Development and Validation of a Headspace Gas Chromatographic (HS-GC) Method for Determination of Residual Solvents in Nitazoxanide API

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INTRODUCTION

Several organic volatile solvents or chemicals are used in the manufacturing of drug substances, excipients and drug products. They are also used to increase the final yield, to enhance the purity or to change the physical form such as polymorphic form and solubility. These solvents or chemicals do not have any therapeutic activity but maybe toxic for humans if consumed more than permitted daily exposure (PDE) (Sitaramaraju et al., 2008). It is necessary to remove them, but some solvents remain in small quantities in the final product. These small quantities of organic solvents remain in the final product is known as residual solvents. Determination of these residual solvents from drug substances, excipients and drug products is a difficult and challenging task. Headspace gas chromatographic technique is most suitable and used for the determination of residual solvents. The acceptance limit

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ABSTRACT
Controlling residual solvents in the drug substances or active pharmaceutical ingredients (API) is mandatory to the specified limits as per the International Conference on Harmonisation (ICH) Q3C guidelines. Residual solvents in pharmaceuticals are mostly determined by Gas Chromatography with Headspace. A simple and sensitive headspace gas chromatographic (HS-GC) method has been developed for the determination of Acetone, Dichloromethane and Cyclohexane in Nitazoxanide API. The separation of analytes was achieved with DB – 624 (30 m length, 0.53 mm inner diameter and 3.0 μm in film thickness) capillary column. Dimethyl formamide was used as a diluent. Nitrogen was used as carrier gas with 3.0 mL/minutes and Flame ionisation detector (FID) for detecting analytes. The oven temperature was set at 60°C for 5 minutes at initial and programmed at a rate of 20°C per minute to a final temperature of 240°C for 2 minutes. Run time was 16 minutes, and total GC cycle time was 25 minutes. The split ratio used as 1:20 to get optimum peak response. The developed method was validated as per the ICH guidelines for specificity, accuracy, precision, linearity, range, the limit of detection, the limit of quantification and robustness. The results of validation were indicated no interference, good recoveries, precise, linear, rugged and robust method, suitable for the determination of residual solvents in Nitazoxanide API for research and routine quality control laboratory.

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for residual solvents is set following the toxicity of solvents and specified in the international conference on harmonisation Q3C guidelines (ICH, 2016; Harold et al., 1997).

Nitazoxanide (Figure 1) is chemically 2-acetyloxy-N-(5-nitro-2-thiazolyl) benzamide and have broad-spectrum antiprotozoal and antiparasitic activity (Rossignol and Cavier, 1976). It is also used to treat helminthic, protozoal, and viral infections. Cryptosporidiosis and giardiasis in immunocompetent patients also be treated with nitazoxanide (Rossignol et al., 2001; Mégraud et al., 1998). It is a prodrug and absorbs from the gastrointestinal tract when administered orally. In humans, it rapidly hydrolysed to its active metabolite tizoxanide (Korba et al., 2008).

Literature survey revealed that several methods by UV – spectroscopy (Pandey, 2009; Gandhi et al., 2008), visible spectroscopy (Narayana and Manohara, 2007) and liquid chromatography (Male-suik et al., 2009; Kumar et al., 2009; Narayan and Mahendra, 2007) are reported for quantitative estimation (assay) of nitazoxanide in bulk alone and combination with other drugs. During the synthesis and purification process of nitazoxanide API, Acetone, Dichloromethane and Cyclohexane were used. This work aimed to develop and validate a simple and sensitive headspace gas chromatographic (HS-GC) method for simultaneous determination of Acetone, Dichloromethane and Cyclohexane in Nitazoxanide API. Acetone, Dichloromethane and Cyclohexane belong to class 3, 2 and 2 respectively. The specifications as per international conference on harmonisation Q3C guidelines are tabulated in Table 1.

**MATERIALS AND CHEMICALS**

GC reference standards of Acetone, Dichloromethane (Methylene dichloride) and cyclohexane were procured from Biosolve Chimie, France. Dimethyl formamide (N, N-Dimethyl Formamide) (Supra Solv, GC grade) was procured from Merck Millipore, India. Nitazoxanide API sample was received as gift samples from Suven Life Sciences Limited, Hyderabad, India.

**METHOD**

The method was developed and validated on Agilent Technologies gas chromatograph (Model No. 7890B) and a headspace sampler (Model No. 7697A) equipped with flame ionisation detector (FID) using Empower 3 software. The separation of analytes was achieved with DB – 624 (30 m length, 0.53 mm inner diameter and 3.0 μm in film thickness) capillary column. The chromatographic parameters were optimised, and optimised chromatographic conditions are shown in Table 2.

**Diluent**

Dimethylformamide.

**Blank**

Use diluent as blank.

**Preparation of Standard solution**

Weigh accurately about 500 mg, 60 mg, 388 mg of Acetone, Dichloromethane and Cyclohexane reference standards respectively into a 100 mL volumetric flask having about 25 mL of diluent. Mix and make up to volume with diluent. Transfer 5.0 mL of above solution into a 100 mL volumetric flask and dilute to volume with diluent and mix well. Transfer 2.0 mL of the above solution into six different headspace vials and seal properly.

**Preparation of Sample solution**

Weigh and transfer accurately about 100 mg of Nitazoxanide API sample into a headspace vial. Add 2.0 mL of diluent, dissolve and seal the vial properly.

**Preparation of System Suitability solution**

Use the standard solution to check the system suitability.

**Procedure**

Inject blank (1 injection), and standard solution (6 injections), sample solution (1 injection) into the chromatograph and record the peak response using the chromatographic and Headspace parameters.

**Acceptance criteria for System Suitability**

The resolution between Acetone and Dichloromethane peaks from the first standard injection from system suitability should be not less than 3.0.

The relative standard deviation (RSD) of area response for Acetone, Dichloromethane and Cyclohexane peaks between the six replicate injections of the standard should be no more than 10 %.

**Method validation**

Validation of the developed method was conducted as per United States Pharmacopoeia general chapter <1225> (USP, 2018a) and International Conference on Harmonization Q2 (R1) (ICH, 2005) guidelines.

**System suitability**

System suitability was evaluated under United States Pharmacopoeia general chapter <621> (USP, 2018b). System suitability of the method was...
established by injecting blank and standard solution for system suitability, calculated the resolution between Acetone and Dichloromethane peaks from first standard injection from system suitability and the relative standard deviation (RSD) of area response for Acetone, Dichloromethane and Cyclohexane peaks from the six replicate injections of the standard solution. The acceptance criteria for resolution between Acetone and Dichloromethane peaks was not less than 3.0 and % RSD for area response of Acetone, Dichloromethane and Cyclohexane peaks were not more than ten from six replicate injections of the standard solution.

Specificity
The specificity of the method was established by injecting blank in triplicate, standard solution, test solution, test solution spiked with analytes at the specification level, Acetone reference standard solution at the specification level, Dichloromethane reference standard solution at specification level and Cyclohexane reference standard solution at the specification level. The chromatograms were evaluated for any interference at the retention time of Acetone, Dichloromethane and Cyclohexane peaks.

Precision
The precision of the method was evaluated by injecting six test sample preparations spiked with Acetone, Dichloromethane and Cyclohexane reference standards at 100% specification level. % relative standard deviation of six test sample preparations spiked with analytes was calculated. Intermediate precision of the method was also evaluated using different analyst, different day, different instrument and different column by injecting six test sample preparations spiked with analytes prepared as same for precision. The acceptance criteria for individual precision % RSD was not more than 5.0, and for 12 preparation results was not more than 7.0.

Accuracy (Recovery)
Recovery study was performed to evaluate the accuracy of the method by spiking method. Recovery study was done by spiking Acetone, Dichloromethane, and Cyclohexane reference standards into the test sample in the concentration of LOQ, 50%, 100% and 150% level of the proposed specification concentration. The recovery samples were prepared in triplicate for 50% & 100% level and six preparations for LOQ & 150%. Injected the prepared recovery samples in the optimised experimental conditions. % recovery of Acetone, Dichloromethane and Cyclohexane peaks were calculated for all the levels. The acceptance criterion for recovery of Acetone, Dichloromethane and Cyclohexane analytes was 80.0 to 120.0% and % RSD for six recovery results at LOQ, and 150% was not more than 5.0.

Limit of detection (LOD) and limit of quantitation (LOQ)
LOD is the lowest amount of analyte that can be detected, but not necessarily quantifiable, and LOQ is the lowest amount of analyte that can be quantitated with acceptable precision and accuracy. The LOD and LOQ were established by injecting a known concentration of serial dilutions of Acetone, Dichloromethane and Cyclohexane under the stated experimental conditions. The LOD and LOQ were established from the Slope and STEYX by plotting the linearity curve of concentration versus area response. LOD and LOQ were estimated by using the following formulae:

\[
LOD = 3.3 \times \left(\frac{\sigma}{S}\right)
\]

\[
LOQ = 10 \times \left(\frac{\sigma}{S}\right)
\]

Where \(\sigma = \text{STEYX of response}\) and \(S = \text{slope determined from the linear plot}\).

Linearity
The linearity of the method was established for Acetone, Dichloromethane and Cyclohexane from LOQ to 150% of the proposed concentration using six calibration levels (LOQ, 25, 50, 100, 125 and 150% of the targeted concentration). The reference standards were used to prepare calibration levels. The calibration curves for Acetone, Dichloromethane and Cyclohexane, were plotted for each level as concentration versus peak area response. The results of linearity were evaluated by regression analysis.

Robustness
Robustness of the method was determined with deliberate changes in the method conditions from the optimised final conditions. Injected blank, standard solution, test sample and spiked test sample solution in each robustness conditions and evaluated the system suitability.

For robustness study, the following parameters were considered such as (i) Change in initial oven temperature 60°C ±5°C (55°C and 65°C) and (ii) Change in nitrogen gas flow rate 3.0 mL/min ±10% (2.7 and 3.3 mL/min).

Solution stability
Solution stability was established for the standard solution and test sample preparations. Bench-top (controlled room temperature) stability was established by injecting standard solution and test sample at regular interval for 48 hours. Solution stability was established by calculating the similarity factor for the standard solution against a new standard.
and % difference for a test sample from the initial value.

RESULTS

System suitability
System suitability of the method was evaluated through resolution between Acetone and Dichloromethane peaks from standard solution and the % RSD of area response for Acetone, Dichloromethane and Cyclohexane peaks from the six replicate injections of the standard solution. The system suitability results were found well within the predefined acceptance criteria. The results are presented in Table 3.

Figure 4: Chromatogram of the test sample

Figure 5: Chromatogram of a spiked test sample

Figure 6: Chromatogram at LOQ level

Figure 7: Chromatogram at 150 % level

Figure 8: Linearity graph of acetone

Specificity
The specificity of the method was performed to check blank interference and confirm the identity
Table 1: Specifications of residual solvents

| Residual Solvent | Solvent Class | Allowable Concentration Limit (ppm) |
|------------------|---------------|-------------------------------------|
| Acetone          | Class 3       | 5000                                |
| Dichloromethane  | Class 2       | 600                                 |
| Cyclohexane      | Class 2       | 3880                                |

Table 2: Optimised chromatographic conditions of the developed method

| Parameter                              | Optimised Condition                                                                 |
|----------------------------------------|--------------------------------------------------------------------------------------|
| **GC Parameters**                      |                                                                                      |
| Column                                 | DB - 624, 30 m length X 0.53 mm inner diameter, 3.0 μm film thickness (6% cyanopropyl phenyl and 94% dimethylpoly siloxane, Part No. 125-1334, Make: Agilent) |
| Carrier gas (Nitrogen) flow            | 3.0 mL/minute                                                                        |
| Oven temperature                       | 60°C (5 minutes hold time) to 240°C (2 minutes hold) at the rate of 20°C/minutes     |
| Run time                               | 16.00                                                                                |
| Nitrogen flow                          | 25 mL/minute                                                                         |
| Hydrogen flow                          | 40 mL/minute                                                                         |
| Airflow                                | 400 mL/minute                                                                        |
| Injector temperature                  | 220°C                                                                                |
| Detector temperature                  | 260°C                                                                                |
| Spilt ratio                            | 1:20                                                                                  |
| **Headspace parameters**               |                                                                                      |
| Oven                                    | 110°C                                                                                |
| Loop                                    | 130°C                                                                                |
| Transfer line                          | 150°C                                                                                |
| GC cycle time                          | 25 minutes                                                                            |
| Vial equilibration time                | 20 minutes                                                                            |
| Pressurise time                        | 3.0 minutes                                                                           |
| Loop fill time                         | 0.5 minutes                                                                           |
| Loop equilibration                     | 0.05 minutes                                                                          |
| Injection time                         | 0.1 minutes                                                                           |
| Vial pressure                          | 11.0 psi                                                                              |

Table 3: System suitability results

| Parameter                          | Acetone Peak Area | Dichloromethane Peak Area | Cyclohexane Peak Area |
|------------------------------------|-------------------|---------------------------|-----------------------|
| Standard solution injection - 1    | 576.9             | 17.3                      | 2008.8                |
| Standard solution injection - 2    | 561.9             | 16.9                      | 1972.4                |
| Standard solution injection - 3    | 545.3             | 16.2                      | 1953.7                |
| Standard solution injection - 4    | 535.3             | 15.9                      | 1898.7                |
| Standard solution injection - 5    | 560.5             | 17.2                      | 1972.2                |
| Standard solution injection – 6    | 595.3             | 17.8                      | 2081.2                |
| Average                            | 562.5             | 16.9                      | 1981.2                |
| % RSD                              | 3.8               | 4.2                       | 3.1                   |
| Retention time (minutes)           | 4.2               | 4.7                       | 6.9                   |
| Resolution between Acetone and Dichloromethane peaks | 5.9 |
### Table 4: Precision results

| Test sample spike No. | Acetone | Dichloromethane | Cyclohexane |
|-----------------------|---------|-----------------|-------------|
|                       | Precision (ppm) | Intermediate precision (ppm) | Precision (ppm) | Intermediate precision (ppm) | Precision (ppm) | Intermediate precision (ppm) |
| 01                    | 5221    | 5345            | 575         | 592            | 3763        | 3865         |
| 02                    | 5278    | 5278            | 583         | 572            | 3814        | 3813         |
| 03                    | 5364    | 5478            | 593         | 602            | 3834        | 3967         |
| 04                    | 5413    | 5289            | 603         | 581            | 3965        | 3784         |
| 05                    | 5340    | 5078            | 589         | 562            | 3822        | 3734         |
| 06                    | 5523    | 5389            | 616         | 596            | 4034        | 3852         |
| Mean (n=6)            | 5357    | 5310            | 593         | 584            | 3872        | 3836         |
| % RSD (n=6)           | 2.0     | 2.5             | 2.5         | 2.6            | 2.7         | 2.1          |
| Mean (n=12)          | 5333    | 589             | 3854        |                |             |              |
| % RSD (n=12)         | 2.2     | 2.6             | 2.3         |                |             |              |

### Table 5: Accuracy results

| Recovery level       | Acetone | Dichloromethane | Cyclohexane |
|----------------------|---------|-----------------|-------------|
|                      | Recovery Mean % RSD | Recovery Mean % RSD | Recovery Mean % RSD |
| LOQ sample - 1       | 90.1    | 88.1            | 94.1        |
| LOQ sample - 2       | 89.4    | 89.9            | 92.7        |
| LOQ sample - 3       | 88.4    | 92.6 4.7        | 90.5 95.3 3.7 |
| LOQ sample - 4       | 95.7    | 93.6            | 97.6        |
| LOQ sample - 5       | 93.3    | 97.6            | 100.3       |
| LOQ sample - 6       | 99.8    | 93.7            | 100.3       |
| 50 % sample - 1      | 93.9    | 97.3            | 100.3       |
| 50 % sample - 2      | 100.5   | 96.9 3.4        | 94.7 97.5 2.9 |
| 50 % sample - 3      | 96.3    | 95.7            | 97.4        |
| 100 % sample - 1     | 99.4    | 96.8            | 96.5        |
| 100 % sample - 2     | 96.5    | 99.0 2.4        | 96.0 97.7 2.3 | 95.5 97.2 2.2 |
| 100 % sample - 3     | 101.2   | 100.2           | 100.3       |
| 150 % sample - 1     | 102.5   | 104.2           | 103.4       |
| 150 % sample - 2     | 101.9   | 100.9           | 102.4       |
| 150 % sample - 3     | 98.6    | 100.7 2.5       | 99.0 100.4 2.8 | 99.8 100.6 2.4 |
| 150 % sample - 4     | 97.1    | 96.3            | 100.7 2.4  |
| 150 % sample - 5     | 100.2   | 99.4            | 100.7 2.4  |
| 150 % sample - 6     | 103.9   | 102.7           | 101.7       |
Table 6: Determination of LOD and LOQ

| Sr. No. | Acetone | Dichloromethane | Cyclohexane |
|---------|---------|-----------------|-------------|
|         | Conc. (ppm) | Area | Response | Conc. (ppm) | Area | Response | Conc. (ppm) | Area | Response |
| 1       | 500.7   | 53.9 | 60.4 | 3.1 | 386.8 | 187.3 |
| 2       | 1001.3  | 106.1| 120.7| 4.6 | 773.5 | 372.6 |
| 3       | 1502.0  | 166.4| 181.1| 6.3 | 1160.3| 586.9 |
| 4       | 2002.6  | 226.2| 241.5| 8.1 | 1547.0| 797.4 |
| 5       | 2503.3  | 268.5| 301.8| 9.2 | 1933.8| 953.2 |

Correlation coefficient: Acetone 0.998, Dichloromethane 0.997, Cyclohexane 0.998
Slope: Acetone 0.1097, Dichloromethane 0.0260, Cyclohexane 0.5059
Steyx: Acetone 5.6425, Dichloromethane 0.1979, Cyclohexane 18.1601
LOD (PPM): Acetone 170, Dichloromethane 25, Cyclohexane 118
LOQ (PPM): Acetone 514, Dichloromethane 170, Cyclohexane 118

LOD = Steyx * 3.3 / Slope; LOQ = Steyx * 10 / Slope

Table 7: Linearity

| Linearity Level | Acetone | Dichloromethane | Cyclohexane |
|-----------------|---------|-----------------|-------------|
|                 | Conc. (ppm) | Area | Response | Conc. (ppm) | Area | Response | Conc. (ppm) | Area | Response |
| LOQ             | 510.4   | 55.0 | 76.4 | 3.3 | 355.5 | 161.1 |
| 50%             | 2501.8  | 269.7| 301.8| 9.2 | 1932.8| 944.6 |
| 75%             | 3752.6  | 373.9| 452.7| 12.1| 2899.2| 1317.8|
| 100%            | 5003.5  | 537.3| 603.6| 16.9| 3865.6| 1893.0|
| 125%            | 6254.4  | 646.4| 754.6| 19.9| 4832.0| 2269.3|
| 150%            | 7505.3  | 811.9| 905.5| 24.7| 5798.4| 2839.1|
| Slope           | 0.1068 | 0.0254 | 0.4860 |
| Intercept       | -5.3654 | 1.2577 | -23.7039 |
| Correlation coefficient | 0.998 | 0.998 | 0.999 |

Figure 9: Linearity graph of dichloromethane

Figure 10: Linearity graph of cyclohexane

of the analytes. The chromatograms confirm (Figures 2, 3, 4 and 5) no interference at the retention time of Acetone, Dichloromethane and Cyclohexane peaks peak due to blank.

Precision

The precision of the method was evaluated by injecting six test sample preparations spiked with Acetone, Dichloromethane and Cyclohexane reference standards at 100% specification level and % RSD for Acetone, Dichloromethane and Cyclohexane (n=6) were found to be 2.0, 2.5 and 2.7 respectively. For intermediate precision (n=6) % RSD was found to be 2.5, 2.6 and 2.1, respectively. % RSD of precision and intermediate precision (n=12) was found
to be 2.2, 2.6 and 2.3, respectively for Acetone, Dichloromethane and Cyclohexane. The results were found well within the acceptance criteria. The results of precision are presented in Table 4.

**Accuracy (Recovery)**

The accuracy of the method was evaluated by calculating the recoveries at LOQ, 50%, 100% and 150% level of the targeted specification concentration. The mean % recoveries for Acetone, Dichloromethane and Cyclohexane at LOQ (n=6), 50% (n=3), 100% (n=3), and 150% (n=6) were found within the acceptance criteria. The % RSD at LOQ was found 4.7, 4.6 and 3.7 respectively for Acetone, Dichloromethane and Cyclohexane. At 150% level the % RSD for Acetone, Dichloromethane and Cyclohexane was found 2.5, 2.8 and 2.4 respectively. The recoveries and precision at LOQ and 150% were found within the acceptance criteria. The results of accuracy are presented in Table 5.

**Limit of detection (LOD) and limit of quantitation (LOQ)**

The LOD and LOQ were established from the Slope and STEYX by plotting the linearity curve of concentration versus area response for Acetone, Dichloromethane and Cyclohexane. The LOD for Acetone, Dichloromethane and Cyclohexane was found 170, 25 and 118 ppm respectively. The LOQ was found 514, 76 and 359 ppm respectively for Acetone, Dichloromethane and Cyclohexane. The results are presented in Table 6.

**Linearity**

The linearity of the method was established for Acetone, Dichloromethane and Cyclohexane from LOQ to 150% of the target specification concentration by plotting concentration versus peak area response. The method was found linear for Acetone, Dichloromethane and Cyclohexane with correlation coefficient 0.998, 0.998 and 0.999 respectively. The linearity results are tabulated in Table 7. Chromatograms at LOQ and 150 % are shown in Figures 6 and 7. Linearity graphs of Acetone, Dichloromethane and Cyclohexane, are shown in Figures 8, 9 and 10.

**Robustness**

The robustness of the method was determined by deliberately changing the initial oven temperature and nitrogen gas flow rate. Evaluated the system suitability results at each robustness condition and were found within the acceptance criteria.

**Solution stability**

Solution stability of the standard solution and test sample solution was established and found to be stable for 48 hours on bench-top (controlled room temperature).

**DISCUSSION**

As per ICH Q3C, it is mandatory to estimate and control residual solvents used for synthesis, crystallisation and purification of drug substances or API. Several trials were taken on HS-GC to optimise the column dimensions, carrier gas flow, oven temperature, detector temperature, gradient programme, split ratio and standard & test concentrations to achieve good peak shape and better retention and resolution of Acetone, Dichloromethane and Cyclohexane peaks. The developed method was very sensitive and straightforward with a shorter run time. The developed method was validated as per the current method validation guidelines and found suitable.

**CONCLUSIONS**

A method was developed for the simultaneous estimation of Acetone, Dichloromethane and Cyclohexane in Nitazoxanide API. The developed method was validated as per ICH Q2 and USP <1225> guidelines for system suitability, specificity, precision, accuracy, LOD & LOQ, linearity and robustness. The method validation results were found meeting the acceptance criteria for all parameters. The proposed method is simple, sensitive, selective, accurate and robust for quantitative estimation of Acetone, Dichloromethane and Cyclohexane in Nitazoxanide API by HS-GC and can be used for routine analysis in quality control and research laboratory.

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**Conflict of interest**

The authors declare that they have no conflict of interest for this study.

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