Application Note

Pharmaceutical

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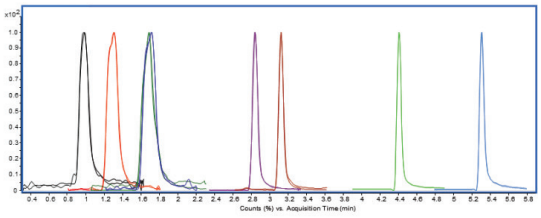
Determination of Nitrosamines in Active Pharmaceutical Ingredients

High flexibility and performance at lowest cost using the Agilent 1260 Infinity II Prime LC with the Agilent Ultivo LC/TQ for LC/MS analysis

Suitable for Agilent
1260 Infinity III LC



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Abstract

This application note describes the analysis of multiple nitrosamine impurities in active pharmaceutical ingredients (API) with high sensitivity to quantify the genotoxic impurities (GTI) nitrosamines as required in the daily dose of the pharmaceutical. The examples indicate the limit of detection (LOD) and limit of quantification (LOQ) of typical nitrosamines as well as the accuracy and precision at a low ng/mL level. Because the Agilent 1260 Infinity II Prime LC has a pressure range of up to 800 bar, modern sub-2 µm columns can be used in combination with lower column temperatures and higher flow rates.

Introduction

During the synthesis of pharmaceutical drug compounds, impurities are generated under reaction or from storage conditions of the involved compounds. These impurities must be identified and monitored during the production process in the API and in the final drug product. Especially important are the GTIs because of their high potential to damage DNA and lead to the generation of tumors. To control the amount of GTIs, the U.S. Food and Drug Administration (FDA) and the European Medicine Agency (EMA) released guidelines for the maximum intake of GTI with the daily dose of a drug in dependence of the duration of application. According to these guidelines, the exposure to an individual genotoxic impurity must be below 1.5 µg/day for an application time longer than ten years.[1] In 2018, the drug Diovan was withdrawn from the market due to an increased amount of the nitrosamine impurity N-nitrosodimethylamine (NDMA) in the included API Valsartan.[2,3]

This application note describes the use of the Agilent 1260 Infinity II Prime LC in combination with the Agilent Ultivo LC/TQ for the sensitive determination of nitrosamines in pharmaceutical compounds. The HPLC system is capable to deliver enough pressure to utilize modern sub-2 µm columns by highest quaternary pump performance. This enables increased flexibility at a lower cost of ownership.

Experimental

Instrument

- Agilent 1260 Infinity II Flexible Pump (G7104C)
- Agilent 1260 Infinity II Multisampler (G7167A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent Ultivo LC/TQ (G6465B)

Software

- Agilent MassHunter workstation
  - LC/MS data acquisition for Ultivo LC/TQ, V1.1
  - Optimizer for LC/TQ, V1.1
  - Source Optimizer for LC/TQ, V1.1
- Agilent MassHunter qualitative software, V10.0
- Agilent MassHunter quantitative software, V10.0

Column

Agilent ZORBAX Eclipse Plus C18, RRHD, 2.1 × 100 mm, 1.8 µm

Standard

2,000 µg/mL, each nitrosamine in MeOH

Calibration

LC/MS standard mixture was diluted to a stock solution of 1 mg/L in methanol Calibration curves were created for 1, 2, 10, 20, 100, and 200 ng/mL.

Sample preparation

80 mg of Valsartan was dissolved in 20 mL of methanol according to the recommended daily dose, then spiked with the nitrosamine mix. The final concentration of each nitrosamine was 50 ng/mL, according to 1 µg per daily dose.

Solvent and chemicals

- All solvents were purchased from Merck, Germany
- Chemicals were purchased from VWR, Germany
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with a LC-Pak polisher and a 0.22 µm membrane pointofuse cartridge (Millipak)

HPLC Method	
Flow Rate	0.35 mL/min
Solvent A	Water + 0.1% formic acid
Solvent B	Methanol + 0.1% formic acid
Solvent C	AcN + 0.1% formic acid
Gradient 1	0 min – 15% B, 4 min – 95% B, 6 min – 95% B Stop time: 6 min Post time: 3 min
Gradient 2	0 min – 15% B, 4 min – 95% B, 6 min – 95% B, 6.1 min – 95% C Stop time: 9 min Post time: 3 min
Injection Volume	3 µL
Needle Wash	3 second, methanol
MS Method	
Agilent APCI source	
Gas Temperature	220 °C
Gas Flow	4 L/min
Vaporizer Temperature	325 °C
Nebulizer Pressure	35 psi
Capillary Voltage	2500 V, positive
Corona Current	5 µA
Time Filter	0.03 min
MRM and DMRM Conditions	see Table 1

Table 1. MRM and DMRM conditions (R.T. window width 1 minute) for the measurement of nitrosamines.

Compound	Formula	Mass	*m/z*	Retention Time (min)	Fragmentor (V)	Fragment Ion	Collison Energy (eV)	Fragment Ion	Collsion Energy (eV)
N-Nitrosodimethylamine (NDMA)	$C_2H_6N_2O$	74.1	75.1	0.987	112	43	16	47	8
Nitrosomorpholine (NMor)	$C_4H_8N_2O_2$	116.1	117.1	1.304	102	87.1	12	45	20
N-Nitrosomethylethylamine (NMEA)	$C_3H_8N_2O$	88.1	89.1	1.685	97	61	12	43.1	12
1-Nitrosopyrrolidine (NPyr)	$C_4H_8N_2O$	100.1	101.1	1.712	102	55.1	20	41	28
N-Nitrosodiethylamine (NDEA)	$C_4H_{10}N_2O$	102.1	103.1	2.840	83	75	8	47	20
Nitrosopiperidine (NPip)	$C_5H_{10}N_2O$	114.1	115.1	3.122	102	69.1	16	41	24
N-Nitroso-*n*-propylamine (NDPA)	$C_6H_{14}N_2O$	130.1	131.1	4.406	88	43	12	89	8
N-Nitroso-*n*-dibutylamine (NDBA)	$C_8H_{18}N_2O$	158.1	159.2	5.306	88	57	12	103	8

Results and discussion

As stated in the EMA guidelines[1] the allowed intake of an individual nitrosamine together with the daily dose depends on the duration of the medication. In the most stringent case, it is not acceptable to take more than 1.5 µg of the GTI with the daily dose of the drug because of threshold of toxicological concern (TTC). Subsequently, the required sensitivity for the quantitative determination of nitrosamines in APIs depends on the allowed daily intake and the recommended daily dose of the drug. In this work, the Diovan case will be used as an example. The API in Diovan is Valsartan, which is recommended with a daily dose of 80 mg. To stay below the acceptable daily intake of the GTI, the concentration of the GTI in the API should be below 18.75 µg/g. A common extraction procedure is to dissolve the daily dose of the API in 20 mL of solvent, which results in a required LOQ of 75 ng/mL. Calibration curves were created for eight nitrosamine compounds in the range of 1 ng/mL to 200 ng/mL. The chromatogram of the calibration point at 10 ng/mL is shown in Figure 1. To minimize peak broadening of the early-eluting compounds, 15% B was taken as the starting composition of the gradient. The later-eluting sharper peaks were eluted in a steep gradient up to 95% B and a hold at that composition to six minutes of total run time. Only two compounds, NMEA (1.304 minutes), and NPyr (1.685) minutes, coeluted.

For all calibration curves, the linearity was better than 0.999 (Table 2). The LOQ was calculated for the quantifier ion signal at a signal-to-noise ratio (S/N) of 10. Typically, the LOQ was at 1 ng/mL or below. The LOD was calculated for the S/N of 3, and was typically below 0.7 ng/mL. For the determination of retention time and area precision, the calibration point at 10 ng/mL was measured ten times. The retention time RSD was typically ≤0.15% and the area RSD was typically better than 3%.

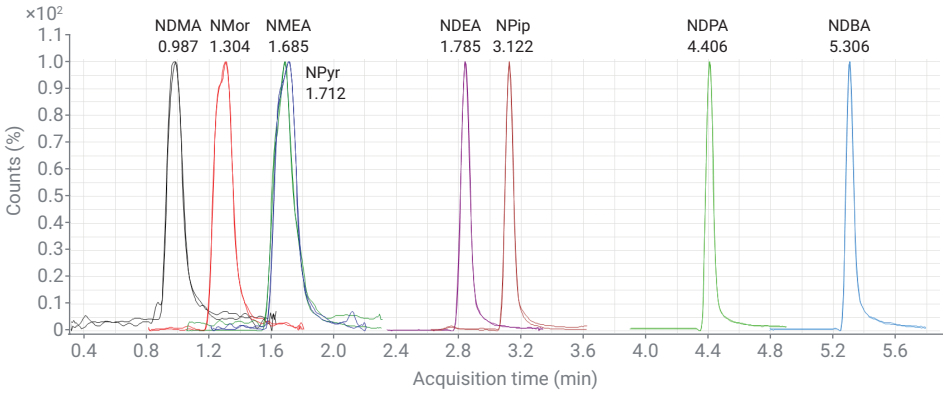

Figure 1. Separation of nine nitrosamines, gradient 1 (see experimental), 10 ng/mL each (normalized).

Table 2. Performance parameters of the measurement of nine nitrosamine compounds.

Compound	Retention Time (min)	RT RSD (%)	Linearity R^2	LOQ (ng/mL)	LOD (ng/mL)	Area RSD (%) (10 ng/mL, n = 10)
N-Nitrosodimethylamine (NDMA)	0.987	0.41	0.9993	5.00	2.00	1.08
Nitrosomorpholine (NMor)	1.304	0.31	0.9990	1.50	0.70	2.84
N-Nitrosomethylethylamine (NMEA)	1.685	0.15	0.9992	0.80	0.30	1.21
1-Nnitrosopyrrolidine (NPyr)	1.712	0.15	0.9992	2.50	0.80	1.78
N-Nitrosodiethylamine (NDEA)	2.840	0.12	0.9992	1.00	0.30	2.84
1-Nitrosopiperidine (NPip)	3.122	0.08	0.9991	1.00	0.40	1.11
N-Nitroso-n-propylamine (NDPA)	4.406	0.01	0.9993	0.40	0.10	2.77
N-Nitroso-n-dibutylamine (NDBA)	5.306	0.08	0.9993	0.25	0.10	3.31

The dynamic MRMs with qualifier and quantifier ion signal for the 10 ng/mL and the 2 ng/mL calibration point for all nitrosamine compounds are shown in Figure 2.

As proof, a solution of the daily dose of 80 mg of Valsartan was dissolved in 20 mL of methanol and spiked with 1 µg daily dose of each GTI nitrosamine. The amount of 1 µg daily dose was chosen to demonstrate the capability for reliable quantification even below the TTC. The amount of 1 µg daily dose in 20 mL led

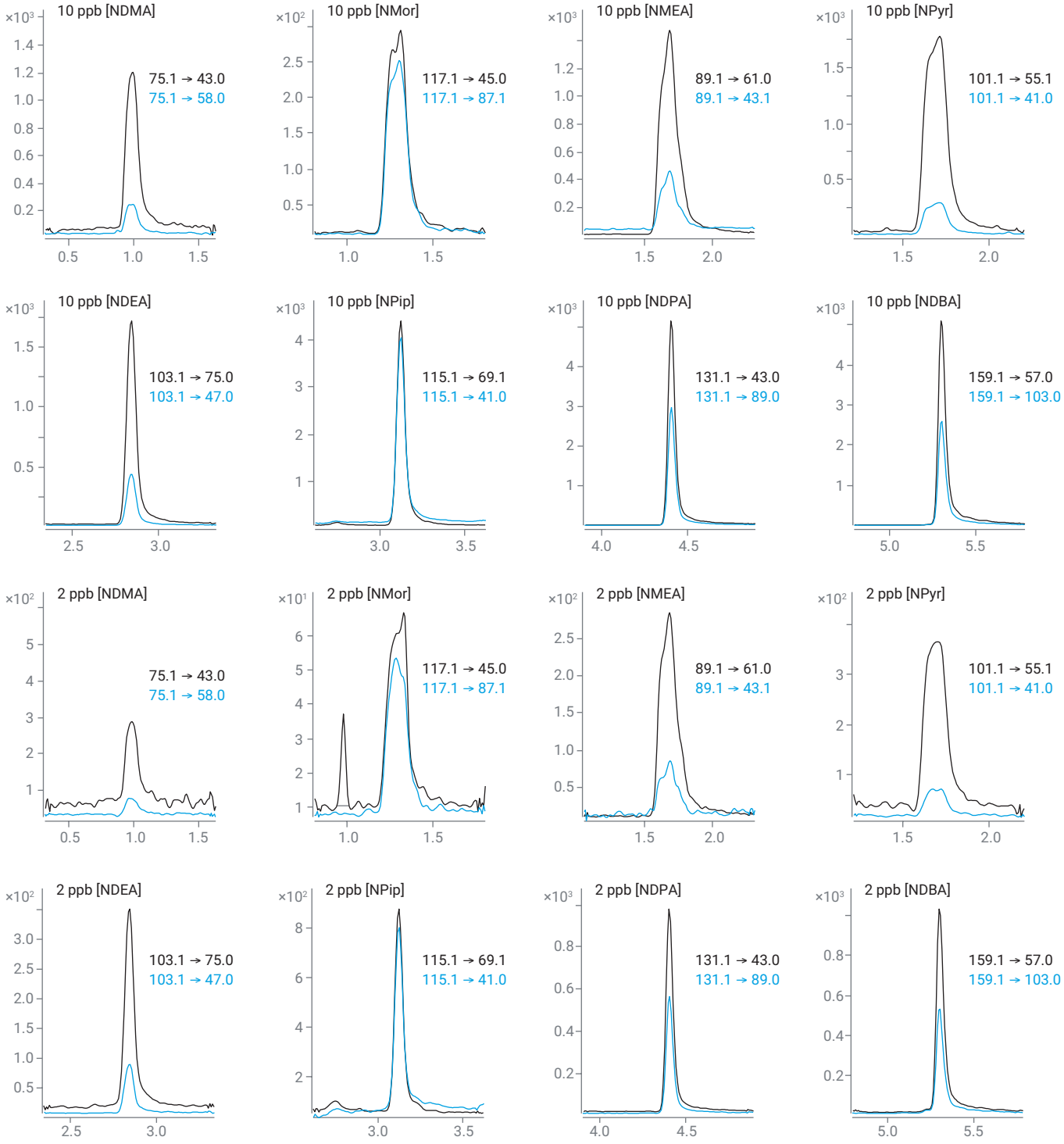

Figure 2. DMRM signals of quantifier and qualifier ions for the 10 ng/mL and the 2 ng/mL calibration points.

to a final concentration of 50 ng/mL. To compare the measurements of the spiked API, a pure dilution of 50 ng/mL nitrosamines in methanol was measured as quality control (Table 3). The pure nitrosamine dilution at the 50 ng/L level showed typical precisions below 1.5% RSD and accuracies between 92 and 104%.

In comparison, the spiked valsartan sample showed precision RSDs typically below 3%. The calculated recoveries were between 93.1 and 99.8% with the exception of NDBA at a recovery of 82.3%. While the matrix effects generally are very low, the decreased recovery of NDBA could be explained by ion suppression in the APCI source due to the coeluting large excess of Valsartan at 4.9 minutes. The retention time of Valsartan was determined in a separate run and measured as TIC (not shown). The large amount of excess Valsartan showed a long tailing in the TIC and requires high organic flushing after the elution of the final analyte compound. For the analysis of the final drug product a column flushing is recommended due to the other substances included in the final dosage form. The cleaning step could be done either by a longer run time with the final solvent concentration of 95% of methanol or by switching to a solvent with a stronger elution power after the elution of all nitrosamine analytes. Since the Agilent 1260 Infinity II Prime LC pump offers the high flexibility of a quaternary pump, it is easy to connect an additional solvent like acetonitrile at channel C for this modification (see experimental, gradient 2).

Table 3. Determination of concentration precision and accuracy of a 50 ng/mL quality control sample for each nitrosamine, and a Valsartan sample spiked with 50 ng/mL of each nitrosamine compound for determination of recovery.

	NDMA		NMor		NMEA		NPyr	
QC Results	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy
Average	51.96	103.92	49.40	95.47	48.74	97.48	46.36	92.73
RSD (%)	2.04		3.25		1.27		1.06	
	NDMA		NMor		NMEA		NPyr	
Sample Results	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy
Average	49.93	99.85	47.26	94.52	47.94	95.87	46.55	93.10
RSD (%)	4.69		4.42		3.08		2.50	
	NDEA		NPip		NDPA		NDBA	
QC Results	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy
Average	50.59	101.18	48.28	96.56	49.36	98.71	48.15	96.30
RSD (%)	1.22		0.71		1.26		1.50	
	NDEA		NPip		NDPA		NDBA	
Sample Results	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy	Final Conc.	Accuracy
Average	49.83	99.67	48.31	96.62	49.61	99.22	41.18	82.36
RSD (%)	2.75		2.08		2.37		2.96	

Conclusion

This application note describes the capability of the 1260 Infinity II Prime LC in combination with the Ultivo LC/TQ to measure genotoxic impurities in pharmaceutical drugs at lowest required levels. The 1260 Infinity II Prime LC is able to deliver solvents at pressures required to operate modern sub-2 µm columns even at lower column temperature and with viscous solvents. The Ultivo LC/TQ provides the highest sensitivity to measure LOQ concentrations of nitrosamines in the single-digit ng/mL range with peak area RSDs below 3%. The 1260 Infinity II Prime LC system in combination with the Ultivo LC/TQ offers a highly sensitive and flexible solution for the pharmaceutical industry for the control of APIs for most critical impurities at a very low cost of ownership.

References

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3. US Food and Drug Adminstration, Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Method for the Determination of Six Nitrosamine Impurities in ARB Drugs, 05/21/2019 (accessed April 2020) https://www.fda.gov/media/125478/download

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