparations are spiked with impurities equivalent to that of specification limit. The remaining six preparations were spiked with LOQ concentrations of the same impurities into the test substance solution. The data are treated as satisfactory if the %RSD of the peak responses for known and unknown impurities is within 10% at all levels.

Intermediate precision was performed similarly to that of method precision on a different instrument, different column by a different qualified analyst.

Accuracy/recovery studies

Standard solutions containing NAT along with impurities were prepared at LOQ, 50%, 100%, and 150% levels. The solutions were analyzed. Equivalent quantities of individual impurities are spiked in the test substance such that the level of impurity matches with that of the reference solution. The % recovery is calculated by taking the ratio of the area responses in the spiked samples to that obtained in the reference solution of the corresponding concentration. SST was performed before the activity. All measurements are conducted in triplicate. The % recovery of NAT along with its known and unknown impurities was calculated. The data is treated as acceptable if the % recovery is within ±15% from their nominal value.

Detection and quantification limits

The limit of detection (LOD) and LOQ are determined by preparing solutions of 10%, 20%, 30%, 50%, and 80% to the specification limit. A linear graph is then plotted to find the slope and standard deviation (σ) of the replicate measurements. LOD = 3.3 σ/slope and LOQ = 10 σ/slope. (n=3). After obtaining theoretical LOD and LOQ values, solutions at concentrations lower than those obtained theoretically were prepared and tested for repeatability and accuracy (n=3).

Robustness

The effect of intentional variation on the overall chromatographic separation is studied. The effect of flow variation (1.0±0.1 mL/min), column temperature oven variation (25±5°C), and pH variation (2.5±0.2 units) was studied.

RESULTS AND DISCUSSION

Method development and method optimization

Before proceeding with the full validation, we initially evaluated the stability of the individual impurity solutions and NAT. Bench top room temperature stability studies for the solutions of salicylic acid and ANT in diluent solution demonstrated good stability during handling and storage. However, degradation peaks spectrally correlating to TIZ were observed when long-standing solutions of NAT in a diluent solution were injected after 24 h period. Since the diluent solution is neutral in pH, it is expected that degradation occurred through oxidation, thermal, and/or photolytic means. To further study the influence of pH,

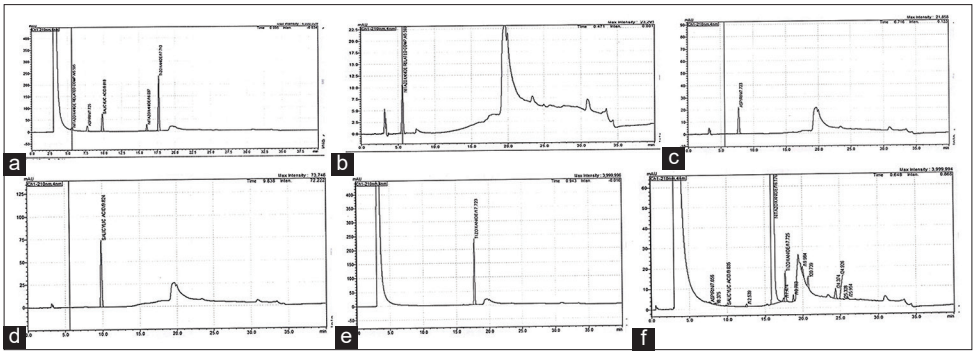

Fig. 2: (a) Chromatogram of the standard solution. (b) Chromatogram of nitazoxanide-related substance A. (c) Chromatogram for aspirin. (d) Chromatogram for salicylic acid. (e) Chromatogram of tizoxanide. (f) Chromatogram of the test substance (batch sample)

Table 3: Results of forced degradation studies

Forced degradation studies on NAT sample (LOD=0.23%w/w)				
Nature of sample	**NAT Assay**	**NAT assay on dried basis**	**Total impurity**	**Mass balance %**
Standard Solution	100%	N.A	0.0	N.A
Sample Solution (Untreated)	99.07	99.3	0.53	100.56
Sample Solution Acid Stress (0.1 M)	94.94	95.15	1.31	96.46
Sample Solution Base Stress (0.1 M)	93.26	93.47	2.86	96.33
Peroxide Treatment	95.83	96.06	5.43	101.48
Diffused Daylight	100.74	100.97	0.55	101.29
Thermal Stress	101.19	101.42	0.71	101.9
UV Treatment	99.5	99.73	0.64	100.37
Humidity	100.34	100.57	0.66	101.23

*N.A: Not applicable. LOD: Limit of detection

Table 4: Results of Linearity for NAT and its related substances

Analyte	Range of the Calibration curve (LOQ-150%) in ppm	Equation of the best-fit line	R^2
ANT	0.269–3.769	Y=56064×−3302.6	0.9958
Aspirin	0.254–3.787	Y=99545×+6453.7	0.9940
Salicylic Acid	0.194–3.794	Y=364843×−29805	0.9919
NAT	0.215–3.820	Y=107086×−4384.4	0.9958
TIZ	0.614–18.791	Y=190022×−78712	0.9940

The values indicated above are for replicate measurements of n=3.
ANT: 2-amino-5-nitro thiazole, TIZ: Tizoxanide, ppm: Parts per million

acid and alkaline degradation studies were conducted as part of forced degradation studies.

Previous literature on the determination of NAT, its process impurities, and degradation products are scarce. NAT in tablets and in powders for oral suspension was estimated spectrophotometrically and the results are compared with an HPLC method [22]. The range of the method was from 2 to 20 ppm and detection was conducted at 345 nm for NAT. Since this was an assay method, LOD and LOQ estimations are out of its scope. In another citation [23], the authors reported three methods namely, a first derivative spectroscopy method with estimation at 364 nm (LOD of 0.95 ppm for NAT), principal component regression method (LOD of 0.78 ppm for NAT) with estimation in the range of 260–360 nm, and a densitometric method of estimation at 254 nm (LOD of 0.1 μg/band). In the densitometric method, the estimation ranged from 0.4 to 2.0 μg/band. Neither of the reported methods considered the estimation of related substances, degradation products, and process impurities. The range of estimation in our method is much below than those reported earlier and includes process impurities and degradation products.

Since NAT and TIZ were structurally related, coelution and/or merged peaks were observed with different compositions of mobile phase under isocratic elution. Since TIZ is the active *in vivo* metabolite of NAT, it is imperative to estimate their contents separately. In routine industrial practice, certificates of analysis for the released batches indicated the contents of TIZ and NAT. When reporting the potency, the additive value of TIZ and NAT was reported since TIZ and NAT are equipotent *in vivo*.

In most of the chromatography experiments performed, the peak parameters such as Tf, and a number of theoretical plates were unaltered. Hence, the separation of TIZ and NAT was most critical for the development. To achieve this separation, a gradient elution program was chosen. Higher carbon load columns such as in Kromasil C18, led to the merging of TIZ and NAT peaks [24]. A carbon load of 7% on a Waters Spherisorb column led to long retention times and unacceptable asymmetry factors for salicylic and aspirin. Columns with a carbon load of 10–12% were found suitable. Final confirmation was taken on a YMC Pack C8 column having a 10% carbon load.

The objective of the method is to increase the sensitivity of detection such that the responses of all known and unknown impurities and degradation products are captured in the chromatogram. In initial experiments, methanol reduced the sensitivity of detection leading to poor peak responses. On the contrary, acetonitrile has a low viscosity and offers higher sensitivity during detection [25]. To retain the stability of solutions during analysis, dimethyl formamide at 1% v/v was added into the diluent solution. This provided stability to the aspirin stock dilutions and the NAT stock dilutions preventing their conversion to their corresponding hydrolyzed products.

Method validation

System suitability

The results of the system suitability performed using the standard solution met the acceptance criteria for NAT and all its related substances and/or impurities. The results are given in Table 1:

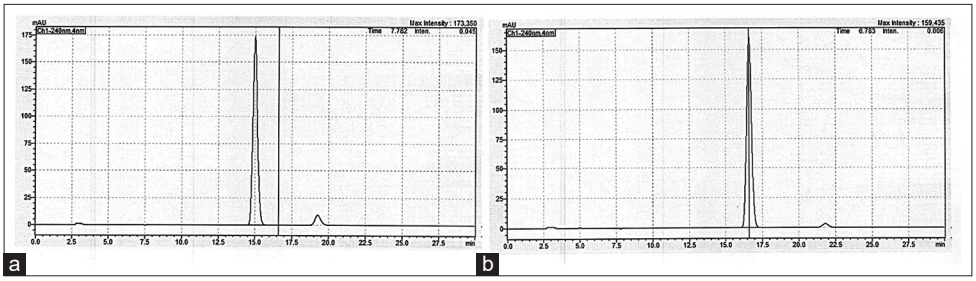

Fig. 3: (a) Chromatogram of base stress sample. (b) Chromatogram of peroxide-treated sample

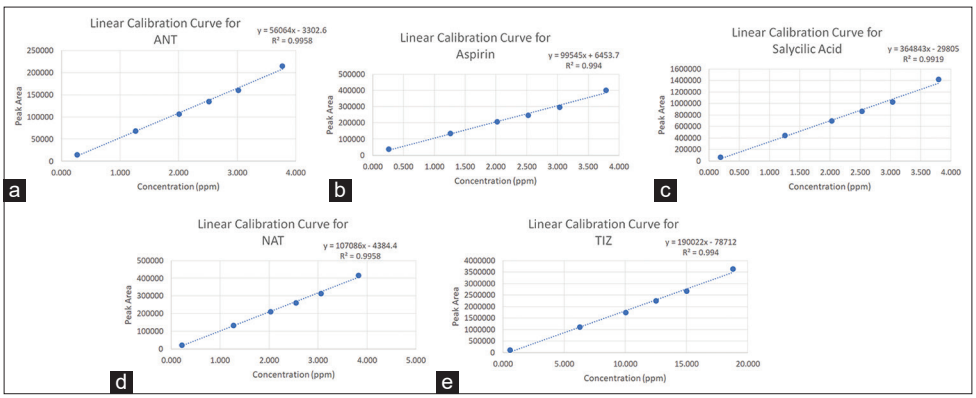

Fig. 4: (a) Linear calibration curve for 2-amino-5-nitro thiazole. (b) Linear calibration curve for aspirin. (c) Linear calibration curve for salicylic acid. (d) Linear calibration curve for NAT. (e) Linear calibration curve for tizoxanide

Table 5: Results of precision for NAT and its related substances

% RSD at various levels				
Analyte	**Standard**	**Test substance**	**100% impurity spiked solution**	**LOQ level spiked solution**
NAT	0.14	0.27	0.27	0.27
ANT	0.38	N.D	1.05	1.66
Aspirin	0.09	n.d	1.28	2.36
Salicylic acid	0.16	5.85	1.07	1.75
TIZ	0.11	5.38	5.06	2.89
SMUI	0.27	N.D	3.02	1.22
Total Impurities		1.51	3.03	1.74

*N.D: Not detected. Values indicated above are for replicate measurements of n=3. LOQ: Limit of quantification, %RSD: % relative standard deviation, ANT: 2-amino-5-nitro thiazole, TIZ: Tizoxanide

Table 6: Results of Recovery studies for NAT and its related substances

% Recovery at various levels				
Analyte	**LOQ**	**50%**	**100%**	**150%**
ANT	107.24±1.34	110.53±6.36	109.38±3.32	106.98±1.18
Aspirin	81.94±0.77	101.56±7.24	104.27±4.43	101.53±0.58
Salicylic acid	101.82±0.28	103.84±2.8	106.48±5.88	102.32±1.62
TIZ	105.59±8.07	102.07±2.44	101.57±1.61	100.14±0.23
SMUI	116.94±1.19	99.55±0.39	99.12±0.29	99.39±0.02

Values of mean±standard deviation indicated above are for replicate measurements of n=3. LOQ: Limit of quantification, ANT: 2-amino-5-nitro thiazole, TIZ: Tizoxanide

Table 7: Results of LOD and LOQ for NAT and its related substances

Analyte	**LOD (ppm)**	**S/N at LOD**	**LOQ (ppm)**	**S/N at LOQ**
NAT	0.07	36.12	0.22	57.82
ANT	0.09	10.65	0.27	32.09
Aspirin	0.08	31.27	0.25	63.28
Salicylic Acid	0.06	111.28	0.19	146.25
TIZ	0.20	99.19	0.61	287.57

Values indicated above are for replicate measurements of n=3. LOD: Limit of detection, S/N: Signal-to-noise ratio, LOQ: Limit of quantification, ppm: Parts per million, ANT: 2-amino-5-nitro thiazole, TIZ: Tizoxanide

Specificity and forced degradation studies

No interference was observed at the retention times of NAT and its impurities. The RRT of replicate injections was highly precise. The forced degradation studies confirmed that NAT is principally susceptible to peroxide degradation. However, some degradation also occurred during alkaline stress. The results of specificity for the untreated samples are given in Table 2. The mass balance results along with peak purity values (from the purity angle and purity threshold values) obtained during forced degradation are tabulated in Table 3: The chromatograms for specificity are given in Fig. 2a-f. The chromatograms for alkaline stress and peroxide exposure are depicted in Fig. 3a and b.

Stability of solutions

Stock solutions of individual impurities and NAT are stable in diluent solution. The stability was achieved after the addition of 1% v/v dimethyl formamide to a 70:30% v/v mixture of acetonitrile and water.

To prevent the formation of TIZ in NAT solutions, the stock solutions are prepared and stored in amber-colored glassware.

Linearity and range

After ascertaining the stability of NAT and known impurities, and also confirming the absence of degradation due to diffused daylight and UV stress, the calibration standards for linearity were prepared. The linearity experiment was performed in triplicate. The results are given in Table 4. The calibration plots of concentration (ppm) versus peak area for each of the analytes are shown in Fig. 4a-e. The unusually high values of the intercepts are due to high noise perhaps caused by acidic substances such as salicylic acid and aspirin [26]. The threshold concentration above which peak responses are captured at the detector is calculated mathematically from the equation of the best-fit line. This threshold concentration is well below the LOQ concentration making the method suitable for its application.

Precision

The precision of the method determines the repeatability of a measurement and is usually expressed as the %RSD. Replicate measurements (n=6) of responses for all the analytes were taken and the % RSD was calculated. The data are compared among the standard solution, solutions of the test substance, solutions spiked with 100% level of impurity standard into test substance and solutions spiked with LOQ levels of impurity standard into test substance solutions. The results of method precision are given in Table 5. Intermediate precision met all the acceptance criteria on different equipment with a different analyst.

Accuracy/recovery studies

Known amounts of the impurity standard solutions are spiked into test substances at LLOQ, 50%, 100%, and 150% levels and analyzed. Similarly, the standard of equivalent concentrations was also analyzed. The responses are compared and the results are tabulated in Table 6. Recovery is the ability of the method to determine the composition of the analyte in the presence of matrix components in solution.

Detection and quantification limits

The LOQ was determined by preparing serial dilutions at 10%, 20%, 30%, 50%, and 80% to the specification limit. The slope and the overall standard deviation were calculated. Acceptable repeatability was found during replicate measurements. After obtaining the empirical values of LOD and LOQ we then prepared solutions individually and as a mixture in solution. The solutions were tested and the signal-to-noise ratio (S/N) was calculated. The results are tabulated in Table 7.

At concentrations below the obtained LOD and LOQ measurement, the % RSD of replicate measurements was out of the limit. The detection and quantification limits finalized are acceptable for compliance with the product.

Robustness

The results of robustness obtained after intentional variations in the chromatographic parameters were compared with those obtained under standard conditions of the method validation. The data such as relative retention time, Tf, theoretical plates, resolution, and peak purity were evaluated. All the results are within 5% confirming the robustness.

CONCLUSION

NAT is a versatile active pharmaceutical ingredient (API) widely used in veterinary applications. To achieve consistent quality, it is imperative to control the overall purity and the residual impurities [27,28]. While assay methods are scarcely available, there is no published method on the estimation of NAT, its related substances, and impurities till date. The objective of our study was to develop and validate a sensitive and robust method for the estimation of NAT, its impurities, and related substances in bulk API.

To achieve the desired robustness, a careful combination of the stationary phase and mobile phase was optimized. Octyl silane bonded to porous silica particles (C8) also called L7 packing with a medium carbon load best suited for the separation. To control the conversion of NAT and aspirin into their hydrolyzed products, the diluent was optimized to contain 1% dimethylformamide. To prevent the merging of the analyte peaks with the degradation products/impurities/related substances, the gradient program was chosen. The spacing between closely eluting peaks was adequate indicating that the selectivity and capacity were optimum. For all the peaks, the purity angle was less than the purity threshold indicating that the peak is pure and devoid of any coeluting substances. The baseline noise in the method is however unavoidable due to the presence of acidic analytes such as aspirin, and salicylic acid and their degradation productions in solution. The S/N ratio at LOD and LOQ correlates to higher intercept values in the calibration curve. Unlike most methods, where noise tends to be maximum resulting in poor repeatability at lower concentrations, this method is superior. The response is uniform at all concentrations. To amplify the signal and achieve greater response, the quantification was done using a PDA detector at 210 nm. Acetonitrile in the mobile phase also amplified the peak response. The pH of 2.5 for the mobile phase provided optimum protection of the end-capped silica.

Overall, the method was validated as per the international conference on harmonization guidelines. Critical validation parameters such as linearity, accuracy, precision, LOD, and LOQ are found to meet the acceptance criteria. The percent recovery from spiked studies was >90% between 50 and 150% of nominal concentration and >80% at LOQ. The method was successfully applied for routine quality control of NAT bulk h2API.

STUDIES INVOLVING PLANTS

Not applicable.

DATA AVAILABILITY STATEMENT

Data included in article/supp. material/referenced in the article.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Given by all the authors.

AUTHOR CONTRIBUTION

The need for the work was identified by Mr. Tata Santosh and further approved by Dr. Prafulla Kumar Sahu. Work execution, results tabulation, and preparation of the manuscript were done by Mr. Tata Santosh. A review of the draft manuscript was done by Dr. Prafulla Kumar Sahu.

CONFLICTS OF INTEREST

None.

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