Rapid Analysis of Genotoxic Nitrosamines by HPLC-MS/MS

Lihai Guo[1], Zhimin Long[1], Xiangyang Leng[1], and James Turner[2]

[1]SCIEX APAC Applications Support Center, Beijing, China

[2]Phenomenex LTD, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire, SK10BN, United Kingdom

Introduction

Potential genotoxic impurities (PGIs) have been a subject of concern for the pharmaceutical industry for the last decade. PGIs are small, reactive molecules, which can react with DNA/RNA causing damage and the potential risk of cancer. These molecules can, however, be required for successful chemical synthesis and it is not always possible to eliminate them from synthetic pathways.

To prevent large-scale recall incidents, such as those involving nelfinavir mesylate (Viracept®) in 2007, or valsartan (Diovan®) in 2018, regulatory agencies including the U.S. Food & Drug Administration (FDA) and the European Medicines Agency (EMA), have issued guidelines on the allowable limits of genotoxic impurities in pharmaceutical products to ensure their safety. These guidelines state exposure to genotoxic impurities (GTIs) must be below the threshold of toxicological concern (TTC) of 1.5 µg per day.

Nitrosamines are known to be carcinogenic and are often found in processed foods such as cured meats although the most prevalent exposure is the smoking of tobacco products. They have the potential to be intermediates in organic synthesis and due to their potent genotoxicity, it has become a requirement to accurately quantitate this group of compounds in pharmaceuticals during drug development and manufacturing. Due to the high polarity and low molecular weight of nitrosamines, developing robust LC-MS methodology is a challenge. The C18 columns conventionally used offer little interaction and retention for molecules of this type. When considering the chromatography of polar compounds, it is also important to consider the ability of the column to retain the analytes away from any potential matrix interferences. This will allow for effective ionization without the complication of matrix generated suppression. In addition, the ability to provide peaks which are not impacted by secondary interactions is strongly required. Peak tailing resulting from secondary interactions will impair peak height and consequently reduce sensitivity and impact accurate quantitation. In this study, a Kinetex® high efficiency core-shell column was utilized, ensuring that analytes are transferred to the MS in narrow bands to enable high sensitivity. The stationary phase is a highly inert pentafluorophenyl phase, offering excellent retention of polar analytes with good peak shape.

In this technical note, a method for the analysis of eight nitrosamine compounds, with a lower limit of quantitation (LLOQ) of 0.05 µg/g in a drug product corresponding to significantly less than the TTC for most pharmaceutical drug products is described.

Methods

The contents of a capsule of the drug product, containing 80 mg of the active pharmaceutical ingredient (API), were dissolved in 40 mL of Methanol/Water (1:1, v/v) and diluted to give a final concentration of 2 mg/mL The mixture was vortexed for 1 minute followed by sonication in an ultrasonic bath for 20 minutes. The solution was allowed to settle for 1 hour at room temperature, then the supernatant was transferred and centrifuged for 5 minutes at 14,000 RPM. Aliquots of the solution were transferred to HPLC vials for analysis by LC-MS/MS (To minimize any impact from micro particulates on the HPLC column and system, we recommend filtering the sample with PTFE or RC syringe filters). Standards were prepared by diluting the stock standard solution for each of the eight compounds with Methanol/Water (1:1, v/v) to yield concentrations of 0.1, 0.2, 1.0, 2.0, 5.0, 10, and 20 ng/mL. The 0.1 ng/mL in the extract corresponds to 0.05 µg/g in the tablet.

LC-MS/MS Conditions

Column: Kinetex 2.6 µm F5
Dimension: 50 x 2.1 mm
Part Number: 00B-4723-AN
Mobile Phase: A: 0.1% Formic acid in Water
B: Methanol

Gradient:

Time (min)	% B
0	15
0.5	15
5.0	95
5.1	15
6.5	15

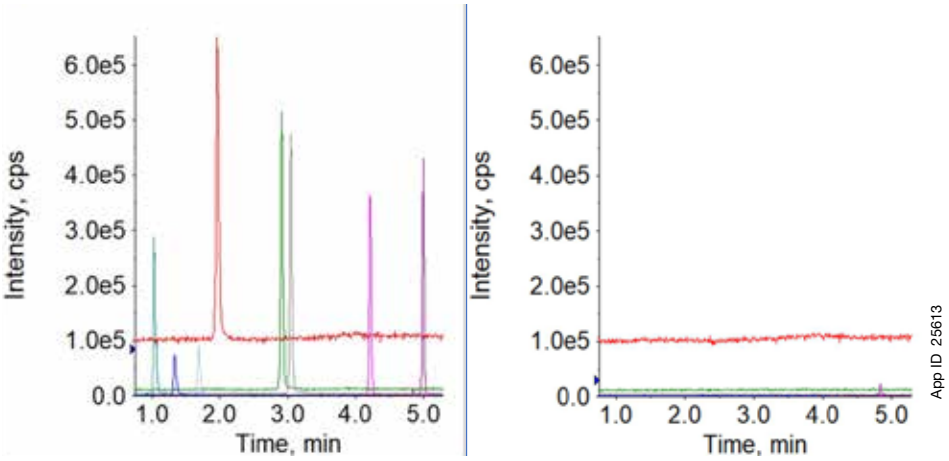
Flow Rate: 0.5 mL/min
Temperature: 40 °C
Injection Volume: 10 µL
HPLC System: SCIEX® ExionLC™ AD HPLC
Mass Spectrometer: SCIEX Triple Quad™ 4500
Scan Type: MRM
Nebulizer Current: 3 µA
Curtain Gas: 30 psi
GS1: 35 psi
CAD: 8
Source Temperature: 350 °C

Table 1.
Compound Retention Times and Dependent Mass Spectrometer Values.

Compound	Q1	Q2	DP	CE	RT (min)
N-Nitrosodiethanolamine (NDELA)	135.1	104.1	40	8	1.03
N-Nitrosodimethylamine (NDMA)	75.1	58.1	40	16	1.34
N-Nitrosomorpholine (NMOR)	117.1	87.1	40	23	1.69
N-Nitrosopyrrolidine (NPYR)	101.1	55.1	40	21	1.97
N-Nitrosodiethylamine (NDEA)	103.1	75.0	40	15	2.91
N-Nitrosopiperidine (NPIP)	115.1	69.0	40	28	3.05
N-Nitrosodi-n-propylamine (NDPA)	131.1	89.1	40	20	4.22
N-Nitrosodi-n-butylamine (NDBA)	159.1	103.1	40	21	5.00

Entrance and exit potentials were set to 10 for all MRM transitions.

Figure 1.
Chromatogram of Eight Nitrosamines (left) and Blank (right)



Results

The chromatogram in **Figure 1** illustrates that all eight nitrosamines were retained and are well separated. The resolving power of the Kinetex® core-shell particle ensured that peaks eluted with very narrow peak widths; this enhanced sensitivity in the detector as the peaks appeared taller, lifting them above any noise from the detection system. The selectivity of the pentafluorophenyl (F5) chemistry provides peaks with good peak shape which are well resolved from each other. The polar nature of the phase provided good interactions with the polar nitrosamines, which results in increased retention. This in turn allowed for elution in a higher percentage of organic solvent, which is advantageous in the ion-source, resulting in better sensitivity.

Specificity

In order that standards of an appropriate concentration were produced the following equation was used. The methodology used allowed for even greater sensitivity, providing greater confidence in the results achievable when using this method.

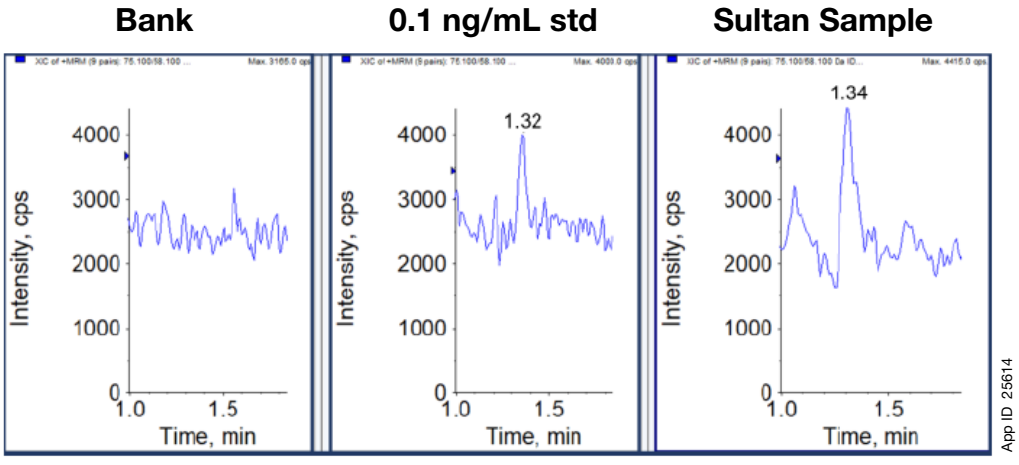
$$\text{Required LLOQ} < \frac{1.5\ \mu g\ \text{allowed nitrosamine}}{0.080\ g\ \text{daily dose}}$$

Required LLOQ <18.75 µg/g

A valsartan standard was dissolved to 2 mg/mL and spiked with working solutions of the eight nitrosamines at 0.5 ng/mL, 5.0 ng/mL, and 15 ng/mL to make solutions equivalent to 0.25, 2.5, and 7.5 µg/g API samples. An additional sample was prepared by dissolving a Sultan (diphenhydramine) capsule to a 2.0 mg/mL concentration. The samples were prepared as described and analyzed by LC-MS/MS. Responses for all nitrosamine spiked samples were linear, with no interferences in the blank. The Sultan sample showed a response for n-nitrosodimethylamine that quantitated to 0.2 ng/mL (shown in **Figure 2**), the equivalent of 0.1 µg/g in the drug product, which is below the threshold of toxicological concern.

None of the other nitrosamine compounds were observed in the sample or in the blank

Figure 2.
Sultan Capsule Analysis

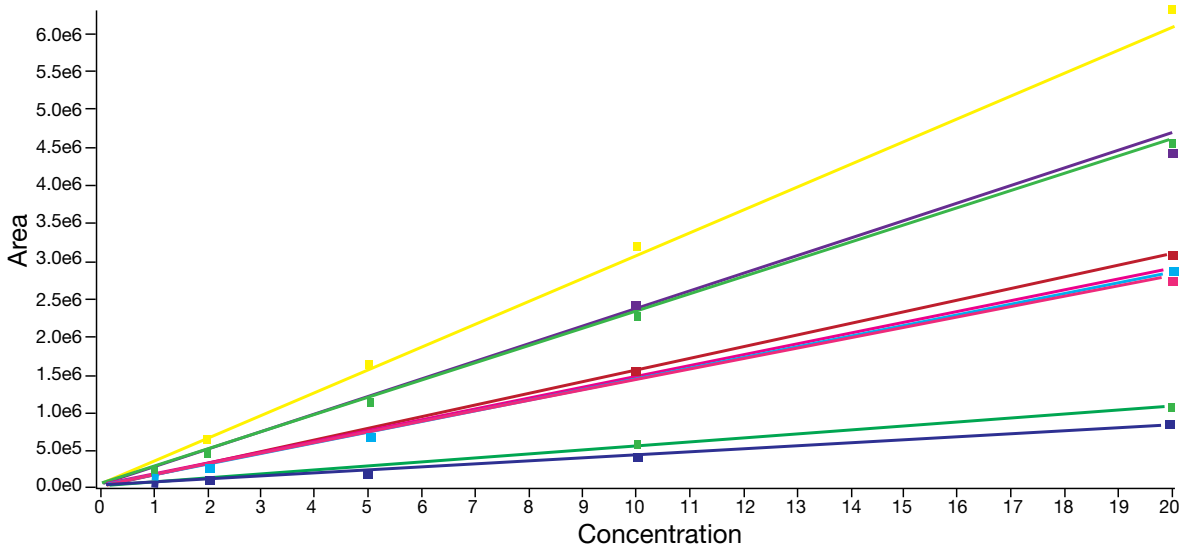


Linear Dynamic Range

Working standard solutions were prepared by serial dilution of the primary stock solution in the sample dilution buffer to 0.1, 0.2, 1.0, 2.0, 5.0, 10, and 20 ng/mL.

As seen in **Figure 3**, the response for all eight compounds is linear over the calibration range from 0.1 to 20 ng/mL with correlation coefficient >0.998.

Figure 3.
Calibration Curves for Eight Nitrosamine Compounds



Recovery and Reproducibility

Recovery and reproducibility were evaluated for the QC samples described in the Specificity section. The recovery of all eight compounds in the three QC levels fell between 89.5% and 112.0%, with %RSD (n=6 at each level) between 0.61% and 4.42%. Data is summarized in **Table 3**. The %RSD (n=6) was evaluated at the LLOQ (0.1 ng/mL) and for the eight compounds which fell between 1.53% and 2.48%, is summarized in **Table 4**.

Table 3.
QC Recovery and Reproducibility

	0.5 ng/mL		5.0 ng/mL		15.0 ng/mL	
Compound	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
NDMA	92.9	2.34	90.4	2.68	89.5	1.44
NDEA	104.4	2.31	108.5	1.22	109.8	1.06
NDPA	100.0	2.67	104.6	2.91	104.5	2.78
NDBA	102.2	2.37	99.9	2,34	98.4	2.26
NPYR	106.2	2.68	111.1	1.84	112.0	0.69
NPIP	101.7	3.49	105.9	2.03	106.0	0.61
NMOR	96.2	3.75	99.2	3.33	100.1	1.31
NDELA	100.6	4.42	92.3	4.12	95.9	1.37

Table 4.
LLOQ Reproducibility

Compound	%RSD (n=6)	Compound	%RSD (n=6)
NDMA	2.11	NPYR	1.67
NDEA	1.53	NPIP	1.91
NDPA	2.48	NMOR	1.78
NDBA	1.62	NDELA	1.62

Summary

In this technical note, an LC-MS/MS method has been described for the quantitation of eight nitrosamine compounds in final drug product using a Kinetex® 2.6 µm F5 column. The results demonstrate good specificity due to the highly polar nature of the stationary phase giving selective interactions with the analytes, with a linear dynamic range from 0.1 to 20 ng/mL. The LLOQ of 0.1 ng/mL is equivalent to 0.05 µg/g of impurity in the drug product, which is lower the threshold of toxicological concern defined by the EMEA and USFDA.

APPLICATIONS

Ordering Information
Kinetex® Core-Shell LC Columns

2.6 µm Minibore Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
F5	00A-4723-AN	00B-4723-AN	00D-4723-AN	00F-4723-AN	AJ0-9322 for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
F5	00B-4723-Y0	00D-4723-Y0	00F-4723-Y0	AJ0-9321 for 3.0 mm ID

2.6 µm Analytical Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 4.6	100 x 4.6	150 x 4.6	3/pk
F5	00B-4723-E0	00D-4723-E0	00F-4723-E0	AJ0-9320 for 4.6 mm ID

1.7 µm Minibore Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
F5	00B-4722-AN	00D-4722-AN	00F-4722-AN	AJ0-9322 for 2.1 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

BE-HAPPY™ guarantee

Your happiness is our mission. Take 45 days to try our products. If you are not happy, we'll make it right.
www.phenomenex.com/behappy

Australia
t: +61 (0)2-9428-6444
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
anfrage@phenomwenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
info@phenomenex.com

China
t: +86 400-606-8099
cninfo@phenomenex.com

Denmark
t: +45 4824 8048
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
anfrage@phenomenex.com

India
t: +91 (0)40-3012 2400
indiainfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
nordicinfo@phenomenex.com

Portugal
t: +351 221 450 488
ptinfo@phenomenex.com

Singapore
t: +65 800-852-3944
sginfo@phenomenex.com

Spain
t: +34 91-413-8613
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
nordicinfo@phenomenex.com

Switzerland
t: +41 (0)61 692 20 20
swissinfo@phenomenex.com

Taiwan
t: +886 (0) 0801-49-1246
twinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
info@phenomenex.com

All other countries
Corporate Office USA
t: +1 (310) 212-0555
info@phenomenex.com

www.phenomenex.com
Phenomenex products are available worldwide. For the distributor in your country/region, contact Phenomenex USA, International Department at international@phenomenex.com

TN7020819_W