Testing Methods for N-Nitrosamines Monitoring in Pharmaceuticals

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Agenda

- Overview of USP Nitrosamine activities
- Method Performance Characteristics & Factors which impact sensitivity and selectivity
- GC <1469> Nitrosamine Impurities: Testing Methods & Analytical Challenges
Overview of USP Nitrosamine activities

1 Documentary Standard
To address the nitrosamine impurities safety concern from a pharmacopeia perspective, USP Joint Expert Subcommittee (JSC) was convened since February 2020 to develop General Chapter <1469> Nitrosamine Impurities.

2 Reference Standard
Eight USP Reference Standards have been established to support General Chapter <1469> Nitrosamine impurities

- N-Nitrosodimethylamine (NDMA) (1 mg/mL in MeOH)
- N-Nitrosodiethylamine (NDEA) (1 mg/mL in MeOH)
- N-Nitrosodimethylamine (NDMA) (1 mg/mL in MeOH)
- N-Nitrosodibutylamine (NDBA) (1 mg/mL in MeOH)
- N-Nitrosoethylisopropylamine (NEIPA) (1 mg/mL in MeOH)
- N-Nitrosomethylaminobutyric Acid (NMBA) (1 mg/mL in ACN)
- Deutero N-Nitrosodimethylamine (NDMA-d₆)

Additional Impurities to be developed
- 1-Cyclopentyl-4-NitrosPiperazine (CPNP)
- 1-Methyl-4-NitrosoPiperazine (MNP)
- N-Nitroso pyrroldidine (NPNYR)
- N-Nitrosodietanolamine (NDELA)
- N-Nitrosodipropylamine (NDPA)
- N-Nitrosomethylamine (NMEA)
- N-Nitrosomorphline (NMOR)
- N-Nitrosopiperidine (NPIP)
Overview of USP Nitrosamine activities

Timeline: GC <1469> Nitrosamines Impurities

1. GC <1469> publication in the PF
   - GC <1469> published in Pharmacopeial Forum 46 Issue 5, available online

2. End commentary
   - Comments period end (ALL stakeholders are encouraged to participate)

3. JSC address comment and review proposal
   - Sub-committee address public comments and revise chapter

4. Standard is balloted
   - GC is balloted for approval by Chemical Analysis General Chapter Expert committee

5. Publish to USP-NF 2021 Issue 3

6. GC <1469> becomes official

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USP is supporting manufacturers and regulators with standards as well as other tools and solutions to test for and assess the risk of nitrosamine impurities.

1. SCIENTIFIC DISCUSSIONS & PUBLICATIONS

- Scientific Webinars
- Nitrosamine Tutorial Videos – Youtube: Playlist
- Review article on Testing Methods (2021)
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   - Review article on Testing Methods (2021)

2. USP EDUCATION
   - Addressing Impurities in Drug Products and Drug Substances: ICH Guidelines Q3-A, B, C, D and M7 (Available on demand)
   - USP Course on Nitrosamines GC <1469> (15 and 16 June 2021)

3. TEST PROCEDURES
   - For Drug Products: Ranitidine, Metformin, Rifapentine
   - For detection of nitrosamine impurities in solvents and water used in the manufacturing process
   - PF 45 (6) Stimuli Article including methods to determine N-nitrosamines and PAHs in elastomeric components for inhalation packaging/delivery systems."

4. OTHER ACTIVITIES
   - Nitrosamine Exchange Knowledge Community - Nitrosamines Risk Assessment
Nitrosamine Exchange Community

Join us at: http://nitrosamines.usp.org
GC <1469> Nitrosamines Impurities

Content and Rationale

1. INTRODUCTION
2. NITROSAMINE IMPURITIES
3. SOURCES OF NITROSAMINES
4. NITROSAMINE RISK ASSESSMENT - DEVELOPMENT OF A CONTROL STRATEGY
5. LIMITS OF NITROSAMINE
6. TESTING FOR THE PRESENCE OF NITROSAMINES
7. TEST METHOD PERFORMANCE CHARACTERISTICS OF NITROSAMINE METHODS
8. ANALYTICAL PROCEDURES
9. ADDITIONAL SOURCES OF INFORMATION
What should be the required sensitivity for analytical methods?

- Limit of Quantification (LOQ) - ICH Q2 (R1): provides the minimum level at which an analyte can be quantified with acceptable accuracy and precision
  - LOQ preferred over LoD for impurity testing and decision-making
  - LOQ should be used to define the required analytical sensitivity for impurity testing.

- Limit of Detection (LOD) - ICH Q2 (R1): LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value
  - Experts recommended not to use LOD for setting limits and not to use technical limits as nitrosamines may not be avoidable completely in many cases

As a minimum requirement the method should have: \( \text{LOQ} \leq \text{AI} \)
and meet regulatory recommendations...

e.g.: NDMA in Valsartan (96ng/day / 320mg/day)
\[ \text{Al} = 0.3 \text{ppm (0.0003mg/mL)} \]
Required sensitivity for analytical methods

- **FDA** Control of Nitrosamines Impurities in Human Drugs - Guidance for Industry - FDA February 2021
  - Products with MDD < 880 mg/day: LOQ ≤ 0.03ppm
  - Products with MDD > 880 mg/day: LOQ as low as reasonably practical
  - LOQ < Test Result ≤ Acceptable Intake

- **EMA** Assessment Report - Nitrosamines Impurities in human medicinal products - EMA 25June20
  - LOQ ≤ Acceptable limit for the respective nitrosamine impurities, taking into account the purpose of testing
    - Routine control: LOQ ≤ Acceptable Limit
    - Justify skip testing: LOQ ≤ 30% of AL
    - Justify omission from the specification: LOQ ≤ 10% of AL
  - Exceptions may be needed depending on the maximum daily dose (MDD) or if more than one nitrosamine is expected to be present. Such cases should be discussed with the relevant competent authorities.
The low levels at which the nitrosamine impurities occur create challenges for testing. To assist in the testing of samples, the US FDA, Official Medicines Control Laboratories (OMCLs) Network of the Council of Europe, Health Canada and have also published several testing methods for nitrosamines.

Published Methods for Nitrosamines monitoring in APIs and DP

Analytical Procedure for Nitrosamines Quantification
Performance Characteristics*

1. Sensitivity:
   - Sensitive to detect and quantify N-nitrosamines in DPs and APIs at ppm level
   - Sensitive enough to meet the proposed regulatory recommendations
   - Neither LOD nor LOQ are constant values and can change over time depending on: equipment, laboratories, personnel, sample preparation and many other factors.

2. Selectivity:
   - Selective for target* nitrosamines (Same method applicable to different DP? And APIs?) *those possible to be formed during the API/DP manufacturing process, storage etc – after risk assessment

3. Accuracy and Precision: Challenges: extraction efficiency suitable recovery of trace impurities, matrix effects

4. Linearity:
   - Linear within the proposed concentration range. Ex.: LOQ, 50%, 75%, 100%, 125%, 150% (including the AI)
   - Note: LOQ as low as reasonably practical or ≤ 10% of AI if possible

5. Robustness: ensure consistent/reproducible results (presence of the drug substance and the extraction efficiencies are the key factors affecting robustness)

ICH guideline Q2(R1) - VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY

“Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

* for quantitative analysis
Analytical Procedure for N-Nitrosamines

Analytical Procedure Development

Risk assessment for method development

1. ANALYTICAL TARGET PROFILE (ATP)
   Predefined objective that stipulates the performance requirements for the analytical procedure

2. COMPOUNDS PROPERTIES EVALUATION
   (Volatility, Solubility, Polarity, pKa, UV-Vis light absorption, Thermally labile? etc...)

   e.g.: ATP - Selective and Robust method for nitrosamines quantification in DS/DP sensitive enough to meet the proposed reporting limit threshold (e.g.: LOQ ≤ 0.03ppm, AI=0.3ppm) by LC-MS

   Risk assessment for method development:
   Prior knowledge on potential nitrosamines impurities presence* excipients, degradation products** and other impurities

Testing methods main challenges:
- Selectivity and sensitivity
- broad coverage of N-Nitrosamines*
- applicability to different APIs/DP

*based on risk assessment of the manufacturing process
** Pilot stress testing?
** In-silico tools to predict degradation products?

METHOD DESIGN

METHOD UNDERSTANDING/KNOWLEDGE

Excipient 1
NDMA
NDEA
Valsartan
Excipient 2
Potential degradation product to be formed > Limit

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Analytical Procedure Development

1. **ANALYTICAL TARGET PROFILE (ATP)**
   - Predefined objective that stipulates the performance requirements for the analytical procedure

2. **COMPONENT PROPERTIES EVALUATION**
   - (Volatility, Solubility, Polarity, pKa, UV-Vis light absorption, Thermally labile, etc...)

3. **CRITICAL QUALITY ATTRIBUTES (CQA)**
   - Analytical responses which represent the quality of the method and reflect method suitable performance

4. **CRITICAL METHOD PARAMETERS (CMP)**
   - Analytical conditions (input factors) which impact significantly on method performance

5. **SAMPLE PREPARATION**
   - Selective extraction of target compounds x Total API/target compounds dissolution?
   - Sonication, extraction time, extraction repetition?
   - Solid Phase Extraction (SPE) to concentrate trace impurities enhancing sensitivity?
   - Reduce/minimize matrix effect

6. **CHROMATOGRAPHY**
   - Stationary phase: chromatographic columns
   - Organic solvent
   - pH of mobile phase
   - Use of additives (ion-pair reagents, acids etc)
   - Column temperature (especially for ionizable compounds)
   - Primary conditions which impact on Selectivity and Retention Factor

7. **MASS SPECTROMETRY**
   - Ionization Source/Parameters (sensitivity and selectivity)
   - MS resolving power (selectivity)
   - Acquisition data strategies (enhance sensitivity and selectivity)
   - Reduce/minimize matrix effect

8. **DATA PROCESSING**
   - Data Processing: mass tolerance settings

**CQA**

1. **Selectivity**
   - Resolution between target compounds and adjacent peaks (chromatography)
   - Resolution between isobaric compounds (MS)

2. **Sensitivity**
   - Sensitive to detect at ppb level

3. **Accuracy & Precision**

4. **Retention Factor (k):**
   - Ideal: 1 < k < 10,
   - Acceptable: 0.5 < k < 20

**IMPURITIES IDENTIFICATION**

**VALIDATION**
Analytical Procedure for N-Nitrosamines Quantification

Mass Spectrometry - Overview

Sample Introduction
- LC
- GC
- ICP
- CE
- Direct Injection
- Flow Injection Analysis (FIA)

Ionization Source
- EI (Electron Ionization)
- CI (Chemical ionization)
- API (Atmospheric Pressure Ionization)
  - ESI (Electrospray Ionization)
  - APCI (Atm. Pressure Chemical Ionization)
  - APPI (Atm. Pressure Photoionization)
- MALDI
- *DESI (Desorption Electrospray Ionization)
- *DART (Direct Analysis in Real Time)
- FAB (Fast Atom Bombardment)

Analyzer
- Quadrupole
- Ion Trap
- ToF (Time-of-Flight)
- FT-ICR (Fourier Transformed-Ion Cyclotron Resonance)
- Orbitrap
- Tandem MS
  - Triple Quadrupole (QqQ)
  - Ion-Trap (IT): 3D or linear
  - QToF/IT-ToF
  - Q-Orbitrap
  - LIT-Orbitrap
  - Qq-Linear Ion Trap
  - Q-Orbitrap-LIT

Detector

Data System

Chromatograms

Data Acquisition Strategies
- Data-Dependent Acquisition (DDA)
- Data-Independent Acquisition (DIA)
- Targeted Data Acquisition (TDA)
- etc...

Data Processing
Mass Analyzers

Resolving Power and Mass Accuracy: Impact on Selectivity

Example: NDMA with DMF

- With low mass, resolving power only peaks differing by 1 mass unit can be separated and the recorded masses are then the nominal masses.
- Mass spectrometers with insufficient mass resolving power do not allow distinguishing ions having the same nominal mass but different exact masses (i.e., isobaric ions).
- The increase in the mass resolving power narrows the peak width, allowing:
  - Peaks differing by a small m/z increment to be resolved
  - Better accuracy of the mass measurement
- With sufficient high mass resolving power, the contaminant could be determined from the experimentally measured accurate mass.

Higher resolving power = greater selectivity
Analytical Procedure for N-Nitrosamines Quantification

Mass Analyzer: Lack of selectivity


Table 2. Comparison of mass spectrometry (MS) conditions used in this study (FDA) and the private laboratory method description

<table>
<thead>
<tr>
<th>MS Conditions</th>
<th>Private laboratory</th>
<th>FDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument</td>
<td>Orbitrap</td>
<td>Q/ToF</td>
</tr>
<tr>
<td>Ionization mode</td>
<td>APCI, positive</td>
<td>APCI, positive</td>
</tr>
<tr>
<td>Data acquisition</td>
<td>Targeted MS2</td>
<td>MRMHR</td>
</tr>
<tr>
<td>MS scan</td>
<td>40–90 m/z</td>
<td>50–450 m/z</td>
</tr>
<tr>
<td>Mass resolution</td>
<td>45,000 ppm</td>
<td>&gt; 25,000 ppm</td>
</tr>
<tr>
<td>Transition(s)</td>
<td>83.0997 – 83.0997</td>
<td>75.0553 – 75.0553</td>
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► High content of NDMA was reported by a private laboratory in metformin drug products
► FDA analyzed the same DPs using MS system with higher resolution
► presence of an interfering substance (DMF) which coeluted with NDMA:
  ▪ insufficient mass resolution or accuracy in data acquisition
  ▪ inappropriate mass tolerance setting in data processing: A mass tolerance window of ±15 ppm or ±30 ppm was applied to obtain the EICs

Table 1. NDMA Amounts in metformin samples reported by FDA (using FDA-1 and FDA-2 methods) and the private laboratory

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<tr>
<th>Sample #</th>
<th>Metformin dosage and formulation</th>
<th>Manufacturer name as per private laboratory</th>
<th>Lot #</th>
<th>FDA-1↑ (ng/mg)</th>
<th>FDA-2 (ng/mg)</th>
<th>Private lab (ng/mg)</th>
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<tr>
<td>1</td>
<td>500 mg IR</td>
<td>ACT Healthcare USA, Inc.</td>
<td>D109061</td>
<td>ND</td>
<td>ND</td>
<td>0.062</td>
</tr>
<tr>
<td>2</td>
<td>500 mg IR</td>
<td>ACT Healthcare USA, Inc.</td>
<td>D10909A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>500 mg IR</td>
<td>ACT Healthcare USA, Inc.</td>
<td>D10909B</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>500 mg ER</td>
<td>Actavis Pharma, Inc.</td>
<td>137639M</td>
<td>0.021↑</td>
<td>0.021</td>
<td>0.364</td>
</tr>
</tbody>
</table>

A minimum resolution of 45,000 and maximum mass tolerance of 15 ppm are required to prevent overestimation of NDMA when quantifying NDMA using the monoisotopic ion.

Isotopic ion
m/z 75.0569 DMF $^{15}$N
m/z 75.0631 DMF $^{13}$C

Monoisotopic exact mass
Analytical Procedure for N-Nitrosamines Quantification

Mass Analyzer: Lack of selectivity


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Data Processing:
- Chromatogram Extraction (EIC)
- INNAPROPRIATE MASS TOLERANCE: DATA PROCESSING

INNAPROPRIATE MASS TOLERANCE: DATA PROCESSING

mass tolerance < 21ppm
mass tolerance > 21ppm

NDMA + Interferent
NDMA + Interferent

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Section 7. Test Method performance characteristics of nitrosamines methods

- Section 7: presents the recommended method performance characteristics for quantitative analysis and limit test.

- The performance criteria for these parameters should be properly set and confirmed through validation to ensure that the method is suitable for its intended use based on the specific analytes, matrices, and required precision and accuracy of the analytical procedures.

- For additional guidance on validation of alternative methods for nitrosamines, see Validation of Compendial Procedures (1225).

**Require validation:**
- existing procedures with modifications changing critical parameters
- existing method is used **outside of its original purpose**

**Require verification:**
- existing method is used **within its original purpose/scope** verify the suitability of the procedures
- For more information on how to conduct verification you can refer to USP chapter 1226 Verification of Compendial Procedures
GC <1469> Nitrosamines Impurities

7.1. CONSIDERATION FOR SAMPLE PREPARATION

► Appropriate sample preparation is a critical step in trace impurity analyses such as those required to evaluate the levels of nitrosamines in drug substances and drug products.

► This is particularly critical to prevent the loss or generation of nitrosamines as artifacts of the analytical procedure itself, as in the following circumstances.

In-situ formation of NDMA in GC-MS analysis

Dialkylamines (dimethylamine):
- degradation product of the API: total dissolution of the DS containing dimethylamino-group should be avoided when applying GC techniques.
- High concentration of the API, when injected in the GC can generate nitrosamines in the injection port if a nitrosating agent is present: sample extractions should be modified to prevent the solubilization of the API while maintaining the extraction efficiency for nitrosamines present in the material.
- process impurity
- counter ion of the salt form of the API

...in the presence of nitrite and acid can lead to in situ formation of nitrosamines as an artifact, especially in GC analyses.

► It is highly recommended that LC-MS be used for determination of NDMA in Ranitidine DS and DP.
8. ANALYTICAL PROCEDURES

Sample Introduction → Ionization Source → MS Analyzer(s)

Liquid Chromatography (LC)
- HPLC - HRMS (QOrbitrap)
  - Procedure 1: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans by HPLC–HRMS
    - LC Column: L43 - PFP

- GC-MS/MS (Direct injection) GC-EI-QqQ
  - Procedure 4: Quantitation of NDMA, NDEA, NDIPA, NEIPA, and NDBA in selected sartans by GC–MS/MS (triple-quad) – (Using internal standards NDMA-C_{13}d6)

Gas Chromatography (GC)
- GC-MS/MS (Headspace) GC-EI-QqQ
  - Procedure 3: Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans by HPLC–MS/M (Using Internal standards NDMA-d6, NMBA-d3, NDEA-D10 NDBA-D18
    - LC Column: L1 – C18

- HPLC-APCI-QqQ
  - Procedure 2: Quantitation of NDMA, NDEA, NDIPA, and NEIPA in selected sartans by GC–MS (using Internal Standard NDMA-d6)
Quantitation of six nitrosamines (NDMA, NDEA, NDIPA, NEIPA, NMBA, NDBA and NMPA) in selected sartans by HPLC-HRMS*

Diluent: Methanol

**Standard solution:** 6.0 ng/mL (0.3 ppm) each in Diluent of USP Nitrosamine Reference Standard (NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA)

**Sensitivity solution:** 1.0 ng/mL (0.05 ppm) each in Diluent of USP Nitrosamine Reference Standard (NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA) from Standard stock solution.

Sample solution: 20 mg/mL of DS in Diluent.

**Chromatographic system:**
- **Mode:** LC
- **Mobile phase A:** 0.1% formic acid in water
- **Mobile phase B:** 0.1% formic acid in methanol
- **Column:** 4.6 mm x 10-cm, 2.6 μm packing L43 (PFP)
- **Column Temperature:** 40º
- **Flow Rate:** 0.6 mL/min
- **Injection Volume:** 3 μL
- **Autosampler Temperature:** 4º

**System suitability requirements**
- Relative standard deviation: NMT 20.0% from 6 replicate injections, **Standard solution**
- Signal-to-noise ratio: NLT 10, **Sensitivity solution**

**Detector:** High resolution mass spectrometer

**MS conditions:**
- **Ionization:** Electrospray Ionization (ESI)
- **Data Acquisition Strategy:** Selected Ion Monitoring (SIM)/Parallel Reaction Monitoring (PRM)

**Scan settings**

<table>
<thead>
<tr>
<th>Impurity</th>
<th>NDMA</th>
<th>NMBA</th>
<th>NDEA</th>
<th>NEIPA</th>
<th>NDIPA</th>
<th>NDBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan Type</td>
<td>SIM</td>
<td>SIM</td>
<td>PRM</td>
<td>SIM</td>
<td>SIM</td>
<td>PRM</td>
</tr>
<tr>
<td>Polarity</td>
<td>POS</td>
<td>NEG</td>
<td>POS</td>
<td>POS</td>
<td>POS</td>
<td></td>
</tr>
<tr>
<td>Scan Start –End (min)</td>
<td>1.0-3.5</td>
<td>3.5-5.5</td>
<td>5.5-7.0</td>
<td>7.0-8.5</td>
<td>8.5-10.0</td>
<td>13.0-15.5</td>
</tr>
<tr>
<td>m/z Isolated for PRM</td>
<td>N/A</td>
<td>N/A</td>
<td>103.0866</td>
<td>N/A</td>
<td>N/A</td>
<td>159.1492</td>
</tr>
<tr>
<td>Resolution</td>
<td>30000</td>
<td>60000</td>
<td>30000</td>
<td>60000</td>
<td>60000</td>
<td>30000</td>
</tr>
<tr>
<td>Isolation Window</td>
<td>N/A</td>
<td>N/A</td>
<td>1.5 m/z</td>
<td>N/A</td>
<td>N/A</td>
<td>1.5 m/z</td>
</tr>
<tr>
<td>Scan Range</td>
<td>m/z 74.3-75.8</td>
<td>m/z 144.3-145.8</td>
<td>m/z 50.0-114.0</td>
<td>m/z 116.4-117.9</td>
<td>m/z 130.4-131.9</td>
<td>m/z 50.0-170.0</td>
</tr>
</tbody>
</table>

**Data Processing:** Peak areas in the extracted ion chromatograms (EIC) with a m/z tolerance of 15 ppm are used for quantitation. The m/z values extracted are listed below.

<table>
<thead>
<tr>
<th>Impurity</th>
<th>NDMA</th>
<th>NMBA</th>
<th>NDEA</th>
<th>NEIPA</th>
<th>NDIPA</th>
<th>NDBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z extracted</td>
<td>75.0553</td>
<td>145.0619</td>
<td>75.0553</td>
<td>103.0866</td>
<td>117.1022</td>
<td>131.1179</td>
</tr>
</tbody>
</table>

*Adapted from the LC-HRMS method published by the US FDA: [https://www.fda.gov/media/125478/download](https://www.fda.gov/media/125478/download)
**Mass Analyzer: QOrbitrap (Tandem MS) – High Resolution**

QOrbitrap:
- **High resolution (Selectivity!)**
  - Higher accuracy in mass measurements
- **Higher cost:** not widely available
- **Data acquisition strategies for quantification:**
  (a) Selected Ion Monitoring (SIM):
  - records the abundance of one or more specific m/z values that are characteristic of the compound of interest in an expected retention time window.
  - In this mode, the MS does not spend time scanning the entire mass range, but rapidly changes between m/z values for which characteristic ions are expected: \( \uparrow \) sensitivity

(b) Parallel Reaction Monitoring (PRM)
- quadrupole selects the precursor ion (selection window is usually m/z\( \leq 2 \)); the precursor ion is fragmented in the collision cell; Orbitrap scans all product ions with high resolution and high accuracy.
- \( \uparrow \) sensitivity and selectivity
- Since PRM does not require prior selection of transitions is relatively easier to build a PRM assay
Quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans by HPLC-APCI-QqQ

Diluent: 1% formic acid in water

Internal standard solution: 10 µg/mL each of NDMA-d6 and NMBA-d3, 1 µg/mL each of NDEA-d10 and NDBA-d18 in water

Standard stock solution: 10 ng/mL each of USP Nitrosamine RS (NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA) in methanol

Standard solutions: Prepare Standard solutions at the concentration levels (L#) given in Table 13.
0.02 – 1.35 ppm NDMA, NDIPA, NEIPA, NMBA, and NDBA
0.01 – 0.89 ppm NDEA

Sample solution: Transfer about 80 mg of the drug substance into a 2-mL lidded centrifuge tube. Add 1188 µL of Diluent and 12 µL of the Internal standard solution. Vortex at 2500 rpm for 20 min (except for losartan potassium, which should be vortexed NMT 5 min). Centrifuge at about 10,000 rpm for 10 min, and filter into a vial using a hydrophilic polytetrafluoroethylene (PTFE) filter of 0.45-µm pore size.

Chromatographic system:
Mode: LC
Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in methanol
Column: 3.0-mm × 15-cm; 2.7-µm packing L1

Chromatographic system:
Temperatures
Autosampler: 18°C
Column: 60°C
Flow rate: 0.5 mL/min
Flow rate to ion source: 0.5 mL/min
Injection volume: 20 µL

Detector: MS/MS (triple quadrupole mass spectrometer)
MS conditions:
Ionization: Atmospheric pressure chemical ionization (APCI)
Scan Settings:

<table>
<thead>
<tr>
<th>Impurity</th>
<th>NDMA</th>
<th>NDMA-d6</th>
<th>NDEA</th>
<th>...</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acquisition mode</td>
<td>MRM</td>
<td>MRM</td>
<td>MRM</td>
<td>...</td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>...</td>
</tr>
<tr>
<td>MRM-1</td>
<td>m/z 75→43</td>
<td>m/z 81.2→46</td>
<td>m/z 103.1→75.1</td>
<td>...</td>
</tr>
<tr>
<td>MRM-2</td>
<td>m/z 75→44.1</td>
<td>m/z 81.2→64.1</td>
<td>m/z 103.1→47.1</td>
<td>...</td>
</tr>
</tbody>
</table>

System suitability requirements
Generate the response versus concentration standard curve for each nitrosamine impurity under test using the corresponding Standard solutions and perform the linear regression analysis.
Correlation coefficient: NLT 0.99
y-Intercept: NMT 25%, Standard solution L4
**MS Analyzer: QqQ (Tandem MS) - Low Resolution**

**SELECTED REACTION MONITORING (SRM)**

- **ANALYZER 1:** PRECURSOR ION SELECTION
- **COLLISION CELL:** FRAGMENTATION
- **ANALYZER 2:** PRODUCT ION SELECTION/MONITORING

**QqQ:**
- **Low resolving power**
  - Lower ability to distinguish between two peaks at \( m/z \) values differing by a small amount
  - Lower accuracy in mass measurements
- **Lower cost:** triple quadrupole MS platform is more widely available than the LC-HRMS platform
- **Data acquisition modes:**
  - (a) Selected Reaction Monitoring (SRM):
    - \( \uparrow \) selectivity
      - Less interference of co-eluting compounds and matrix
      - Works like a double mass filter which drastically reduces noise and increases selectivity
    - \( \uparrow \) sensitivity:
      - Better Signal-to-Noise ratio (S/N) allowing quantitation with lower limits of quantitation
      - Wider linear range of quantitation
  - (b) Multiple Reaction Monitoring (MRM):
    - Multiple SRM transitions are measured within the same experiment
    - \( \uparrow \) Selectivity: allows additional selectivity by monitoring the chromatographic coelution of multiple transitions for a given analyte.
Challenges and Recommendations

- **Sample Preparation – Dispersion in Extraction Solvent**
  - The sartans are insoluble in the diluent (1% formic acid in water).
  - The sartans must be fully dispersed for efficient extraction.
  - Clumps or dry spots will cause artificially lower quantification results.
  - Using larger vessels allows for more of the sartans to deposit on the walls, limiting the interaction with solvent.
Sample Preparation – Extraction Efficiency Considerations

- Drug substance is **soluble** in extraction solvent.
  - No extraction efficiency issues.
  - Must prevent the API from entering the MS by diverting LC flow to waste during elution.

- Drug substance is **somewhat soluble** in extraction solvent.
  - Extraction time and mixing are critical for proper extraction.
  - Accuracy spike experiments will be lower than target if the nitrosamines are adsorbing onto the drug substance.

- Drug substance is **insoluble** in extraction solvent.
  - Caution must be exercised due to the high potential for nitrosamines to adsorb onto the API yielding lower quantification results. This may be corrected by use of an internal standard and appropriate equilibration. Inefficient extraction will affect LOD/LOQ.

- Extraction efficiency issues? Use of different calibration procedures (Internal standards/ Matrix-matched calibration) can minimize issues?
Sample Preparation – The Matrix Precipitation Strategy

- Dissolve the drug substance in an appropriate solvent to achieve the desired high concentration, then add an “anti-solvent” to precipitate the drug substances.

- Advantages of matrix precipitation:
  - Full dissolution of the drug substance guarantees nitrosamines are transferred into solution.
  - Precipitation of the drug substance prevents potential contamination of or damage to the mass spectrometer, since it is removed from the sample solutions.
  - Can be more reproducible and less time-consuming than extraction using a solvent in which the drug substance is somewhat soluble or insoluble.

- Sample preparation in this manner must be validated before use.

Use of Internal Standard

- The analyte-to-internal standard response ratio can compensate the matrix effect and ion suppression during analysis providing for a more accurate and precise method.

- ISTD can be used:
  - during sample preparation (account for extraction AND ionization efficiency issues)
  - or prior to sample injection into the LC–MS or GC-MS (account for ionization efficiency issues due to matrix effects)

- ISTD must have ionization properties and retention time similar to the analyte:
  - isotopically labelled compounds
  - structural analogue or
  - another compound that is similar to the analyte under investigation.
Challenges and Recommendations

- **Use of Internal Standard**

  - **Isotopically labelled compounds (IL-STD):**
    - IL-STD will behave almost identically to the analyte during sample preparation, chromatographic separation and MS ionization.
    - Same degree of ion suppression* or enhancement will be observed for the target analyte and its isotopically labeled analogue: the ratio of the two signals should not be affected, and correct quantification can still be achieved.

  - **Challenges for using IL-STD:**
    - high cost and difficult to obtain and/or synthesis (often unavailable)
    - lack of confidence in the isotopic purity and integrity

**USP GC <1469>: Analytical Procedures**

- **Procedure 2:** GC-MS/MS (Headspace) GC-EI-QqQ, ISTD: NDMA-d6
- **Procedure 3:** HPLC-APCI-QqQ
  ISTD: NDMA-d6, NMBA-d3, NDEA-D10
  NDBA-D18
- **Procedure 4:** GC-MS/MS (Direct injection) GC-EI-QqQ
  ISTD: NDMA-13C2-d6

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Heavy Isotope Effect on Chromatography*  


Fig. 5. Illustration of when a standard and internal standard are affected by different levels of ion suppression. This shows a 32% difference in signal response over a 9 second period of analysis.
GC <1469> Procedure 2: (HS) GC-MS/MS (QqQ)

Headspace (HS) GC-MS/MS (QqQ) - Quantitation of NDMA, NDEA, NDIPA, and NEIPA in selected sartans.

Sample solution: 200 ± 10 mg of DS and 100 mg of imidazole in a headspace vial. Add 1.0 mL of internal standard solution (0.016 µg/mL of NDMA-d6 in methanol) and 1.0 mL of acetonitrile. Apply the stopper, cap and crimp tightly.

Chromatographic conditions:
Mode: GC
Injector: Headspace
Injection type: Split (Split ratio, 1:1 or 1:3)
[Note—Split ratio can be modified to optimize sensitivity.]
Detector: MS/MS (QqQ mass detector)
Column: 0.32-mm × 30-m fused-silica coated with a 1.0-µm layer of phase G16

Column temperature:

<table>
<thead>
<tr>
<th>Initial Temperature (°C)</th>
<th>Temperature Ramp (°C/min)</th>
<th>Final Temp. (°C)</th>
<th>Hold Time at Final Temp. (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>0</td>
<td>45</td>
<td>3</td>
</tr>
<tr>
<td>45</td>
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<td>-</td>
</tr>
<tr>
<td>190</td>
<td>40</td>
<td>240</td>
<td>10</td>
</tr>
</tbody>
</table>

Carrier gas: Helium
Gas Flow: Constant flow at 1.8 mL/min (adjustment and verification are necessary for other carrier gases)
Purge Flow: 3.0 mL/min or default value

MS conditions:
Ionization: Electron Impact
Scan Settings:

<table>
<thead>
<tr>
<th>Impurity</th>
<th>NMDA</th>
<th>NDMA-d6</th>
<th>NDEA</th>
<th>NEIPA</th>
<th>NDIPA</th>
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<tbody>
<tr>
<td>Acquisition mode</td>
<td>MRM</td>
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<td>MRM</td>
<td>MRM</td>
<td>MRM</td>
</tr>
<tr>
<td>MRM 1</td>
<td>m/z 74 → 44</td>
<td>m/z 80 → 50</td>
<td>m/z 102 → 85.1</td>
<td>m/z 116 → 99.1</td>
<td>m/z 130.0 → 42</td>
</tr>
<tr>
<td>MRM 2</td>
<td>m/z 74 → 42</td>
<td>m/z 102 → 56.1</td>
<td>m/z 99.0 → 44.1</td>
<td>m/z 130.0 → 43.1</td>
<td></td>
</tr>
</tbody>
</table>

System suitability:
Suitability requirements
Relative standard deviation: NMT 20.0% for the ratios of the impurity standard peak response to the internal standard peak response from six replicate injections, Standard solution
Signal-to-noise ratio: NLT 10 for each nitrosamine,
Sensitivity solution Blank: No interfering peaks from the blank

Analysis
Standard solution and Sample solution Calculate the concentration (ppm) of each specified nitrosamine impurity in the portion of Drug Substance taken: Result = (1/W) x (RU/ RST) x CST
Quantitation of NDMA, NDEA, NDIPA, NEIPA, and NDBA in selected sartans by GC-MS/MS (triple-quad)

Sample solution: Transfer 500 mg of the drug substance into a disposable 10- to 15-mL glass centrifuge tube. Add 5.0 mL of the Internal standard solution (50 ng/mL of NDMA:13C2-d6 in methylene chloride). Cap the tube. Vortex the sample for 1 min, and then place in the centrifuge. Centrifuge the sample at 4000 rpm for 2.5 min. Transfer 2 mL of the bottom methylene chloride layer to a 5-mL syringe fitted with a 0.45-µm nylon filter. Filter 1 mL of sample extract into a 2-mL GC autosampler vial and cap.

Chromatographic conditions:
Mode: GC
Injector: Split/splitless
Injection type: Splitless with purge, Purge time: 0.5 min
Column: 0.25-mm x 30-m; fused-silica coated with a 1.0-µm layer of phase G16
Carrier gas: Helium, Flow rate: Constant flow at 1.0 mL/min
Injection volume: 2 µL
Temp. Injector: 250°
Temp. Transfer line to MS detector: 220°
Temp. Ionization source: 250°
Column: See Table 17.

Detector: MS/MS (triple quadrupole mass spectrometer)
MS conditions
Ionization: Electron impact
Scan Settings:

<table>
<thead>
<tr>
<th>Impurity</th>
<th>NMDA</th>
<th>NDMA-C13d6</th>
<th>NDEA</th>
<th>NEIPA</th>
<th>NDIPA</th>
<th>NDBA</th>
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<tr>
<td>Acquisition mode</td>
<td>MRM</td>
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<td>MRM</td>
<td>MRM</td>
<td>MRM</td>
</tr>
<tr>
<td>MRM 1</td>
<td>m/z 74 → 44</td>
<td>m/z 82 → 48</td>
<td>m/z 102 → 85.1</td>
<td>m/z 116 → 99</td>
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<td>m/z 158 → 99</td>
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<tr>
<td>MRM 2</td>
<td>m/z 74 → 42</td>
<td>m/z 102 → 56</td>
<td>m/z 99.0 → 44.1</td>
<td>m/z 130.0 → 42</td>
<td>m/z 84 → 56</td>
<td></td>
</tr>
</tbody>
</table>

System suitability Samples: Generate the response versus concentration standard curve for each nitrosamine impurity under test using the corresponding Standard solutions and perform the linear regression analysis.

Suitability requirements
Correlation coefficient: NLT 0.98
Signal-to-noise: NLT 10 for the impurity peak, Standard solution Cal 2
### Summary of Factors Impacting Sensitivity & Selectivity

#### Selectivity

**Mass Analyzer**

**Low Resolution MS**
- Poor accuracy in mass measurement may lead to false detection of nitrosamines
- Multiple Reaction Monitoring (MRM) will enhance selectivity
- Chromatographic method with good resolution between target compounds and adjacent peaks!

**High Resolution MS**
- High accuracy in mass measurement
- QOrbitrap hybrid systems: Parallel Reaction Monitoring mode (PRM) QOrbitrap MS systems: provides greatly increased selectivity and confidence compounds identification
- QToF: Multiple Reaction Monitoring

**Data Processing**
- Targeted data extraction strategy = Extract the ion Chromatogram for the specific ion (m/z)
- LC-HRMS: Use a suitable mass tolerance window to obtain the EICs (not so broad window in ppm/amu)

#### Sensitivity

**Mass Analyzer**

**QqQ**
- Improve analysis sensitivity by acquiring data in Multiple Reaction Monitoring (MRM) mode allowing quantitation with lower limits of quantitation

**High Resolution MS**
- Improve analysis sensitivity by acquiring data in: PRM/SIM (eg.: QOrbitrap) / MRM (QToF)

**Ionization Source**

- **ESI**
  - Majority of nitrosamines are not basic - lower ionization efficiency compared to APCI, exception: NMBA (acidic group) great ionization efficiency in ESI(-) mode, MNP/CPNP: ESI+
  - Less tolerant to Matrix effects (ME). Is ME impacting on ionization efficiency?

- **APCI**
  - Higher ionization efficiency for SOME nitrosamines when compared to ESI (exception: NMBA in ESI- mode and with basic moiety:MNP/CPNP)
  - More tolerant to matrix effect

**Chromatographic method: may improve ionization efficiency**

#### Sensitivity and Accuracy

**Sample Preparation Protocol**

- Matrix effects? Can we improve sample preparation to reduce matrix effects? Are they being totally extracted from matrix?
  - Selective extraction x total dissolution extraction
  - Concentration step using SPE after selective extraction?
- Good repeatability? Good recovery?

#### Accuracy/Precision

- GC-MS/MS: Inlet: high temperature - Degradation of API? Formation of nitrosamines in-situ (inlet/headspace)
- LC-MS/MS: broader range of applicability; preferred if the API may degrade due to high temperature (APCI: use ↓ temperature)

**Use of internal standard:**
- Compensate for extraction issues: Account for possible losses during workup or due to the thermal instability inherent to several N-nitrosamines
- Compensate for ionization efficiency due to matrix effect
- Minimize repeatability issues
Thank You

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https://www.usp.org/chemical-medicines/nitrosamine-impurities