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

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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 spilt 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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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 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 (Malesuik *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 of eluting peaks 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:

$LOD = 3.3 \times (\sigma/S)$

$LOQ = 10 \times (\sigma/S)$

Where σ = STEYX of response and S = 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.

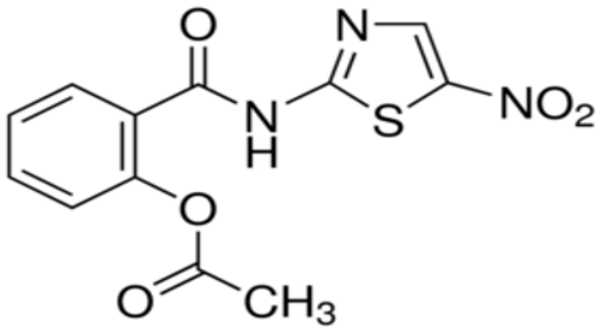

Figure 1: Chemical structure of nitazoxanide

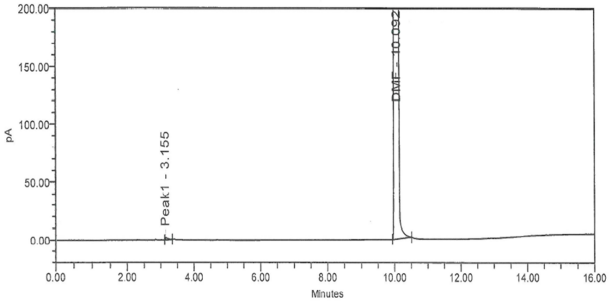

Figure 2: Chromatogram of blank

Specificity

The specificity of the method was performed to check blank interference and confirm the identity 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 Ace-

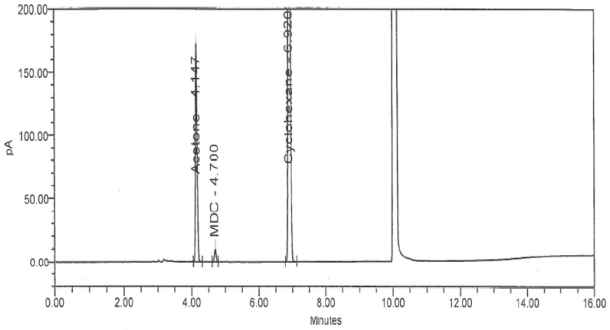

Figure 3: Chromatogram of standard

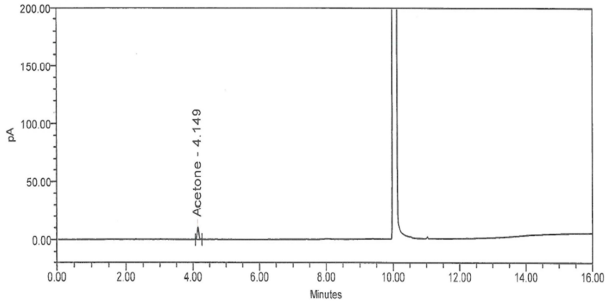

Figure 4: Chromatogram of the test sample

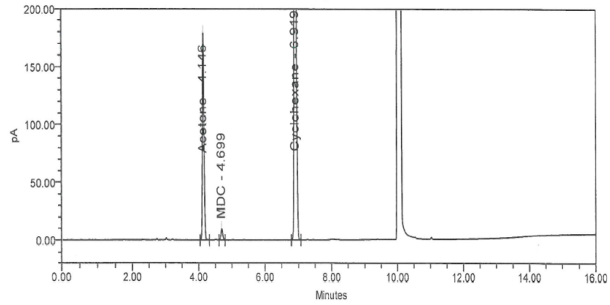

Figure 5: Chromatogram of a spiked test sample

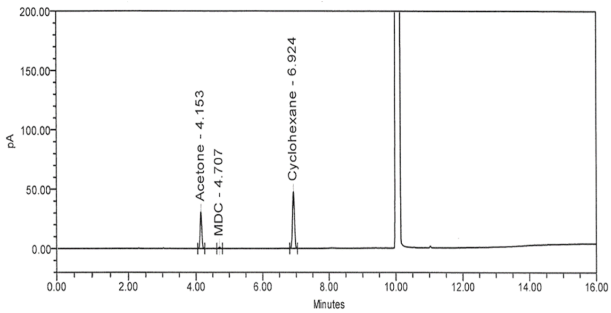

Figure 6: Chromatogram at LOQ level

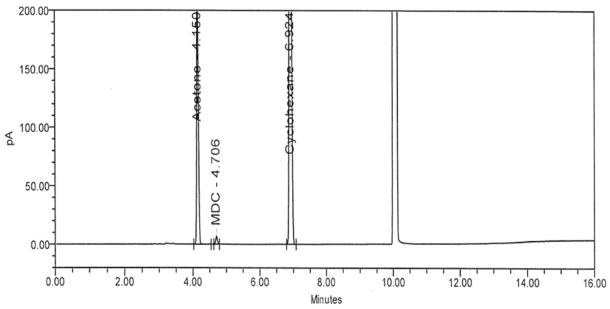

Figure 7: Chromatogram at 150 % level

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/minutes
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 Six replicate standard	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 spike sample 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	86.1	91.5	4.6	90.5	95.3	3.7
LOQ sample - 4	95.7			93.6			96.5		
LOQ sample - 5	93.3			97.6			100.3		
LOQ sample - 6	99.8			93.7			97.6		
50 % sample - 1	93.9			97.3			100.3		
50 % sample - 2	100.5	96.9	3.4	89.8	94.3	4.2	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			99.6		
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			96.7		
150 % sample - 5	100.2			99.4			99.7		
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	0.998		0.997		0.998	
Slope	0.1097		0.0260		0.5059	
Steyx	5.6425		0.1979		18.1601	
LOD (PPM)	170		25		118	
LOQ (PPM)	514		76		359	

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	

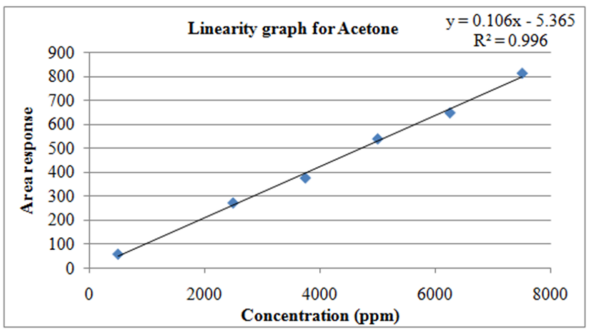

Figure 8: Linearity graph of acetone

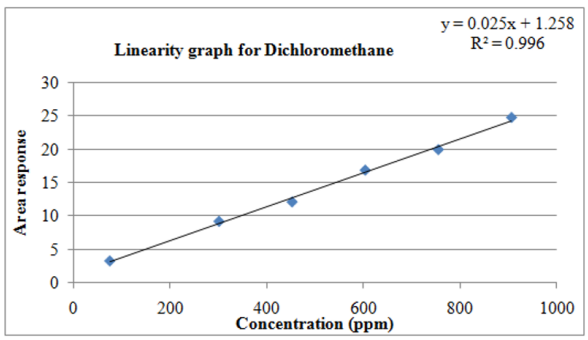

Figure 9: Linearity graph of dichloromethane

tone, 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

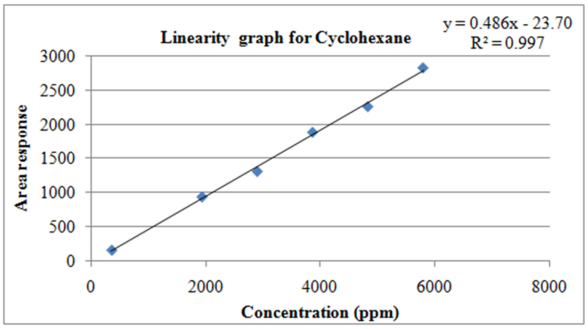

Figure 10: Linearity graph of cyclohexane

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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