Natural Resources for Human Health
eISSN: 2583-1194

Original Research

Received 12 May 2026
Revised 20 June 2026
Accepted 12 July 2026

Natural Resources for Human Health 2026, Vol. 6 (8s): 1467–1475

KEYWORDS:
Dapagliflozin propanediol monohydrate, N-Methyl-N-Nitrosopyridin-4-amine (DMAP-nitroso), Validation, LCMS/MS.

DMAP-NITROSO Impurity Method Development and Validation in Dapagliflozin Propanediol Monohydrate Drug Substance

Sri Siva Kumar Gubbala[1], K. Gowri Shankar Rao[2], Tummuri Sudheer Reddy[3], Paul Douglas Sanasi[4]

[1,2,3,4]Andhra University, Department of Engineering Chemistry, AU College of Engineering, Visakhapatnam-530003, India.

ABSTRACT: N-Nitrosamine, the probable carcinogen has been reported with an type-II diabatic drug. dapagliflozin propanediol monohydrate, *N*-Methyl-*N*-Nitrosopyridin-4-amine (DMAP-nitroso) impurities play a vital role in the pharmaceutical in the separation, purification and cleaning process for manufacturing of dapagliflozin propanediol monohydrate drug Substance. According to the FDA and EMA guideline (EMA Q&A document EMA/40981/2020), the drug manufacturing process are potential root cause of N- Nitrosamine impurity. Hence, monitoring of N-Nitrosamine for manufacturing of dapagliflozin propanediol monohydrate drug substance. A sensitive LCMS/MS an essential analytical tool for low level N-Nitrosamine quantification in dapagliflozin propanediol monohydrate drug substance, method development by multiple reaction monitoring mode (MRM). A novel method was developed to optimize the critical parameters of LCMS/MS according to the dapagliflozin propanediol monohydrate samples. The method validation was performed through the following parameters, specificity, sensitivity, linearity, accuracy, precision and stability. The quantification of *N*-Methyl-*N*-Nitrosopyridin-4-amine (DMAP-nitroso) in dapagliflozin propanediol monohydrate drug substance ranged from 0.18 ppm to 2.12 ppm with respect to the sample concentration of 1 mg/mL with good sensitivity in LOQ level. Hence, it will be useful to quantify the trace level DMAP-nitroso in dapagliflozin propanediol monohydrate drug substance.

1. INTRODUCTION

N-Methyl-*N*-Nitrosopyridin-4-amine (DMAP-nitroso) are organic chemical compounds that may be present in low level in a variety of products that people are exposed to every day in air, water, consumed products such as processed meat, alcoholic beverages, cosmetics and cigarette smoke all contain N-Nitrosamines. N-Nitrosamines (NAs) have been classified as 'probable human carcinogens' by international Agencies. Research on Cancer were listed as a respective "cohort of concern" of mutagenic carcinogens (in addition to aflatoxin like compounds and alkyl azoxy compounds) in the international council for harmonization of technical requirements for pharmaceuticals for human use (ICH) guidance for industry M7(R1). In 2018, the nitrosamine N-Nitroso diethyl amine (NDEA) and N-Nitroso dimethyl amine (NDMA) were detected in several active pharmaceutical ingredients (APIs) used in the treatment of hypertension, diabetic and related medicines. After thorough investigations, regulatory agencies have outlined that the formation of nitrosamines is possible in the presence of secondary, tertiary, quaternary amines and nitrite salts under acidic reaction conditions. The starting materials, intermediates, reagents, solvents, catalysts, active pharmaceutical ingredient (API), or API degradants may participate in reactions and form nitrosamines in certain processing conditions. Contaminated reaction materials in the manufacturing process such as recycled solvents are potential root cause of nitrosamines in drug substances. Excipients and packing materials may contain nitrosamine precursors to generate nitrosamines in drug products. Regulatory agencies have guided manufacturers to follow risk assessment, acceptable intake (AI) limits and multiple analytical test procedures to control nitrosamines. As a non-profit, public standards setting organization, the USP has been making continuous efforts to protect public health and strengthen, the global medicine supply chain. In response to the

nitrosamine crisis, USP has been providing support to manufacturers and regulators with tools and solution for testing, assessing risk and identifying potential sources related to nitrosamines. Specifically, USP has released several nitrosamine reference standards and published anew United states Pharmacopeia National Formulary (USPNF), General chapter <1469> nitrosamine impurities to support the industry in analysing monitoring nitrosamines in the drug supply chain. T current study is aimed at providing solution for determining multiple nitrosamines at trace levels in the NDSRIs in Dapagliflozin propanediol monohydrate.

Controlling nitrosamines in drug substance will help in eliminating or reducing the process of nitrosamines in drug product and in turn improves the quality of the dug product, Chemical name and structure of Dapagliflozin propanediol monohydrate in Figure-1.

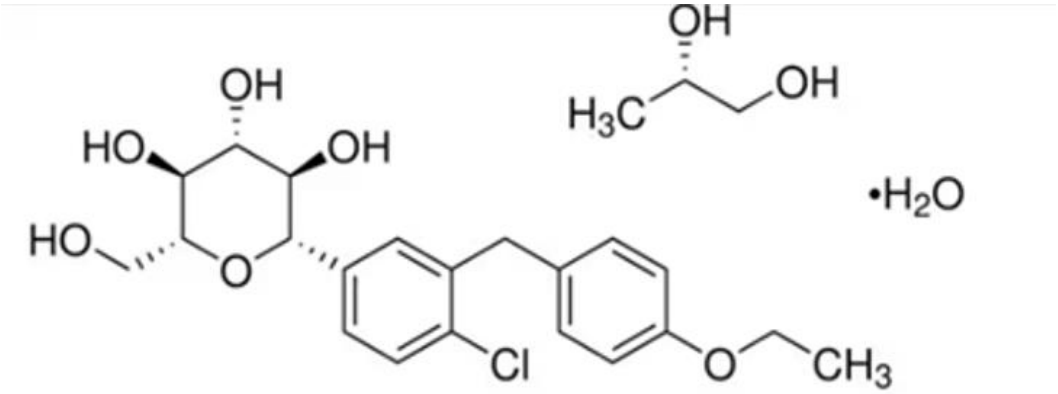

Figure 1: (1S)-1,5-Anhydro-1-C-[4-chlore-3-[(4-ethoxypheny1) methy1] pheny1]-D-glucitol(S)-propane-1,2-diol (1:1) monohydrate

Current year, impurities especially Nitrosamine analysis and control strategies development in final products become an epicentre in the pharmaceuticals and regulatory authorities. Active pharmaceutical ingredients. The risk of carryover into the drug substance should be assessed for identified impurities that are present in starting materials / intermediates and impurities that are reasonably expected by products in the route of synthesis from the starting material to the drug substance.

4-Dimethylaminopyridine is a key reagent used in the preparation of dapagliflozin propanediol monohydrate. It can form *N*-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso), a structurally related N-nitrosamine impurity. The chemical name and structure of this impurity are shown in Figure 2. The acceptable limit of 1.8 µg/g was calculated using the threshold of toxicological concern (TTC) approach, based on a maximum daily dose of 80 mg and lifetime treatment duration, in accordance with ICH M7[13] and EMEA guidelines.

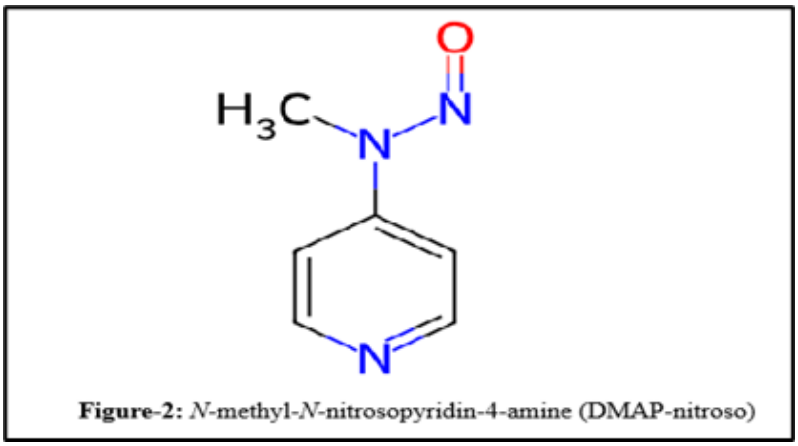

Figure-2: *N*-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso)

The goal of this research study is to develop a sensitive, selective, accurate, reproducible and simple method to analyse these N-Nitrosamine impurities in Dapagliflozin propanediol monohydrate drug substance. For the sensitivity of impurity level, we have chosen LCMS/MS technique. To the best of our knowledge, *N*-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso)

determination of by LCMS/MS in Dapagliflozin propanediol monohydrate drug substance has not been reported in literature till date. This paper describes the development, optimization of LCMS/MS method for the determination of subjected impurities and method validated accordance with ICH guidelines.

Table-1: Mass spectrometer conditions (MRM1 transitions, CE and Dwell time).

Name of the Nitrosamine	**Quantifier (MRM1 and Collision energies)**	**Dwell time (msec)**
N-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso)	138.00 > 108.15 (CE= -14.0)	100

MATERIAL AND METHODS

Chemicals and Reagents

N-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso) was prepared Raghava Life Science Pvt. Ltd., Hyderabad, India.

Dapagliflozin propanediol monohydrate commercial batches from Raghava Life Sciences Pvt. Ltd., Unit-I, Kamareddy, Telangana, India.

Instrument and Analytical Parameters

Analysis was carried out on a Shimadzu triple quadruple LCMS/MS system, LCMS/MS-8045 with Nexera X2 HPLC system having LCMS solution. Separations were carried out on a Zorbax SB-Phenyl, (250 × 4.6) mm, 5 µm, column from waters corporation. Flow rate: 0.8 mL/min, Injection volume: 5µL, Column oven temperature: 40 ºC, Sample cooler temperature: 15 ºC, Mobile phase-A was prepared 0.1% formic acid in water and Mobile phase-B is methanol, Diluent: Methanol.

Elution mode is gradient, run time is 20 min.

Table-2: Gradient elution

Time (min)	**% Mobile Phase-A**	**% Mobile Phase-B**
0.01	75	25
3.0	75	25
10.0	5	95
14.0	5	95
15.0	75	25
20.0	75	25

Blank solution:

Inject diluent as a blank.

Preparation of Impurity Stock Solution-I:

Weigh accurately about 25.0 mg of DMAP-Nitroso into 25 mL volumetric flask and add about

10 mL of diluent sonicate to dissolve and dilute to volume with diluent and mix well.

Preparation of Impurity Stock Solution-II:

Dilute 0.2 mL of above Impurity Stock Solution-I into 25 mL Volumetric flask and dilute to volume with diluent and mix well.

Preparation of Impurity Stock Solution-Ill:

Dilute 0.14 mL of above Impurity Stock Solution-II into 25 mL Volumetric flask and dilute to volume with diluent and mix well.

Standard solution:

Dilute 1.0 mL of above Impurity Stock Solution-Ill into 25 mL Volumetric flask and dilute to volume with diluent and mix well.

Sensitivity solution:

Dilute 2.5 mL of above standard solution into 25 mL Volumetric flask and dilute to volume with diluent and mix well.

Sample solution:

Weigh about 25 mg of Dapagliflozin propanediol monohydrate sample into a 25mL volumetric flask add 10 mL diluent sonicate to dissolve and make up to volume with diluent.

RESULTS AND DISCUSSION

Method validation

The developed and optimized method was then validated for the following parameters.

- Specificity
- Limit of Detection (LOD) and Limit of Quantitation (LOQ)
- Linearity
- Accuracy
- Precision (System Precision and Method Precision)
- Stability of Sample Solution

The results of each of the above validation experiments indicated the conformity to the stipulated acceptance criteria and these experimental results have been discussed in this research article.

Specificity:

The Specificity parameter was performed by preparing and injecting Blank (diluent) once, Sensitivity Solution in single, Standard solution six replicates, individual impurity solution in single and Test preparation (un spike) in single into LC-MS/MS system as per the test method. Prepared Test solution spiked with impurity at specification level and injected into the LC-MS/MS system.

Evaluated the effective separation between the blank and impurity from the principal peak (Dapagliflozin).

LCMS/MS total ion chromatogram. Typical LCMS/MS total ion chromatograms of specificity experiments are shown in in Figure-3 and Figure-4

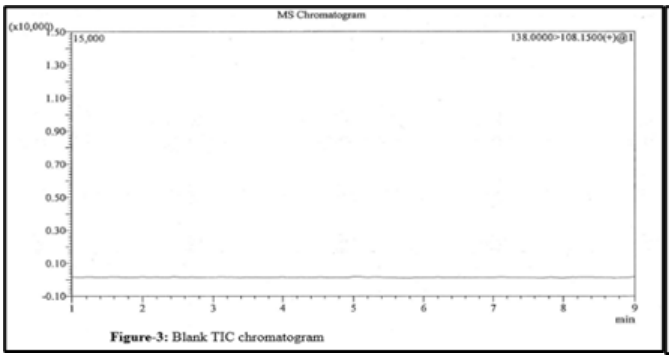

Figure 3

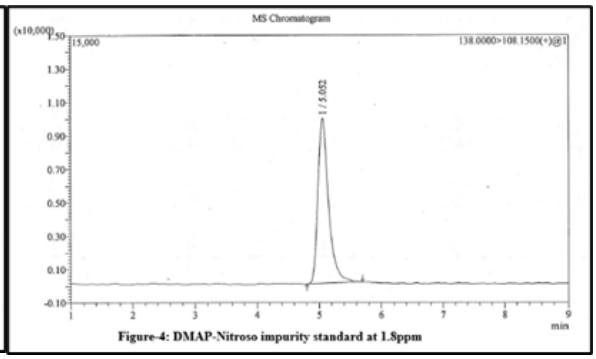

Figure 4

LOD and LOQ

The sensitivity for detection can be demonstrated by determining the limit of detection (LOD) and limit of quantitation (LOQ). LOD/LOQ values of desired analytes were determined from based on response of analytes. The predicted concentrations of LOD and LOQ for these contents were verified for precision by preparing the solutions containing impurities at about predicted concentrations. Each of these solutions six times injected into the LCMS/MS system.

Table-3: LOQ Precession statistical data

Blank	0
Injection	DMAP-Nitroso Area
1	11754
2	12222
3	12482
4	12391
5	11744
6	12243
Mean	12139
SD	317
% RSD	2.60
95% CI(±)	333

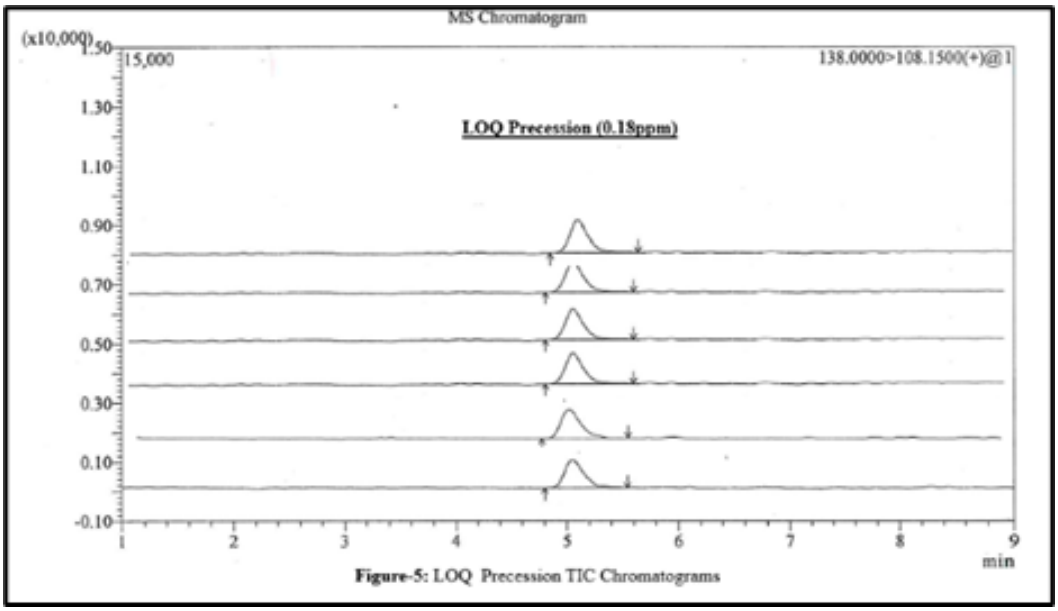

Figure 5

Linearity

The linearity was to be determined by injecting the standard solution in duplicate ranging from LOQ to 120% level of the standard concentration. Prepare the required solutions as per seirs of dilutions.

Plotted the graph of average area response of the duplicate injections for the concentration verses concentration on Y and X axis respectively and evaluated the correlation coefficient, Y Intercept, Slope of the regression line and Residual sum of square.

. The linearity, LOD and LOQ experiments data is shown in Table 4 & Figure-6.

Natural Resources for Human Health
eISSN: 2583-1194

Table 4: Linearity, LOD & LOQ experiments data

***N*-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso) (µg/mL)**	**Area**	**Statistical analysis**	
0.18	11697	Slope	69755.55
0.44	26938	Intercept	-716.21
0.88	67435	Residual sum of squares	5364
1.41	96860	Correlation Coefficient	0.99523
1.76	114648	Limit of Detection (ppm)	0.06
2.12	151620	Limit of Quantification (ppm)	0.18

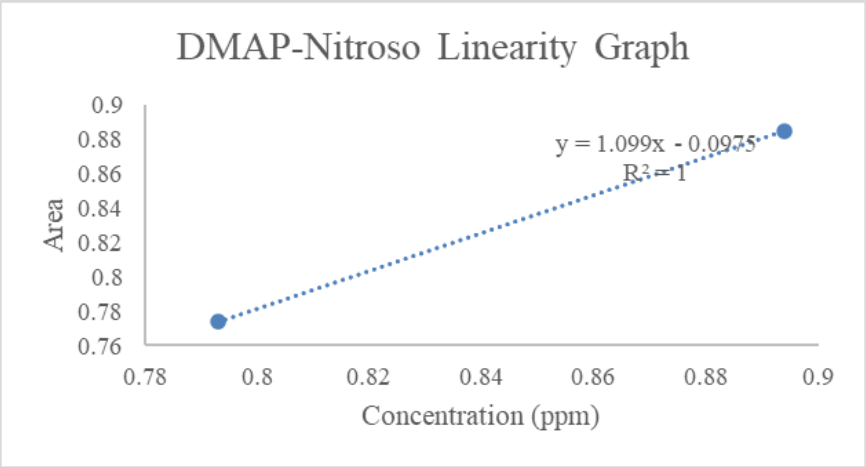

Figure-6: Linearity graph for DMAP-Nitroso

Accuracy

The recovery parameter was performed by preparing the triplicate preparation from the sample as per test method and spiking each of the test preparation at LOQ, 50%, 100% and 120% of the targeted concentration (Specification) for impurity. Prepared and injected the Blank in single, system suitability solution and each recovery level solution in single. Separately prepared and injected the control test preparation (without spiking) in single. Calculated the % of Impurity and subtracted the amount achieved from the recovery data and reported % impurity recovered. Calculated the amount recovered and % recovery for each test preparation for each of the level and calculated the % RSD for the triplicates of each level and for all recovery and calculated each level. The results are mentioned below recovery results are shown in Table-5.

Table-5: Accuracy data of N-methyl-N-nitrosopyridin-4-amine (DMAP-nitroso)

% Level / Sample ID	Amount Added (µg/g)	Amount Found (µg/g)	% Recovery
LOQ Level Sample - 1	0.18	0.18	100
LOQ Level Sample - 2	0.18	0.17	94.44
LOQ Level Sample - 3	0.18	0.2	111.11
Statistical Analysis			
Mean	**101.8**	%RSD	**8.3**

50% Level Sample - 1	0.92	0.85	92.39
50% Level Sample - 2	0.92	0.82	89.13
50% Level Sample - 3	0.92	0.84	91.3
Statistical Analysis			
Mean	**90.9**	%RSD	**1.8**
100% Level Sample - 1	1.83	1.77	96.72
100% Level Sample - 2	1.83	1.6	87.43
100% Level Sample - 3	1.83	1.56	85.25
Statistical Analysis			
Mean	**89.8**	%RSD	**6.8**
120% Level Sample - 1	2.2	2.07	94.09
120% Level Sample - 2	2.2	2.14	97.27
120% Level Sample - 3	2.2	2.08	94.55
Statistical Analysis			
Mean	**95.3**	%RSD	**1.8**

Precision:

Prepared and injected the Blank (diluent) once, Sensitivity Solution in single, Standard solution six replicates, six different Spike Sample preparation from the single homogeneous sample as per the test method and injected each of the preparation into the LC-MS/MS system as per test method. Calculate the % RSD of impurity as per the test method.

The results are mentioned below **Table-6a&Table-6b.**

Table 6a : System Precision experiments

Name of Preparation	DMAP-Nitroso
Standard solution-1	132231
Standard solution-2	126551
Standard solution-3	126965
Standard solution-4	125323
Standard solution-5	124589
Standard solution-6	121835
Average	**126249**
SD	**3449**
%RSD	**2.7**

Table 6b: Method Precision experiments data

Name of Preparation	DMAP-Nitroso
Spiked Sample-1	1.77
Spiked Sample-2	1.6
Spiked Sample-3	1.56
Spiked Sample-4	1.63
Spiked Sample-5	1.57
Spiked Sample-6	1.62
Average	**1.63**
SD	**0.07**
%RSD	**4.3**

Solution stability

For the determination of stability of the standard and sample solutions, Dapagliflozin propanediol monohydrate drug substance spiked *N*-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso) at specification level was prepared as per methodology and analysed initially and at different time intervals by keeping the solutions at room temperature (~ 25°C) conditions. to determine stability acceptance criteria, the following difference has been considered, "Percentage difference between the area obtained at initial and different time interval should be no more than 30.0" for *N*-methyl-*N*-nitrosopyridin-4-amine (DMAP-nitroso).

Therefore, the stability of sample solution at room temperature (~ 25°C) can be considered stable for 22 hours. The experimental data is tabulated in Table 9.

Table 7: Solution stability experiments data.

Name of the Solution	Name of standards	Initial	After 22hrs	% Difference
Spiked Sample	**N-methyl-N-nitrosopyridin-4-amine (DMAP-nitroso)**	1.55	1.56	-1.6

CONCLUSION:

The LCMS/MS method was developed, optimized and validated for the determination of with N-methyl-N-nitrosopyridin-4-amine (DMAP-nitroso) contents at trace level in Dapagliflozin propanediol monohydrate drug substance and the results of various validation parameters proved that the method is specific, sensitive, precise and accurate and the method can be introduced into routine testing.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the management of Raghava Life Sciences Pvt. Ltd., for allowing us to carry out the present work. The authors are also thankful to the colleagues of Analytical Research Department and Chemical Research Department for their co-operation.

REFERENCES

[1] Savale SK. Genotoxicity of drugs: Introduction, prediction and evaluation. Asian

[2] J Biomater Res 2018;4:1-29.

[3] Gosar A, Sayyed H, Shaikh T. Genotoxic impurities and its risk assessment in drug compounds. Drug Design Intellect Properties Int J 2018;2:227-32.

[4] EFSA Scientific Committee. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA J 2011;9:2379.

[5] Shah SU. Importance of genotoxicity and S2A guidelines for genotoxicity testing for pharmaceuticals. IOSR J Pharm Biol Sci 2012;1:43-54.

[6] Guideline IH. Impurities in new drug substances Q3A (R2). In: Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. Geneva, Switzerland: 2006. Available from: https://www.database. ich.org/sites/default/files/Q3A_R2__Guideline.pdf. [Last accessed on 2019 Oct 31].

[7] International Conference on Harmonisation Guideline on Impurities in New Drug Products, Q3B(R2); 2006. Available from: https://www.ema. europa.eu/en/documents/scientific-guideline/ich-q-3-b-r2-impuritiesnew-drug-products-step-5en.pdf. [Last accessed on 2019 Oct 31].

[8] International Conference on Harmonisation Guideline for Residual Solvents, Q3C(R4); 2019. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/international-conferenceharmonisation-technical-requirements-registration-pharmaceuticalshuman-use_en-33.pdf. [Last accessed on 2019 Nov 01].

[9] European Medicines Agency. Guideline on the Specification Limits for Residues of Metal Catalysts or Metal Reagents, Doc. Ref. EMEA/ CHMP/SWP/4446/2000. Netherlands: European Medicines Agency; 2013. Available from: https://www.ema.europa.eu/documents/ scientific-guideline/guideline-specification-limits-residues-metalcatalysts-metal-reagents_en.pdf. [Last accessed on 2019 Oct 31].

[10] European Medicines Agency. Guideline on the Limits of Genotoxic Impurities, CPMP/SWP/5199/02, EMEA/CHMP/QWP/251344/2006. European Medicines Agency; 2007. Available from: https://www.ema.

europa.eu/documents/scientific-guideline/guideline-limits-genotoxicimpurities_en.pdf. [Last accessed on 2019 Oct 31].

[11] Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, et al. Structure-based thresholds of toxicological concern (TTC): Guidance for application to substances present at low levels in the diet. Food Chem Toxicol 2004;42:65-83.

[12] Muller L, Mauthe RJ, Riley CM, Andino MM, Antonis DD, Beels C, et al. A rationale for determining, testing and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity, regul. Toxicol Pharmacol 2006;44:198-211.

[13] Center for Drug Evaluation and Research, Food and Drug Administration. Guidance (Draft) for Industry Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches. United States: Center for Drug Evaluation and Research, Food and Drug Administration; 2008.

[14] Connelly JC. ICH guideline residual solvents. Counc pharmeur 1997;1:S1-68.

[15] Callis CM, Bercu JP, DeVries KM, Dow LK, Robbins DK, Varie DL. Risk assessment of genotoxic impurities in marketed compounds administered over a short-term duration: Applications to oncology products and implications for impurity control limits. Org Process Res Dev 2010;14:986-92.

[16] Leighton JK, Olejniczak K, Onodera H. ICH S9: Nonclinical evaluation of anticancer pharmaceuticals: A perspective from regulators on the development of the guideline. In: Global Approach in Safety Testing. New York: Springer; 2013. p. 283-98.

[17] Sultan S, Tengli A, Akhila G. Analytical assessment of genotoxic impurities in pharmaceuticals by gas chromatographic techniques. Res Rev 2019;1:22-32.

[18] Mohamed SA, Sabita U, Rajendra S, Raman D. Genotoxicity: Mechanisms, testing guidelines and methods. Glob J Pharm Pharm Sci 2017;1:1-6.

[19] Guideline IH. Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals To Limit Potential Carcinogenic RISK M7. Geneva: International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH); 2018. Available from: https://www.fda.gov/regulatoryinformation/search-fda-guidance-documents/m7r1-assessment-andcontrol-dna-reactive-mutagenic-impurities-pharmaceuticals-limitpotential [Last accessed on 2019 Nov 02].

[20] Pierson DA, Olsen BA, Robbins DK, DeVries KM, Varie DL. Approaches to assessment, testing decisions, and analytical determination of genotoxic impurities in drug substances. Org Process Res Dev 2009;13:285-91.

[21] Helmy R, Strickfuss S, Al-Sayah M, Hamilton S, Bu X, Lee C, et al. Quantification of Genotoxic Impurities in Active Pharmaceutical Ingredients. United Kingdom; Taylor & Francis Group; 2015. p. 293.