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A validated LC-MS method for the estimation of genotoxic nitroso amines impurities in empagliflozin

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Abstract---Background: Nitrosamine impurities are known to be mutagenic and carcinogenic, and even minor exposure to these impurities can result in genotoxicity. Objective: The objective of this study was to develop and validate simple, robust and accurate Liquid Chromatography coupled with Mass spectroscopy (LC-MS) method for estimation of nitroso amine impurities [N-nitroso dimethyl amine (NDMA), N-nitroso diethylamine (NDEA), N-nitroso ethyl isopropylamine (NEIA), N-Nitroso diisopropylamine (NDIPA)] in empagliflozin. Method & Results: Aqueous ammonia buffer (0.10%) in a mixture of methanol (5:95 v/v), flow rate, and injection volume were 0.4mL/min, 20µL, respectively. Linearity was observed in the concentration range of 0.05-0.5 ppm ($r^2 = 1$). The retention time of NDMA, NDEA, NEIA and NDIPA were 6.412, 7.172, 7.486 and 7.719, respectively. The method was validated according to ICH guidelines with good reproducibility, and the limits of detection were 0.03 ppm for NDMA, NEIA and NDIPA and 0.02 ppm for NDEA, respectively. The limit of quantitation for NDMA, NEIA and NDIPA was 0.09 ppm and 0.06 ppm for NDEA, respectively. Conclusion: The proposed method is helpful for the best analysis of nitrosamine impurities in dosage forms of empagliflozin within a shorter duration of time.

Keywords---Nitrosamine impurities, LC-MS method, ICH, Empagliflozin

1. Introduction

Nitrosamine impurities are classified as Class 1 by ICH M7 (R1), which is known to be mutagenic and carcinogenic (ICH, M7 R1, 2017). Impurities are produced throughout the API production process from several sources, including intermediates, starting materials, intermediates, reagents, and solvents. Primary, secondary, tertiary, or quaternary ammonium salts and nitrosating substances such as sodium nitrite are thought to be precursors for forming Nitrosamine impurities (ICH Q3 A, 2006). Similarly, when carbamate, amides, and N-alkyl are nitrosated, they might generate Nitrosamine impurities Fig. 2. To calculate its limit, the median toxic dose TD50 is used. The TD50 is well-accepted by ICH M7 (R1) for the calculation of the acceptable excess risk to calculate acceptable intake (AI) for mutagenic and carcinogenic impurities, and it is a well-recognized international standard (USFDA 2019a, b,c,d). The nitrosamine impurity production is mainly determined by the kind of reagent, structure, and concentration of the nitrosating agent. (Jireš, 2021) Empagliflozin is a selective inhibitor of sodium-glucose cotransporter two approved for type 2 diabetes (NCBI, Pubchem, 2021) Fig. 1. Given either monotherapy or add-on therapy, the drug is reported to reduce glycosylated haemoglobin levels in patients with type 2 diabetes, including those with stage 2 or 3a chronic kidney disease (Buse et al., 2020; Guo et al., 2020). Several regulatory agencies like EMA FDA has declared Nitrosamine impurities as highly toxic, and stringent control limits were implied for the nitrosamine class of impurities (Sedlo, 2021 & Tuesuwan, 2021). Literature reveals that many countries recalled several antihypertensives under sartan class due to the Nitrosamine-based impurities (Bharate, 2021). The trace amount of these impurities may be formed due to the decomposition of solvent or other materials used to synthesize drug substances (Vyas, 2021). Similarly, by-products formed in the drug synthesis process may be carried forward to the drug substances as Nitrosamine impurities (Ngongang et al., 2015). Solvents such as Dimethylformamide, Dimethylacetamide (DMF) or Diethylacetamide (DEA) may form potential NDMA and NDEA impurities (Liu et al., 2021). The use of DMF in the synthesis might lead to the generation of nitrosamine impurity (Parr & Joseph, 2019). DMF is used as a solvent in the synthesis of Empagliflozin, which might increase the risk of nitrosamine impurity in empagliflozin. As a majority of the diabetic patients having type II diabetes mellitus have been prescribed Empagliflozin to reduce the plasma glucose level, it is essential to check the presence of nitrosamine-related impurities Empagliflozin (Ayoub & Mowaka, 2017). In recent times LC-MS and GC-MS methods have been reported to estimate nitrosamine impurities in dosage forms of sartans (Schmidtsdorff & Schmidt, 2019). As of our knowledge, analytical methods are not established to estimate nitrosamine impurities in empagliflozin. Four Nitrosamine based impurities like N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA), N-Nitrosoethylisopropylamine (NEIA), N-Nitrosodiisopropylamine (NDIPA) were considered for the study. The objective of the present work is to study the presence of four nitrosamines in empagliflozin by a validated LC-MS method with good linearity, accuracy and sensitivity.

2. Experimental

2.1 Instrumentation and Reagents

N-nitrosodimethylamine (NDMA), chemical purity: 98.0% and N-nitrosodiethylamine (NDEA), chemical purity: 98.0%, were obtained from Yarrow Chem, Mumbai (Sigma Aldrich, Germany). N-Nitrosoethylisopropylamine (NEIA), N-Nitrosodiisopropylamine (NDIPA) was purchased from Yucca Enterprises, Mumbai (Sigma Aldrich, Germany). Methanol $\geq 99.9\%$ and Ammonia 98–100% were obtained from Bros Scientifics (Qualigens, Mumbai). Ultrapure water was obtained using a Milli-Q® purification system from Millipore Corporation (Bedford, MA, USA). Chromatographic separations were performed on a Zorbax SBC18, 75 x 4.6 mm, 3.5 μm from Agilent (US).

The liquid chromatography system consisted of a binary LC pump equipped with Waters Quattro Micro tandem mass spectrometer with Z spray source (Waters, Hertfordshire, UK) was used for the study. The mass spectrometer maintained positive ion mode, with a desolvation gas flow of 630 L/h. Chromatographic separations were performed by gradient elution with 0.4 mL/min flow rate at ambient temperature with 0.1% Ammonia in water (A) and methanol (B). Gradient for determination: 0-0.3 min: 40 to 90 % B, 0.3-1.0 min: 90 % B, 1.0-1.01 min: 90 to 40 % B, 1.01-3.0 min: 70 % B. NDMA, NDEA, NEIA and NDIPA were quantified using a Waters Quattro Micro tandem quadrupole mass spectrometer fitted with a heated nebulizer interface (API), both from Micromass MS Technologies (Milford, USA). High purity nitrogen gas was employed as a crash gas, nebulizer, curtain, and auxiliary gas. The mass spectrometer was operated in positive ion mode using the following settings: nebulizer current: 3A, probe temperature: 500°C. Multiple reaction monitoring was used for quantification. (MRM). Data acquisition was performed with Software MassLynx™ (Milford, USA). Calculations were carried out using Microsoft Excel 2010 from Microsoft Co (Redmond, WA, USA, 2010).

3. Methods

3.1 Preparation of mobile phases and standard solutions

For the preparation of 0.10% ammonia, 2 mL of ammonia 98–100 % were added to 1998 mL of Milli-Q® -water and sonicated for 15 min. Methanol was sonicated for 15 min. NDMA (1990 g/mL), and NDEA (1990 g/mL), NEIA (1990 g/mL), NDIPA (1990 g/mL) are certified standard solutions. Following dilution, with methanol, a final standard solution containing 999.0 ng/mL NDMA, 999.0 ng/mL NDEA, 999.0 ng/mL of NEIA, and 999.0 ng/mL of NDIPA was obtained.

3.2 Preparation of Test solution

Accurately weighed, about 250 mg of the test sample was placed into a headspace vial; after adding 1.0 mL of diluent, the vial was closed with a septum and crimped. Column is equilibrated for 16 minutes with the mobile phase, and then the blank solution was injected into the system, and the chromatogram was recorded. The Data processor was programmed to inhibit the peaks due to blank.

Standard solutions were injected into the system separately six times, and the chromatograms were recorded.

3.3 Method Validation

The quantification method of four N- nitrosamines through LC-MS with MRM mode was validated using system suitability, specificity, sensitivity, linearity, LLOQ, LLOD, accuracy, precision and stability. The following equation was used to determine the matrix effect.

$$\text{Matrix Impact} = A/B \times 100$$

Where A is the concentrations of sample in the matrix and B is the mobile phase/blank.

Specificity The specificity of the method for NDMA, NDEA, NEIA and NDIPA was verified by comparing the chromatograms of contaminants free samples before and after spiking with the respective analytes. No peak from the matrix should coelute at the retention times of NDMA, NDEA, NEIA and NDIPA, respectively.

3.4 Linearity, accuracy and precision

To determine the linearity of the NDMA, NDEA, NEIA and NDIPA assay, three seven-point calibration curves were prepared, including a contaminants' free sample, which was not used for linear regression. The calibration curves were evaluated individually by linear regression, and the calibration standards' concentrations were back-calculated. The matching individual curves' slopes, intercepts, and coefficients of determination were determined. For acceptable linearity, the coefficient of determination of each calibration curve should be ≥ 0.999 . Additionally, the precision (reported as the relative standard deviation) and accuracy (expressed as the relative bias of the measured concentration from the nominal concentration) shouldn't be greater than 15% at each concentration level except for the lowest concentration level, a deviation of 20% was allowed.

To determine the inter-day precision and accuracy of the NDMA, NDEA, NEIA and NDIPA assay, two sets of samples were prepared and analyzed for NDMA, NDEA, NEIA and NDIPA in three different runs on two different days. Moreover, the intra-day precision was also tested on the empagliflozin matrix positively tested for NDMA, NDEA, NEIA and NDIPA, respectively, prepared in triplicate.

3.5. LLOQ

The LLOQ represents that concentration that can be still quantified with acceptable accuracy and precision. This is achieved when the relative bias of the measured concentration from the nominal concentration and the relative standard deviation of 3 repetitive measurements do not exceed a deviation of 20%. At the same time, the S/N ratio of the LLOQ for NDMA, NDEA, NEIA and NDIPA should be greater than 5.

3.6. Sample preparation

To reach an exceptionally high sensitivity, between five tablets (Empagliflozin, 10mg) were accurately weighed and ground to a fine powder. A tenth of the weights of the products was accurately weighed in a 1.5 mL centrifuge tube and suspended with 500 L of methanol containing the internal standard (NDMA), subsequently shaken vigorously (5 min) and sonicated for 5 min. The suspension was diluted to a final volume of 1000 L using MilliQ® -water, subsequently shaken again vigorously (5 min) and sonicated for 5 min. The suspended samples were centrifuged at 5000 rpm for 35 min at 4°C. There was no filtration step. The supernatants were transferred into glass vials for injection.

4. Results

The LC-MS/MS method developed for the quantitation of the four contaminants NDMA, NDEA, NEIA and NDIPA revealed to be suitable for the determination of even traces of the four analytes, as depicted from the validation results summarized below.

4.1. Validation results

The matrix effect was found in 96.44 ± 3.43 to $98.98 \pm 1.21\%$. The result of extraction recovery and matrix effect is within the acceptable limit, and there is no measurable interference of Nitrosamine impurities in Empagliflozin.

4.1.1 Linearity, Accuracy and Precision

Good linearity for each analyte could be achieved by applying weighted ($1/\text{concentration}^2$) linear regression over the following calibration ranges: NDMA 0.093-0.464 ppm, NDEA 0.062-0.461ppm, NEIA 0.090-0.449 ppm, and NDIPA 0.088-0.439ppm. The coefficients of determination of the weighted calibration curves were ≥ 0.999 for all analytes Fig. 3,4,5,6. Representative chromatograms for analyte and mass spectral data are presented in Fig. 7, 8. Blank chromatogram ensures the specificity of the method Fig 9. The ranges of the inter-day precisions and accuracies of the calibration standards for each analyte are summarized in Table 2. At the LLOQ, the inter-day precision and accuracy were found to be 1.3 and 102.1% for NDMA, 3.4 and 95.1% for NDEA, 2.2 and 114.8% for NEIA, and 2.7 and 115.9% for NDIPA, respectively. Fig 11,12

4.1.2 LLOD and LLOQ

The LLOD values for NDMA, NEIA and NDIPA, was found to be 0.03ppm and NDEA 0.02 ppm; furthermore, the LLOQ values of NDMA, NEIA and NDIPA were found to be 0.09ppm and NDEA 0.06 ppm the repetitive analysis of two sets of SQC samples verified the accuracy (within-run and between-run bias within -5.2% – 3.2%) and precision (within-run and between-run coefficients of variation $\leq 6.5\%$) in case of all tested concentrations for all analytes (Table 3), fulfilling thus the acceptance criteria of no more than $\pm 20\%$ deviation at LLOQ and no more than $\pm 15\%$ deviation for quality controls above LLOQ.

4.13 Accuracy

After all, the triplicate analysis of the same finished product yielded excellent precisions for each analyte ranging in sum between 0.8 and 15.3%, further

verifying the repeatability of the assay when used on finished products with various amounts of the relevant analyte. The mean absolute recovery in-ground tablets were found to be $102.2 \pm 1.05\%$ for NDMA, $95.13 \pm 5.60\%$ for NDEA, $114.80 \pm 2.82\%$ for NEIA, and $115.91 \pm 5.99\%$ for NDIPA Table 3. Hence the complete recoveries for ground tablets and the tablet extract are comparable, indicating the efficiency of the extraction procedure. On the other hand, the reduced mean absolute recoveries determined for NDEA and NDIPA in the tablet extracts compared to analytes in methanol/water (1:1, v/v) may be an indication for some ion suppression resulting from the tablet matrix Fig. 13 However, because the quantification was performed against matrix-matched standards to correct for any possible ion suppression, the observed ion suppression did not affect the accuracy of the four analytes' quantification results.

Figures

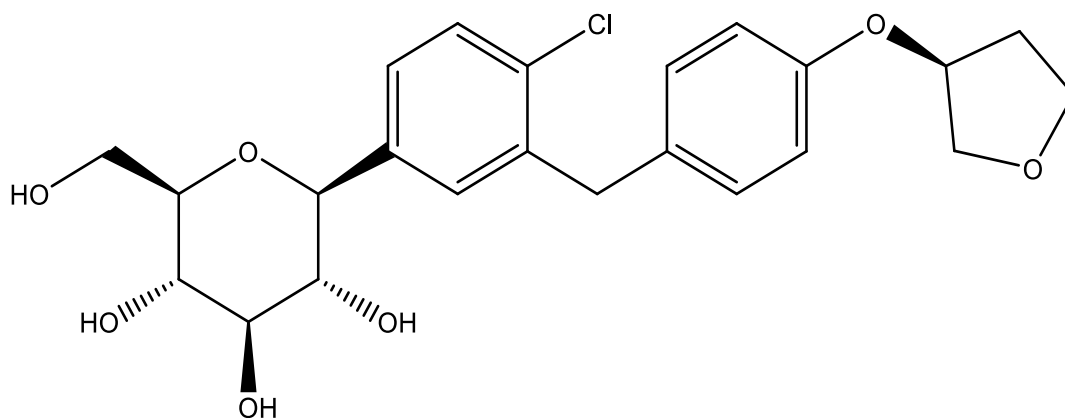
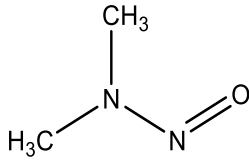
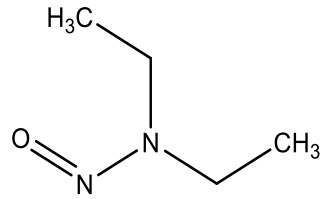


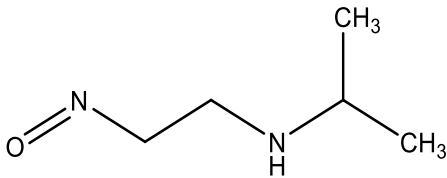
Figure 1: Chemical Structure of Empagliflozin



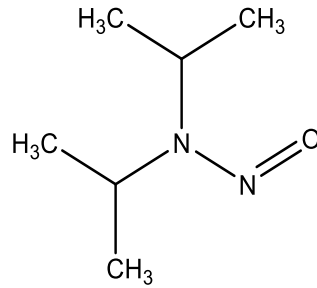
N-Nitrosodimethylamine (NDMA)



N-Nitrosodiethylamine (NDEA)



N-Nitrosoethylisopropylamine (NEIA)



N-Nitrosodiisopropylamine (NDIPA)

Figure 2: Nitrosamine Impurities

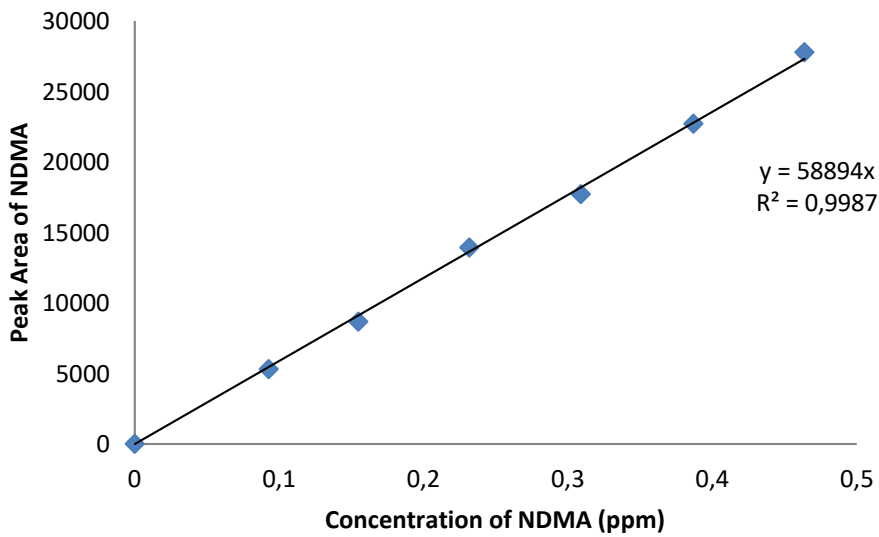


Figure 3: Linearity curve of NDMA

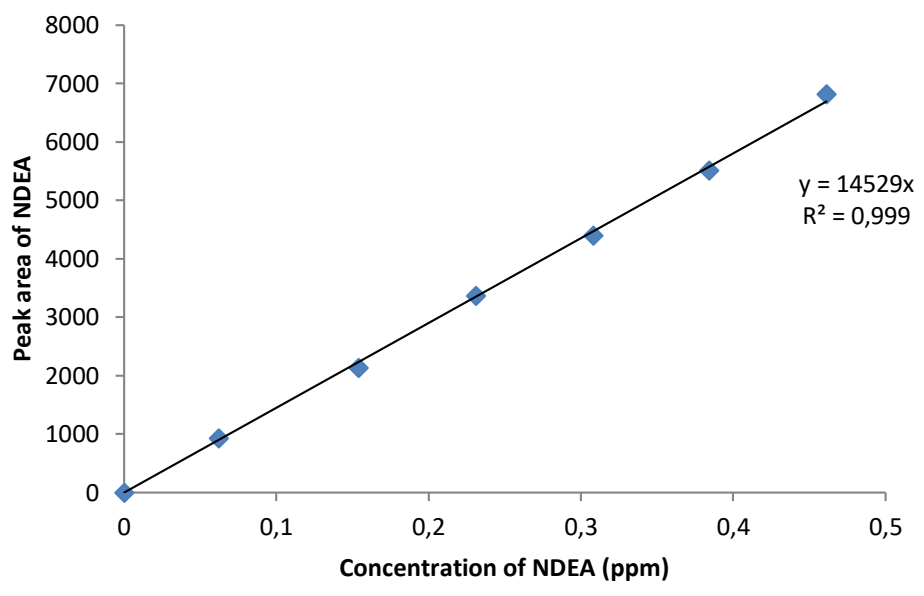


Figure 4: Linearity curve of NDEA

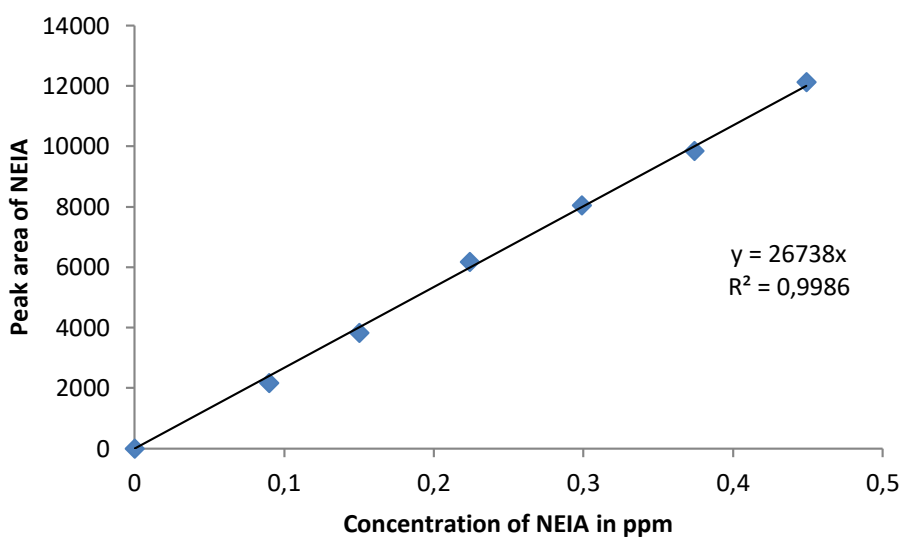


Figure 5: Linearity curve of NEIA

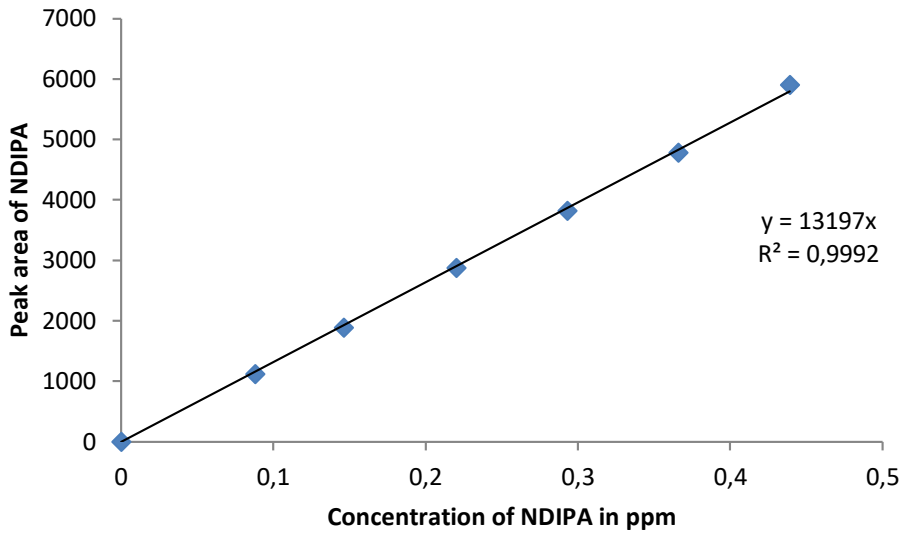


Figure 6: Linearity curve of NDIPA

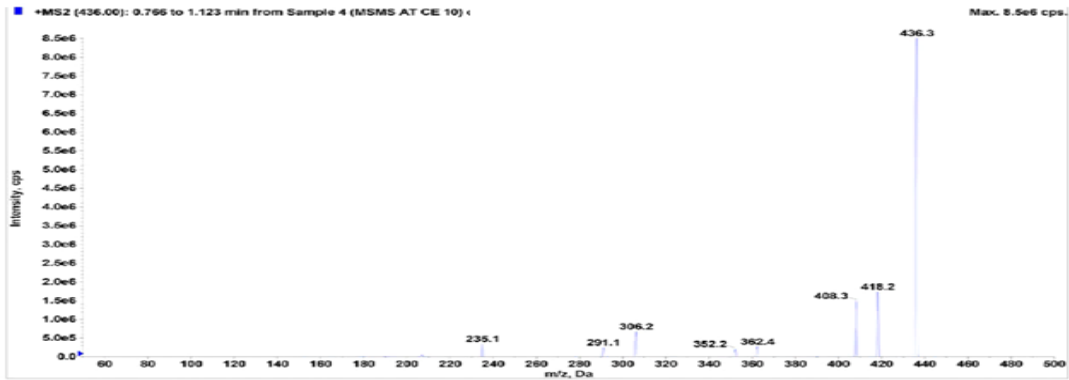


Figure 7: Mass Spectra of Emphagliflozin

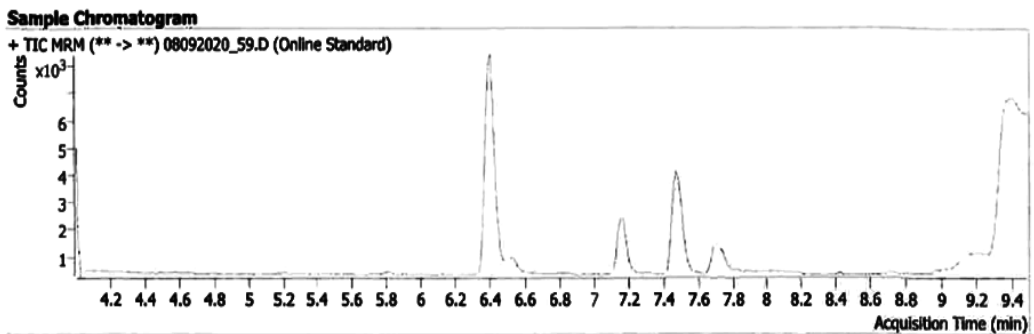


Figure 8: Standard solution chromatogram

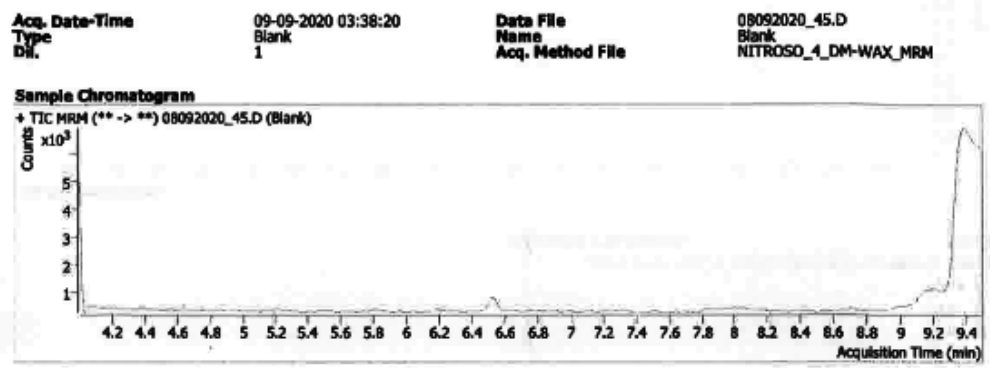
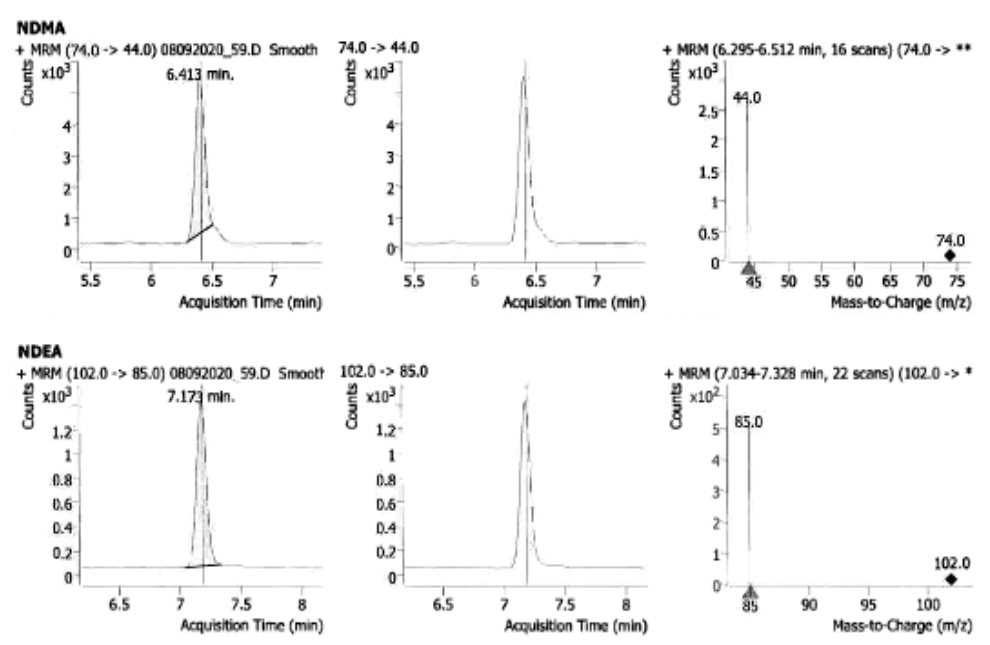


Figure 9: Blank Chromatogram



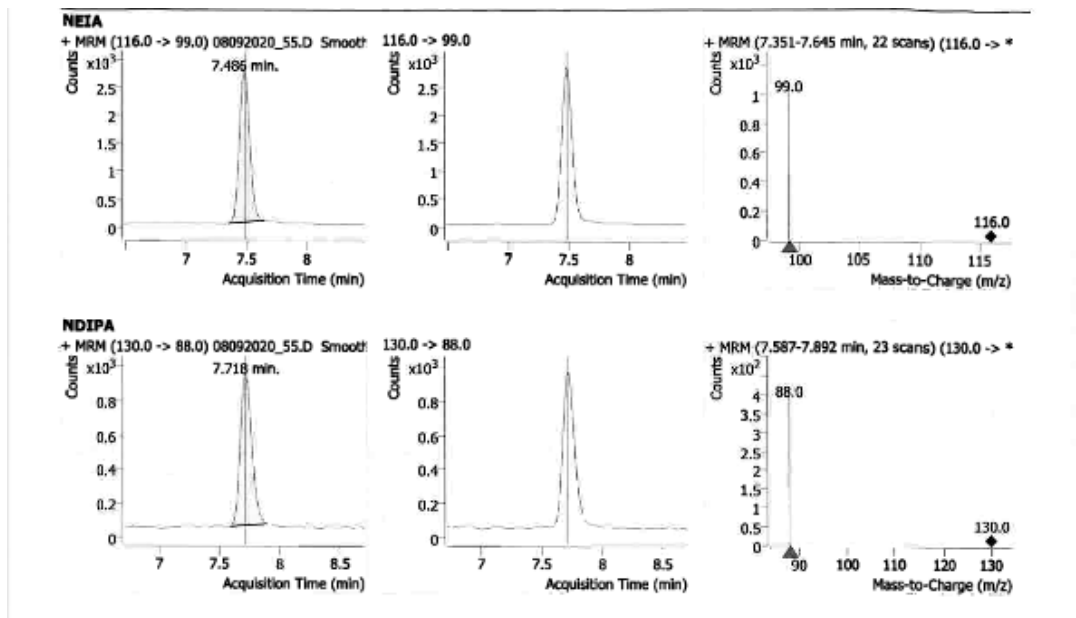


Figure 10: Representative chromatogram for Nitrosamine impurities

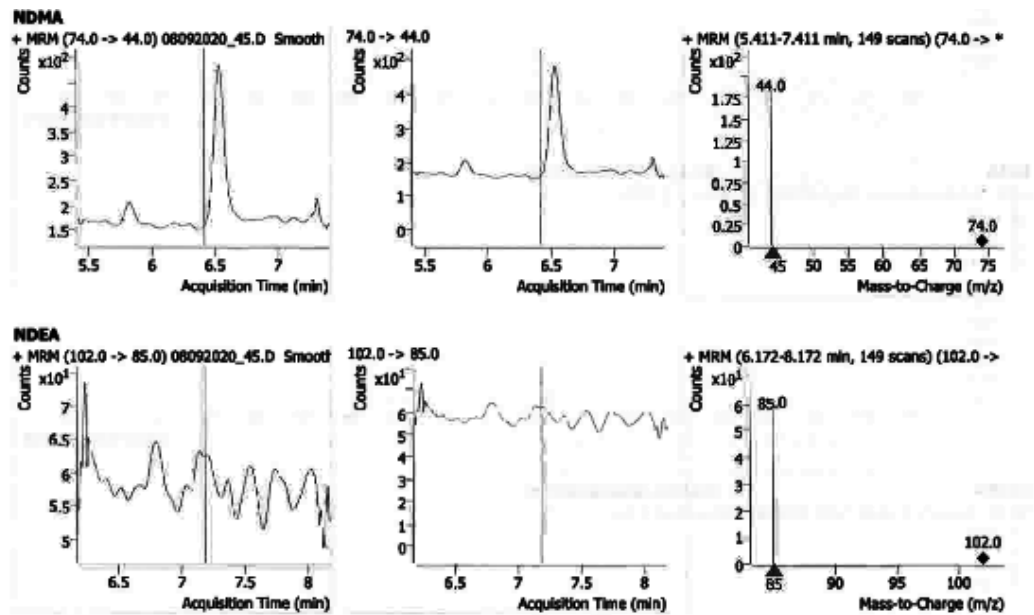


Figure 11: Representative chromatogram for LOQ

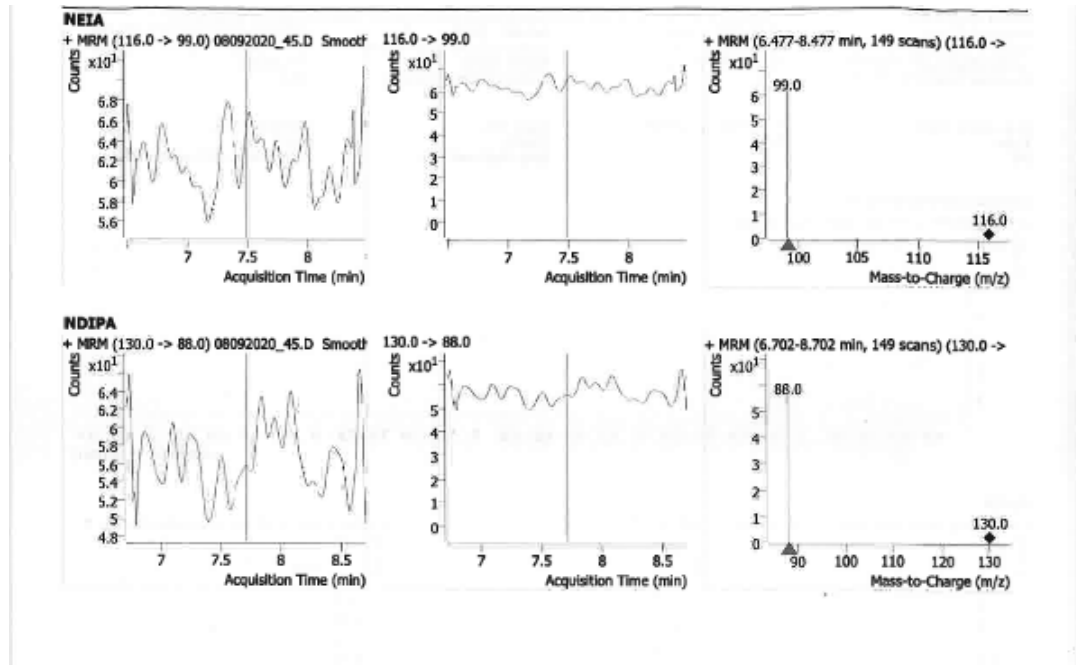


Figure 12: Representative chromatogram for LOD

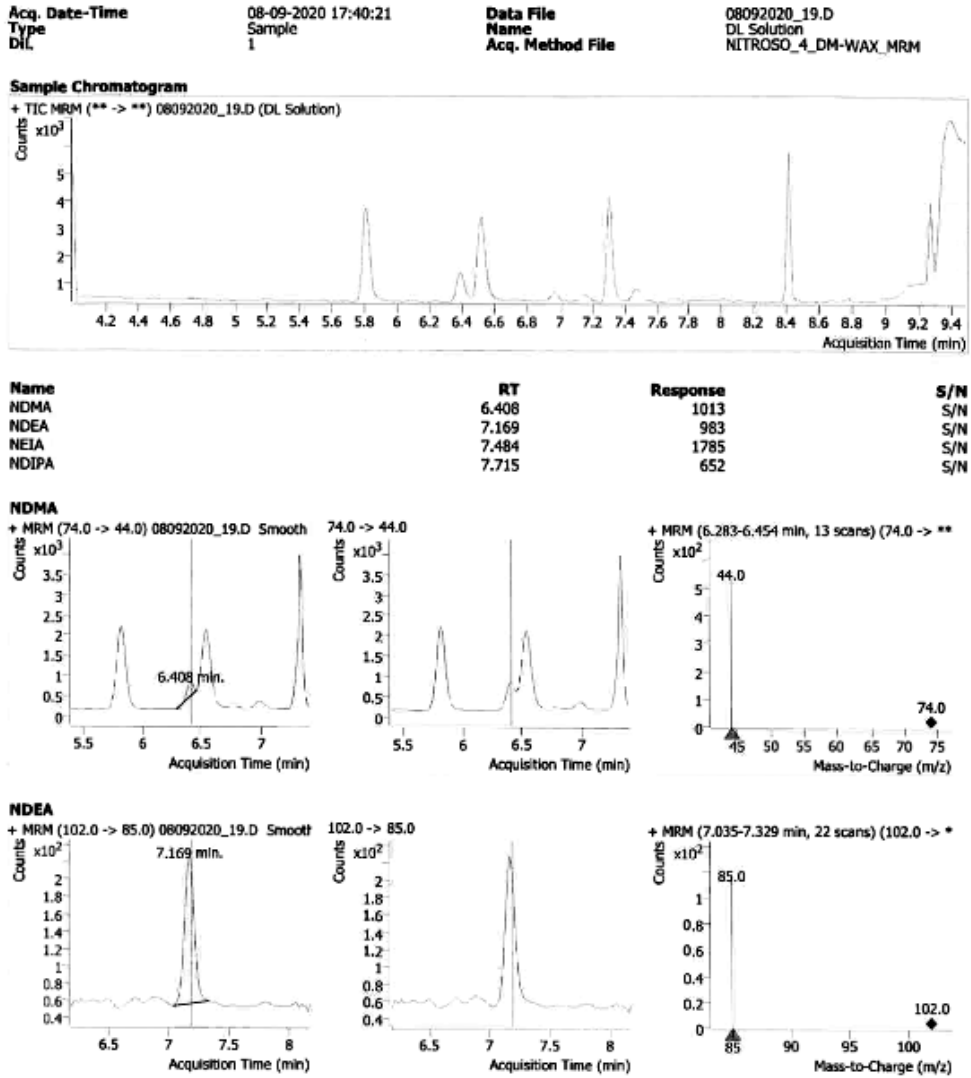


Figure 13: Representative Chromatogram for Accuracy

Table 1: System Suitability

Injection No.	Areas of NDMA	Areas of NDEA	Areas of NEIA	Areas of NDIPA
1	18850	4884	8508	4122
2	17923	4543	8199	3994
3	19185	4747	8541	4139
4	16863	4456	7438	3594
5	17050	4034	7646	3329
6	16392	3952	7268	3500
% RSD	6.4	8.5	7.0	9.2

Table 2: Results for Precision at QL Level

QL Level	Peak Areas of NDMA	Peak Areas of NDEA	Peak Areas of NEIA	Peak Areas of NDIPA
Injection-1	5423	1365	2188	1186
Injection-2	5214	941	2153	1052
Injection-3	5103	863	2348	1149
Injection-4	5590	933	2568	1191
Injection-5	5191	976	2112	1069
Injection-6	5208	885	2387	1111
Average	5288.167	993.833	2292.667	1126.333
%RSD	3.4	18.7	7.6	5.2

Table 3: Summary of % Recoveries at QL level

Accuracy level	% Recovery Of NDMA	% Recovery Of NDEA	% Recovery of NEIA	% Recovery Of NDIPA
QL level	103.2	91.9	114.4	111.4
	101.1	91.9	112.2	113.6
	102.2	101.6	117.8	122.7
Average	102.16	95.13	114.80	115.91
%RSD	1.05	5.60	2.82	5.99

5. Conclusion

This study successfully developed an LC-MS method to screen and determine four nitrosamines in empagliflozin. The proposed method was validated and provided the satisfactory validation result for target nitrosamines in most APIs and final products, which demonstrated exemplary performance and specificity for the method in screening and qualification of nitrosamines. The developed method can be used for routine analysis of nitrosamine impurities in empagliflozin formulations.

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Conflict of Interest

The authors declare that there are no Conflicts of Interests

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Nil

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