**RESEARCH ARTICLE**

# New Validated LC-MS Method Development and Estimation of Nitrosamine impurities in Canaglifozin

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## ABSTRACT

**Background:** Canagliflozin is most prescribed anti-diabetic drug, as diabetic drugs are consumed daily there may be a chance of consuming nitrosamine impurities beyond their limits hence, the Food and Drug Association (FDA) recommends checking for carcinogenic impurities in routine use drugs.

**Aims and Objectives:** The study aims to simultaneous estimation of 4 nitrosamines (NMDA, NEIA, NIEA, and NDIPA) by liquid chromatography-mass spectrometry (LCMS) to detect the limit of detection (LoD) and limit of quantitation (LoQ). estimation of these impurities (NMDA, NEIA) in canagliflozin tablet dosage form with specificity parameters.

**Methods:** Drug canagliflozin was collected from hetero labs, methanol, LC-MS grade acetonitrile, formic acid four impurities (NMDA, NEIA, NDIPA, NIEA) purchased from Merck Pharmaceuticals Mumbai. The Zorbax SB C18 column (250 x 4.6 mm, 3 mcm) is used as the stationary phase, 0.1% formic acid and acetonitrile in the ratio of 70:30 is used as the mobile phase, methanol is used as diluent flow rate was maintained at 1-mL/min, the injection volume was fixed to 10 mcl, which is run at 10 minutes. The electron ionization ion source, multiple reaction monitoring, and acquisition mode is used for the study. The time required for the solvent delay and detector off is 4 and 9.5 minutes, respectively. The product ions peaks of impurities were observed at 44 for NDMA, 99 for NEIA.

**Results:** The %RSD for the peak areas of NDMA, NEIA obtained from six replicate injections of standard solutions were 2.5, 9.8, 1.4 and 7.7, respectively. The %recovery of NDMA and NEIA were within the limit. The %RSD for the results obtained from the method precision study was within the limit.

**Conclusion:** The above observations indicate that the LC-MS method meets the acceptance criteria for the parameters selected for the validation study. Hence, the method is suitable for the determination of NDMA, NEIA in a Drug Substance by LC-MS.

**Keywords:** Canagliflozin, Electron ionization, LC-MS method, NMDA, NDEA, NEIPA, NDIPA.

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**Conflict of interest:** None

## INTRODUCTION

The study of nitrosamine impurities gained a lot of exposure since 2019 when FDA and EMA agencies recalled some antihypertensive drugs like sartans from the market due to the presence of them beyond their limits.[1] These impurities classified as class 1 according to ICH M7 Guidelines as mutagenic and carcinogenic.[2] The agencies declare to check the presence of these impurities in daily consumed drugs. Impurities may get incorporated through intermediates, starting materials, APIs, reagents,[3] Solvents, drugs containing functional groups carbamates, amides, N alkyl are nitrosated,[4] DMF is used as solvent in many drug synthesis including Gliflozins.[5]These nitrosamines are very carcinogenic and effects toxicity to the human body.[6,7]

Since Gliflozins (canagliflozin) is one of the most prescribed drug after metformin, and it is consumed on daily base it is important to check the presence of impurities to check their limits.[8] Drug canaglifozin reduces the reabsorption of filterd glucose and lowers renal threshold for glucose and shows maximal effect in patients with uncontrolled type 2 diabetes mellitus (T2DM)[9] this is a unique mechanism of action approved by USFDA in 2017. It also improves the sensivity of liver which is beneficiary for T2DM patients as serum insulin level declines.[10,11] The drug helps in the reduction

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of BP and shows diuretic action. The chemical structure of canagliflozin is shown in Figure 1. In order to determine its limit, the median toxic dose (TD50) is used. ICH M7 (R1) recognizes the TD50 for the calculation of acceptable excess risk, which allows the calculation of acceptable intake (AI) for mutagenic and carcinogenic impurities,[12] A lot of literature review on canagliflozin reported some analytical methods a UV method for estimation of canagliflozin in bulk and formulation, the developed method obeys beer lamberts law with in concentration range from 5 to 10 mcg, lamda max was found at 290 nm. RS[13] method which is checked for purity of the drug by using solvent methanol, Lamda max was detected at 290 nm, and linearity concentration ranges from 5 to 25 mcg/mL A HPTLC study was reported by sheet team for determination of canagliflozin bulk by using mobile phase toluene, ethyl aceate, methanol in ratio 2:2:1 and silica gel $60F_{254.}$ as stationary phase the spots were identified by densitometric analysis at UV detector 290 nm.[14]

Some RPHPLC methods were also developed for the estimation of canagliflozin in bulk and formulations, using different mobile phases and stationary phases according to ICH guide lines.[15] Another few methods were reported on the simultaneous estimation of canagliflozin with other oral hypoglycemic agents like metformin. Simultaneous estimation of metformin and canagliflozin was developed by the murugasen, team they developed an RPHPLC method for simultaneously estimating empagliflozin and canagliflozin they used Grace mart C18 column as stationary phase and acetonitrile: ammonium acetate buffer as mobile phase, PDA as detector and nanometer fixed at 252 nm,[16] this method found to be effective and less time-consuming. No analytical method was reported for the estimation of nitrosamine impurities in canaglifozin drug hence the present study concentrates on this and tried to develop a new LC-MS method development to check their limits in bulk and formulations. Figure 2 describes nitrosamine impurities.

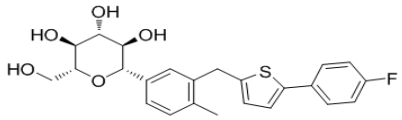

**Figure 1:** Canaglifozins structure

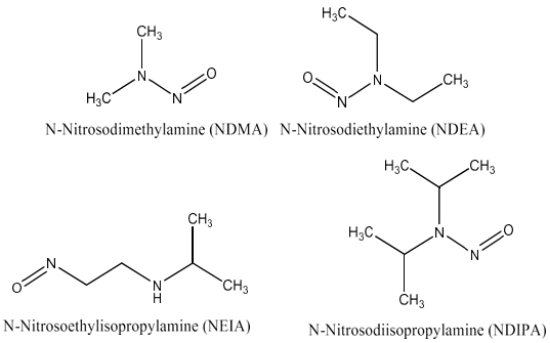


**Figure 2:** Various impurities of nitrosamine

## Experimental Procedure

*Chemicals and instruments used*

NDEA (N-Nitrosodiethylamine) having purity of 98% and NDMA (N-Nitroso-di-methylamine) having purity of 98 percent purchased from Yarrow Chemicals, Mumbai (Sigma Aldrich-German).

There was a solution of N-Nitroso-ethyl- isopropylamine (NEIA) and N- Nitroso-di-isopropylamine (NDIPA) from Yucca Enterprises, Mumbai (Sigma Aldrich, German) used in this experiment. In this research, Acetonitrile (99.9%) and Formic acid (98–100%) were procured from Bros Scientifics (Qualigens, Mumbai). In this investigation, ultrapure water was generated using Millipore Corporation's Milli-Q® purification system (Bedford, MA, USA). An Agilent (US) Zorbax SBC18 column (250 x 4.6 mm, 3.5 µm) served as a stationary phase for the chromatographic separations. Water (A) and acetonitrile (B) were mixed in a 70:30 ratio to produce the mobile phases. Methanol was used as a diluent to maintain a 1mL/min flow rate. There were two liquid chromatography systems: a binary LC pump and a waters quattro micro tandem mass spectrometer (Waters, Hertfordshire, United Kingdom.). Chromatographic separation was performed using gradient elution using the mass spectrometer in positive ion mode. In addition to N-Nitroso-di-methylamine, N-Nitrosodiethylamine, N-Nitroso-ethyl-isopropylamine, and N- Nitroso-di-isopropylamine, the multiple reaction monitoring (MRM) system of the Waters Quattro Micro tandem quadrupole mass spectrometer was utilized in this study. Calculations were performed with Microsoft Excel 2010 and data acquisition was performed with MassLynxTM software.

## METHODOLOGY

### Mobile Phase and Standard Solution Preparation

The mobile phase employed in this study consisted of acetonitrile and formic acid (0.1%) in a 70:30 ratio. To prepare formic acid (0.1%), 2 mL of formic acid was dissolved in 1000 mL of MilliQ ultra-pure $H_2O$ and mixed for 15 minutes. Acetonitrile and methanol solvents were also sonicated for 15 minutes. Certified standard solutions of NDMA (1-mg/mL), NDEA (1-mg/mL), NEIA (1-mg/mL), and NDIPA (1-mg/mL) were used. N-Nitroso-di-methylamine, N-Nitrosodiethylamine, N-Nitroso-ethyl-isopropylamine, and N-Nitroso-di-isopropylamine final standards were prepared and diluted with methanol to 999 ng/mL.

### Sample Solution Preparation

After adding 1-mL of diluent methanol to the headspace vial, 10 mg of the test sample was weighed accurately and placed inside the septum-sealed vial. Injecting the blank solution into the system followed the 16-minute equilibration with the mobile phase, leading to the recording of the chromatogram. Due to its programming, the data processor is able to inhibit peaks easily due to blanks. Chromatograms were recorded after injecting six times the standard solutions separately into the system.

**Validation Procedure**

We validated LC-MS measurements of four N-nitrosamines through MRM mode by considering system suitability, specificity, sensitivity, linearity, LoQ, LoD, accuracy, precision, and stability. The following equation was used to determine the matrix effect.

$$Matrix\ Impact = \frac{A}{B} X100$$

where,

- A- Matrix concentrations of sample
- B- blank/mobile phase

**Detection of Specificity**

Comparing the chromatograms of contaminants-free samples before and after being spiked with the respective analytes verified the specificity of the method for N-Nitroso-di-methylamine, N-Nitrosodiethylamine, N-Nitroso-ethyl- isopropylamine, and N- Nitroso-di-isopropylamine. When N-Nitroso-di-methylamine, N-Nitrosodiethylamine, N-Nitroso-ethyl- isopropylamine, and N- NNitroso-di-isopropylamine are retained at different retention times, no peak should co-elute with them.

**Accuracy, Precision and Linearity**

For the N-Nitroso-di-methylamine, N-Nitrosodiethylamine, N-Nitroso-ethyl-isopropylamine, and N-N-Nitroso-di-isopropylamine assays, three seven-point calibration curves were established, incorporating canaglifozin, a contaminants-free drug, which was excluded from the linear regression. Concentrations of calibration standards were determined by evaluating the calibration curves individually through linear regression, ensuring that they shared slope, intercept, and a coefficient of determination of at least 0.999 for linearity. Each calibration curve was required to exhibit precision of less than 15% (expressed as the relative standard deviation), accuracy of less than 15% (expressed as the relative bias of measured concentrations from nominal concentrations) at each concentration level, with a permissible deviation of 20% at the lowest concentration level. To assess inter-day precision and accuracy, assays for NDMA, NDEA, NEIA, and NDIPA were conducted in triplicate on three different runs across two distinct days. Additionally, intra-day precision of empagliflozin matrix was examined in triplicate in the presence of NDMA, NDEA, NEIA, and NDIPA, as identified by positive tests.

**Limit of Quantification**

LoQ is a parameter that represents the concentration at which acceptable accuracy and precision can still be achieved. The deviation of three repetitive measurements from the nominal concentration cannot exceed 20%, whereas the relative bias from the nominal concentration cannot exceed 20%. Additionally, the S/N ratio should be greater than 4 for N-Nitroso-di-methylamine, N-Nitrosodiethylamine, N-Nitroso-ethyl- isopropylamine, and N- Nitroso-di-isopropylamine.

**Sample Preparation**

In order to increase the sensitivity of the results, a precise amount of finely powdered canagliflozin tablets (10 mg) was obtained. In a 1.5 mL centrifuge tube, one-tenth of this weight was accurately weighed and then mixed with 500 mL of methanol containing the internal standard (NDMA). An additional 5 minutes were spent sonicating the mixture after vigorous shaking for 5 minutes. Following vigorous shaking for 5 minutes and sonication for 5 minutes, the suspension was diluted to a final volume of 1000 mL with MilliQ® water. Following centrifugation at 3°C at 5000 rpm for 35 minutes, the suspended samples were collected. As soon as the supernatants were centrifuged, they were carefully transferred into glass vials for further injection.

## RESULTS

According to the validation results below, the LC-MS/MS method developed for the quantification of the four contaminants N-Nitroso-di-methylamine, N-Nitrosodiethylamine, N-Nitroso-ethyl- isopropylamine, and N- Nitroso-di-isopropylamine is suitable for the determination of even traces of the four analytes.

**Systemic Validation**

There was a matrix effect of 96.44 ± 3.43% to 98.88 ± 1.21%. There are no measurable interferences with canagliflozin caused by nitrosamine impurities in extraction recovery and matrix effects. Table 1 shows the system suitability parameters.

**Precision, Accuracy, and Linearity**

Each analyte had different calibration ranges: NDMA 0.092 to 0.463 ppm, NDEA 0.065 to 0.465 ppm, NEIA 0.095 to 0.445 ppm, and NDIPA 0.089 to 0.440 ppm. Weighted (1/concentration^2) linear regression was applied to achieve good linearity. Figures 3-6 show the coefficients of determination of calibration curves for all analytes. Figures 7-12 illustrate representative chromatograms and mass spectrometer data. A blank chromatogram confirms the specificity of the methods. According to the results, NDMA, NDEA, NEIA, and NDIPA were found to be suitable for 2.5, 9.8, 1.4, and 7.7, respectively shown in Table 2. For precision and accuracy in NDMA, the standard deviations were 2.4 and 2.9, for NDEA they were 7.9 and 3.5, NEIA they were 4.0 and 3.3, and for NDIPA they were 4.8 and 3.7.

**LoQ and LoD**

We found that the LLoD values for NDMA, NEIA, NDIPA, and NDEA were 0.02, 0.015, and 0.13 ppm, respectively. A further 0.09 ppm of LLoQ was found for NDMA, NEIA, and NDIPA, while 0.06 ppm was found for NDEA. Two sets of SQC samples were repeated for accuracy (bias within and between runs, -5.6 to -3.2%) and precision (coefficient of variation, 6.5%) for all tested concentrations of analytes (Table 3). A deviation of no more than ± 20% is acceptable for quality control above LoQ and no more than ± 15% is acceptable for LoQ.

**Accuracy**

The triplicate analysis of the same finished product demonstrated excellent precisions, ranging between 0.8 and 15.3% for each analyte. The assay's repeatability was further confirmed by using varied amounts of the relevant analyte in finished products. The mean absolute recovery in ground tablets was

**Table 1:** System suitability results

| *Injection Number* | *Curve area of NDMA* | *Curve area of NDEA* | *Curve area of NEIA* | *Curve area of NDIPA* |
|---|---|---|---|---|
| 1 | 13584 | 5652 | 9623 | 4526 |
| 2 | 13523 | 5896 | 9442 | 4412 |
| 3 | 13588 | 5748 | 9564 | 4896 |
| 4 | 13963 | 5847 | 9847 | 4231 |
| 5 | 14050 | 6012 | 9632 | 4452 |
| 6 | 14392 | 4521 | 9547 | 3865 |
| Average | 13850.0 | 5612.7 | 9609.2 | 4397.0 |
| SD | 344.3 | 548.9 | 135.0 | 340.4 |
| % RSD | 2.5 | 9.8 | 1.4 | 7.7 |

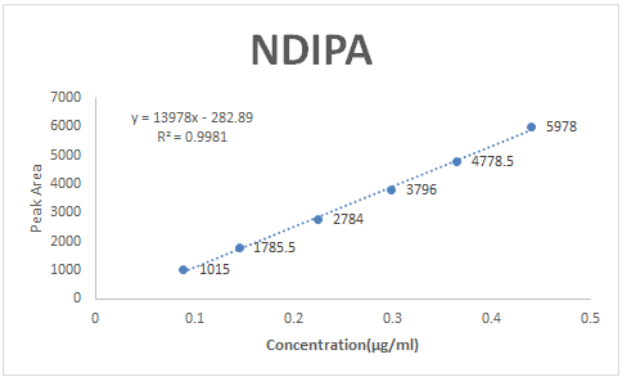


**Figure 6:** NDIPA Linearity curve

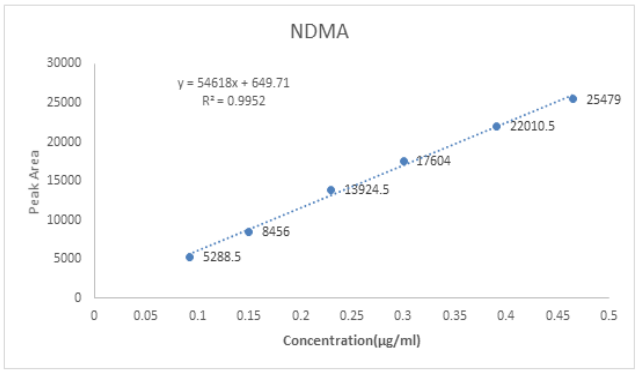


**Figure 3:** NDMA Linearity curve

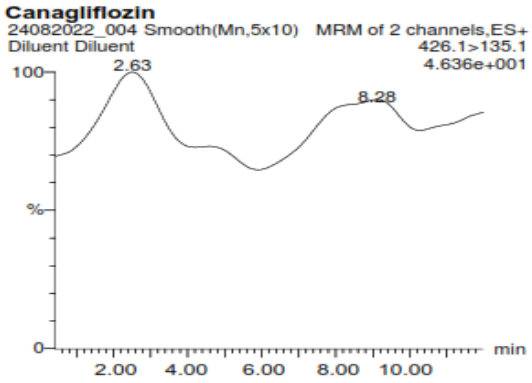


**Figure 7:** Chromatogram of blank injection

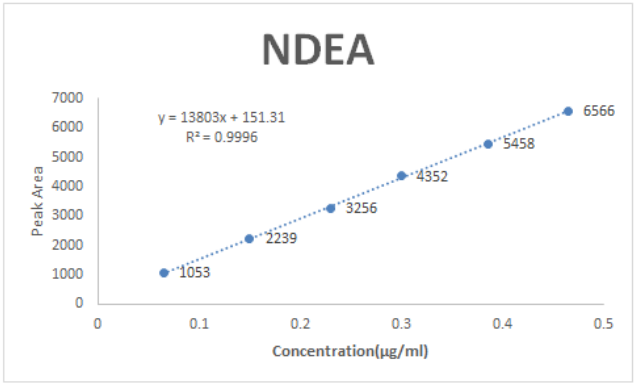


**Figure 4:** NDEA Linearity curve

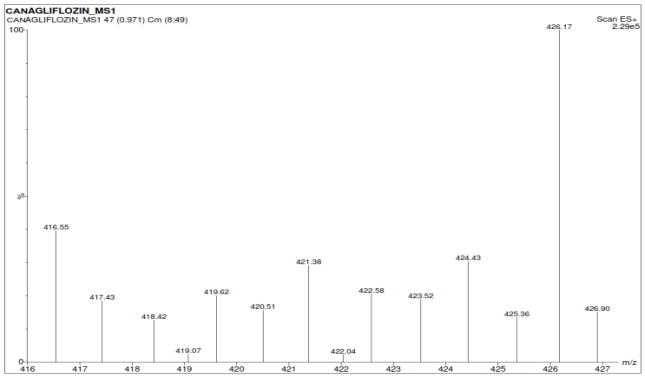


**Figure 8:** Mass spectra of canaglifozin drug

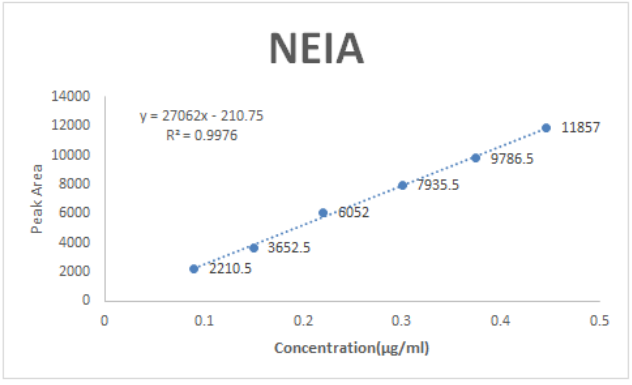


**Figure:**5 NEIA Linearity curve

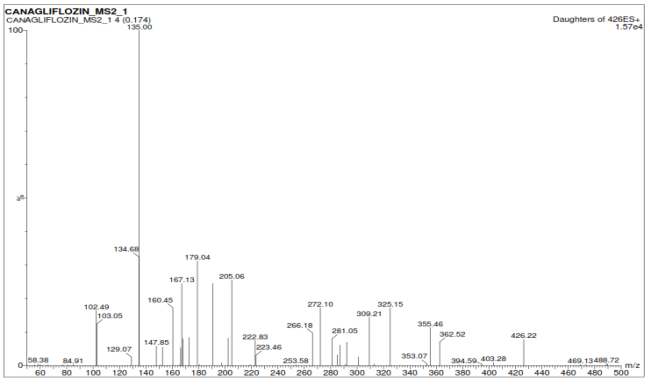


**Figure 9:** Mass spectra of canaglifozin

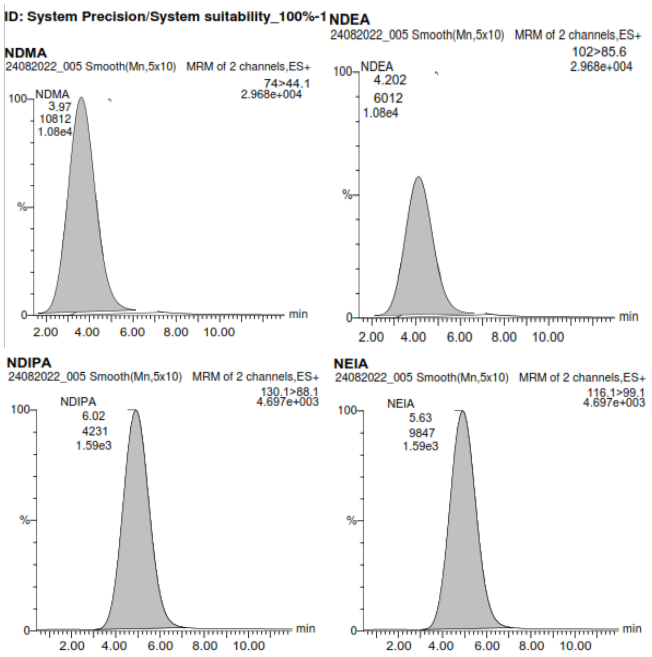


**Figure 10:** Chromatograms of impurities

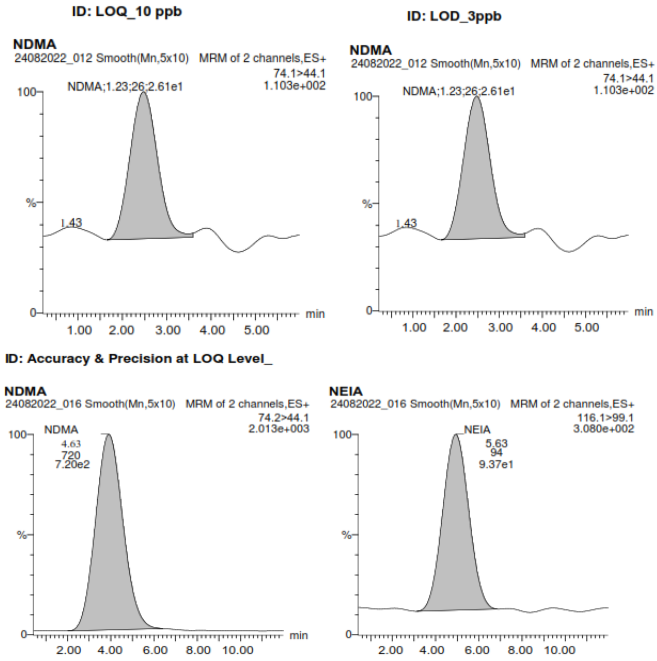


**Figure 11:** LOD chromatograms

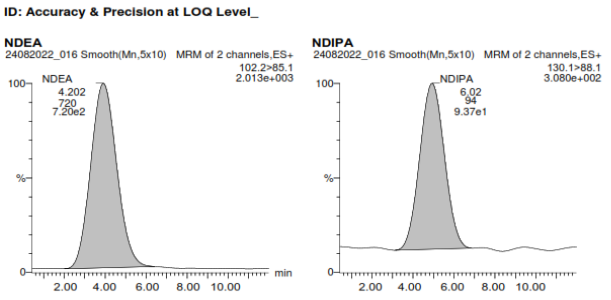


**Figure:** 12 Chromatogram of precision at QL

**Table 2:** QL precision level

| *QL Level* | *NDMA-Peak area* | *NDEA-Peak area* | *NEIA-Peak area* | *NDIPA-Peak area* |
|---|---|---|---|---|
| Injection-1 | 5322 | 1124 | 2235 | 1235 |
| Injection-2 | 5314 | 1025 | 2214 | 1245 |
| Injection-3 | 5103 | 1036 | 2256 | 1123 |
| Injection-4 | 5490 | 1048 | 2214 | 1189 |
| Injection-5 | 5291 | 976 | 2112 | 1153 |
| Injection-6 | 5208 | 885 | 2387 | 1111 |
| Average | 5288.0 | 1015.7 | 2236.3 | 1176.0 |
| SD | 129.1 | 79.9 | 88.9 | 56.5 |
| %RSD | 2.4 | 7.9 | 4.0 | 4.8 |

**Table 3:** % recoveries at 3 concentrations

| *Accuracy level* | *Preparations* | *% Recovery of NDMA* | *% Recovery of NDEA* | *% Recovery of NEIA* | *% Recovery of NDIPA* |
|---|---|---|---|---|---|
| | 1 | 105.6 | 103.6 | 112.05 | 115.6 |
| 50% | 2 | 104.5 | 102.5 | 112.5 | 115.6 |
| | 3 | 104.3 | 10.3.4 | 110.4 | 118.7 |
| | 1 | 108.7 | 108.1 | 111.4 | 117.1 |
| 100 % | 2 | 107.1 | 106.8 | 109.7 | 115.7 |
| | 3 | 106.1 | 102.9 | 105.7 | 109.9 |
| | 1 | 106.3 | 104.7 | 110.2 | 112.2 |
| 150% | 2 | 104.5 | 105.8 | 102.5 | 114.2 |
| | 3 | 105.4 | 104.2 | 104.5 | 112.5 |

determined to be 104.3 ± 1.05% for NDMA, 103.4 ± 0.09% for NDEA, 110.80 ± 2.02% for NEIA, and 115.6 ± 2.09% for NDIPA (Table 3). This indicates that the complete recoveries for ground tablets and tablet extracts are comparable, underscoring the efficiency of the extraction method. NDMA, NDEA, NEIA, and NDIPA exhibited percentage recoveries between 70 to 130% across three different concentrations.

## CONCLUSION

An LC-MS method has been developed for screening and quantifying four nitrosamines in canagliflozin in this study. As a result of the development, the simultaneous estimation of nitrosamines can also be applied to other drugs. It was validated against the target nitrosamines both in APIs and finished products, with satisfactory results obtained. It has therefore proven to be an excellent screening and qualification method for nitrosamines due to its exemplary performance and specificity. Canagliflozin formulations can be routinely analyzed for nitrosamine impurities using the developed method.

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