FULL METHOD VALIDATION OF AN AUTOMATED SOLUTION FOR NITROSAMINES IN IRBESARTAN AND METFORMIN BY LC-MS-MS ANALYSIS

APPLICATION NOTE AS-239

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Abstract

This application note follows the preliminary work undertaken at Anatune to address the measurement of a wide range of nitrosamines in drug substance as outlined in Application Note AS234. Here we demonstrate the optimisation and validation of a fully automated solution for the sample preparation and LC-MS/MS analysis of seven target nitrosamines in two classes of drug substance, Irbesartan (angiotensin II receptor antagonist) and Metformin (biguanide analogue antidiabetic).

The inherent benefits of automated sample preparation are clearly demonstrated including:

- High throughput, unattended operation
- Improved data accuracy and precision for greater confidence in results
- Minimal analyst interaction with the sample and potentially harmful chemicals for enhanced health and safety
- Reduced risk of contamination leading to false positives and the resulting costly delays or erroneous product recalls.

This solution will be of interest to pharmaceutical drug substance and drug product manufacturers to support their research, process development and quality control programs. CRO organisations offering outsourced, high throughput, testing will also benefit greatly from the quality and efficiency gains associated with this automated analysis.

INTRODUCTION

Following the discovery of nitrosamines contamination in certain classes of drug products over the past two years, regulators have mandated worldwide stringent reviews of the pharmaceutical industry portfolio, including challenging analytical testing to meet the recently established zero-risk environment requirements.

This application note builds upon preliminary work undertaken at Anatune to address the detection of nitrosamines in drug substance as outlined in Application Note AS234.

Initial evaluation highlighted the significant benefits of a fully automated solution for this analytical challenge, specifically to reduce risks of contamination and the occurrence of false positives. Automation of sample preparation can help significantly in addressing some of the many challenges to be faced in the analysis of nitrosamines thanks to its inherent qualities of convenience, control, and consistency.

Convenience

AS234 described how false positives could be obtained if samples have been manually prepared due to contamination introduced by common lab consumables such as nitrile gloves. Automation delegates tasks to a robot. This is not only valuable from a health, safety, and time perspective, but it can be extremely beneficial in controlling and containing sources of contamination.

In terms of throughput and speed performances, the system was set up as an offline solution, that is samples were prepared in batches and then injected onto the LC-MS-MS. This configuration gives the flexibility of coupling the automated sample preparation with whichever instrumental system might be needed. Cycle time per sample was approximately 15 min.

However, the same solution can be configured online, that is allowing direct injection of each sample as soon as prepared. A function known as "PrepAhead" allows the preparation and injection of every sample promptly, thereby staggering the sample preparation and analysis time in the most efficient way possible. Every sample is injected as soon as preparation is complete.

Since the cycle time per sample is approximately 15 min and so is the instrumental run time, using this option would allow perfect overlap of the preparation and analysis. A visualization of the operation mode of the PrepAhead function in comparison to the standard approach is shown in Figure 1. The PrepAhead option is particularly useful when sample stability is a concern.

Figure 1: *Automation PrepAhead function in comparison to a standard approach*

Control

Automating the sample preparation allows the accurate control of the experimental variables involved such as timing, temperatures, and speeds (e.g. mixing, centrifuging and liquid handling). This control will have a strong effect on the extraction efficiency of the tested workflow.

Consistency

Automation can reduce biases since it will always perform a task in a consistent way. Method consistency will increase robustness and accuracy while minimising analytical variability – figures of merit which determine success or failure of method validation.

Here we describe the optimisation and validation of a solution for the determination of seven target nitrosamines in two drug substances, Irbesartan and Metformin. This solution encompasses fully automated sample preparation and LC-MS/MS analysis for the selected matrices and analytes.

EXPERIMENTAL

Instrumentation

LC-MS/MS: Agilent 1260 Infinity Binary pump coupled to Agilent 6470 QqQ equipped with APCI source.

Automation: GERSTEL Dual Head MPS Robotic/Robotic Pro configured with:

- Anatune CF200 Solvent Safe Robotic centrifuge

- GERSTEL QuickMix
- GERSTEL Filtration Unit with VT40-2mm filters tray
- Ultrasonication bath
- 180 mL Solvent Reservoirs
- VT12-10mL trays and R60-10mL trays
- VT40-2mL trays
- Fast wash
- LC injection valve

Figure 2: *Automated sample prep configuration for the analysis of nitrosamines in selected drug substances* [Link to video]

Materials

Reference material standard solutions in methanol for all target nitrosamines and relative labelled internal standards were obtained from LGC Standards, UK. Pharmaceutical Secondary Standard Certified Reference Materials for both chosen drug substances (Supelco PHR1443 Irbesartan and PHR1084 Metformin Hydrochloride) were purchased from Sigma Aldrich, Merck, UK. Ultra LC grade solvents (Water, Methanol, Formic Acid) were obtained from ROMIL, UK.

Methods

Optimised LC-APCI- MS/MS Method

Column: Reverse Phase
(provided by Crawford Scientific)
Mobile phase:
Water, Methanol, Formic Acid
Injection volume: 100 µL
Flow rate: 0.6 mL/min
Column temperature: 40 ºC
Elution: Gradient

Table 1 and Table 2 summarise details for the ISTDs and target nitrosamines addressed in this study. Figure 3 shows as an example the Extracted Ion Chromatogram for spiked Irbesartan drug substance extract.

Table 1: *ISTDs and target nitrosamines name and abbreviation*

Compound Name	Abbrev
N-nitrosodimethylamine	NDMA
N-nitroso-4-methyl-4-aminobutyric acid	NMBA
N-nitrosodiethylamine	NDEA
N-nitrosoethylisopropylamine	NEIPA
N-nitrosodiisopropylamine	NDIPA
N-nitroso-di-n-propylamine	NDPA
N-nitroso-di-n-butylamine	NDBA
N-nitrosodimethylamine d6 (IS)	NDMA-d6
N-nitrosodiethylamine d10 (IS)	NDEA-d10
N-nitrosodiisopropylamine d14 (IS)	NDIPA-d14

Table 2: *Retention time and MRM transitions for the investigated nitrosamines*

Analyte	Retention Time [min]	MRM Transitions
NDMA	4.520	75.1>43.0 Qual 75.1>58.0
NMBA	5.751	147.1.>44.2 Qual 147.2>87.2
NDEA	7.566	103.1>75.1 Qual 103.1>47.1
NEIPA	8.615	117.1>75.1 Qual 117.1>47.1
NDIPA	9.422	131.1>89.0 Qual 131.1>43.1
NDPA	9.807	131.1>89.0 Qual 131.1>43.1
NDBA	11.228	159.1>57.2 Qual 159.1>41.1
NDMA-d6	4.464	81.1>46.1 Qual 81.1>64.1
NDEA-d10	7.500	113.0>81.0 Qual 113.0>49.0
NDIPA-d14	9.316	145.2>97. Qual 145.2>50.2

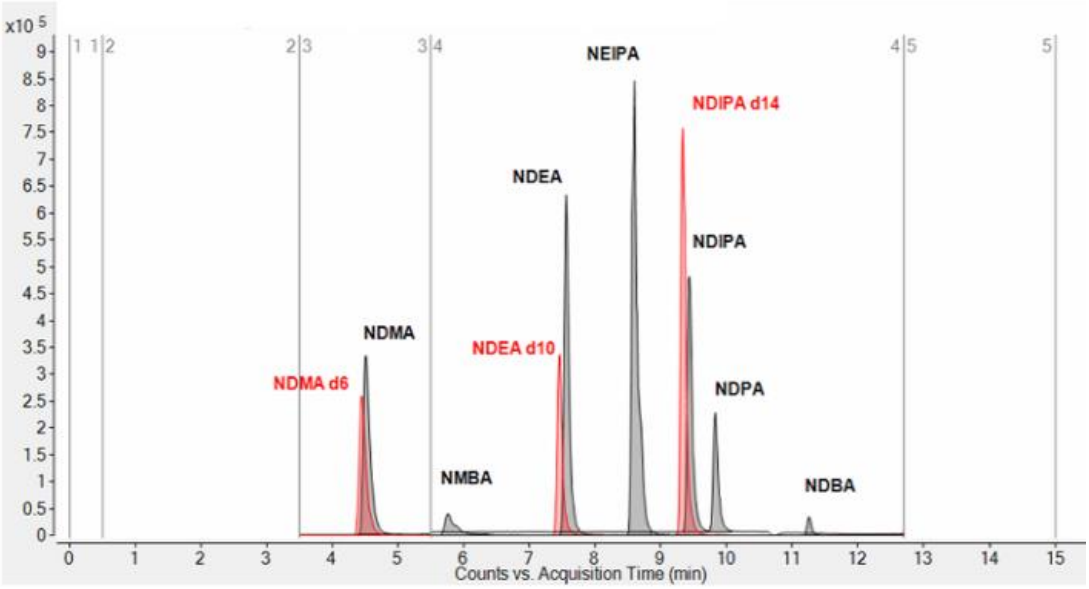

Figure 3: *MRM Extracted Ion Chromatogram for an Irbesartan extract spiked with the seven target nitrosamines at 1250 ng/g in drug substance*

Optimised Automated Sample Preparation

200 mg of CRM were manually weighed and transferred into a 10 mL screw cap glass vial.

Appropriate volume of Ultra LC water was spiked with methanolic solutions of 1 ug/mL ISTD and target nitrosamines and mixed to achieve the chosen final solution volume.

The sample was mixed, sonicated, and centrifuged to separate supernatant from residual excipients. An aliquot of 350 µL of the supernatant was filtered directly into 2mL injection vials.

In contrast to Metformin, Irbesartan is water insoluble and most of the drug substance is not dissolved in the extracting solvent but suspended as solid which is then separated via centrifugation (Figure 4) followed by filtration (Figure 5).

As shown by the comparison between Figure 4 and Figure 5, filtration was necessary to obtain a clear extract for Irbesartan.

Extraction of target nitrosamines using an extracting solvent in which the drug substance is not soluble has been justified and applied by Mark Harrison's team at AstraZeneca.

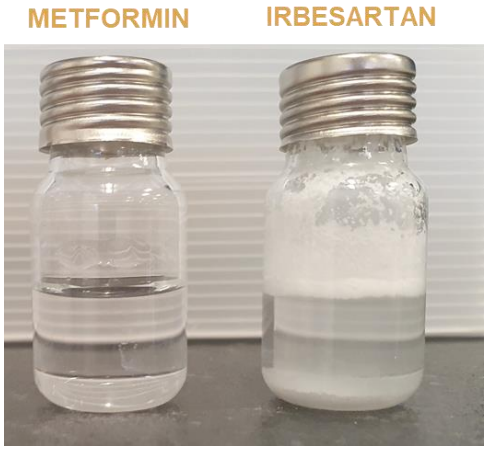

Figure 4: *Example of drug substance preparation for Metformin and Irbesartan after mixing, sonication and centrifugation*

Figure 5: *Irbesartan drug substance extract after filtration*

RESULTS AND DISCUSSION

Method optimisation

The preliminary approach described in AS234 involved an LC-ESI-MS/MS method with focus on ranitidine as drug substance. The sample preparation showed good RSDs% but the instrumental analysis was heavily affected by ion suppression and sensitivity did not meet regulatory requirements.

To address ion suppression resilience, APCI was selected as source of choice. Nevertheless, due to the heavy matrix load when working with drug substance it was also necessary to optimise the chromatographic separation to guarantee robustness of the final method.

Different HPLC stationary phases were evaluated to achieve increased retention for the early eluting nitrosamines (e.g. NDMA and NMBA) and allow use of the divert valve to reduce the impact of the matrix on the APCI source. One column provided the best performances in terms of peak retention, resolution, and peak shape together with successful separation of the matrix load for both investigated drug substances and it was therefore selected for the finalised method.

Once chromatographic conditions were finalised, the APCI source conditions were fully evaluated and optimised to provide the best sensitivity.

Method Validation

The developed and optimised solution for the sample preparation and LC-MS-MS analysis of seven target nitrosamines in two selected drug substances, Irbesartan and Metformin, was validated performing the following procedure[3,4,5].

Linearity

Linearity was demonstrated by preparing and analysing spiked test solvent solutions across the range of the analytical procedure. The results were then evaluated by least squares regression line, correlation coefficient (R^2), y-intercept, slope, and deviation of actual data points from regression line are calculated.

Seven concentrations levels of solvent standard plus blank, evenly spaced over the concentration range, were prepared in triplicate (n=3) and run in duplicate at the beginning and at the end of the batch, for a total of six replicates for concentration level.

Standard solution concentration expressed as ppb (ng/g) of nitrosamines in drug substance were as follows:

25,125, 250, 625, 1250, 1875, 2500 ppb

Acceptance Criteria: R^2 >0.99

Evaluation of the residuals by row plot for the standard least-squares linear regression of the analyte calibration curves highlighted heteroscedasticity i.e. increasing absolute error with increasing concentration. However, the relative error (percent relative standard deviation %RSD) for the data throughout the curve was confirmed to be relative constant. Since heteroscedasticity is a violation of the assumptions required to apply least-squares linear regression, curve weighting is a way to compensate and give a better fit of the experimental data by weighting the data inversely with the concentration[1]. The FDA's guidelines for validation of bioanalytical methods[2] contains this statement: "*Standard curve fitting is determined by applying the simplest model that adequately describes the concentration–response relationship using appropriate weighting and statistical tests for goodness of fit*". A weight of 1/x was therefore applied to all calibration curves. Figure 6 lists the calibration curves for all seven analytes. Analysis of variance and lack of fit evaluations confirmed good fit of the weighted linear regression to the experimental data. Summary of the slope, intercept and R^2 for all analytes are is reported in Table 3.

Table 3: *Summary of slope, intercept and R^2 for target nitrosamines*

Analyte	Slope	Intercept	R^2
NDMA	0.0249	-0.0045	0.9992
NMBA	0.0040	-0.0011	0.9956
NDEA	0.0356	-0.0058	0.9994
NEIPA	0.0681	-0.0168	0.9987
NDIPA	0.0570	-0.0135	0.9985
NDPA	0.0282	-0.0087	0.9960
NDBA	0.0203	-0.0060	0.9807

Of the seven target analytes, only NDBA showed R^2 less than 0.99. It is noteworthy that only three of the seven analytes had corresponding labelled

internal standard so improved performance would be expected when labelled IS is used for all analytes.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were demonstrated by preparing six replicate procedural blanks spiked with internal standard (IS). LOD was calculated using the standard deviation of the blank (σ) and the calibration cure slope (S) as described by the formula below:

$$LOD = \frac{3.3\sigma}{S}$$

Acceptance Criteria: 30 ppb nitrosamines in drug substance. Concentrations are expressed as ppb (ng/g) of nitrosamines in drug substance.

LOQ was calculated using the standard deviation of the blank (σ) and the calibration cure slope (S) as described by the formula below:

$$LOQ = \frac{10\sigma}{S}$$

Acceptance Criteria: 100 ppb nitrosamines in drug substance. Concentrations are expressed as ppb (ng/g) of nitrosamines in drug substance.

Table 4 lists the calculated LODs and LOQs for the seven target nitrosamines.

Table 4: *Limits of Detection (LODs) and Limits of Quantification (LOQs) for the investigated nitrosamines expressed as ppb (ng/g) of nitrosamines in drug substance.*

Analyte	LOD	LOQ
NDMA	1	4
NMBA	0.4	1
NDEA	1	3
NEIPA	1	4
NDIPA	1	4
NDPA	2	5
NDBA	3	9

LODs and LOQs for all seven target nitrosamines were significantly lower than the target agreed in the acceptance criteria (30 ng/g in drug substance).

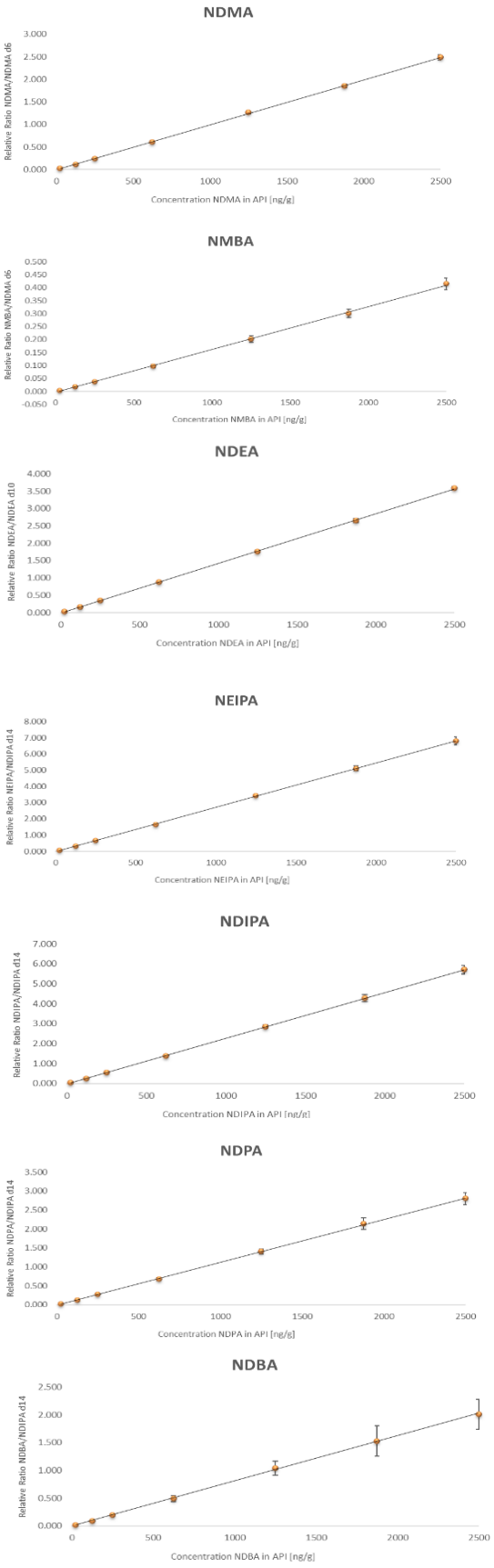

Figure 6: *Calibration curves in solvent for the seven target nitrosamines*

However in order to assess impact on detection and confirmation in the presence of the drug substance matrix, additional LOD and LOQ values were calculated based on the signal-to-noise (peak-to-peak based on peak height) observed for the low spike matrix (125 ng/g) used for the accuracy and precision evaluation.

Table 5 reports the calculated LODs and LOQs for the seven target nitrosamines based on the average (*n*=3) S/N ratio obtained for each analyte in the low spike matrix for both investigated drug substances. The results for NDBA in Irbesartan matrix reflect the poor recovery of NDBA with this matrix.

Figure 7a and 7b show the MRM extracted ion chromatograms for all the target analytes in the two drug substance matrices, Irbesartan and Metformin, spiked at the low level (125 ng/g).

Table 5: *Limits of Detection (LODs) and Limits of Quantification (LOQs) for the investigated nitrosamines expressed as ppb (ng/g) of nitrosamines in drug substance calculated based on S/N ratio in low spike matrix for Irbesartan and Metformin.*

	Irbesartan		Metformin	
Analyte	**LOD**	**LOQ**	**LOD**	**LOQ**
NDMA	2	7	3	10
NMBA	7	23	9	31
NDEA	2	5	1	4
NEIPA	1	2	3	11
NDIPA	1	3	2	5
NDPA	3	11	4	14
NDBA	22	74	2	5

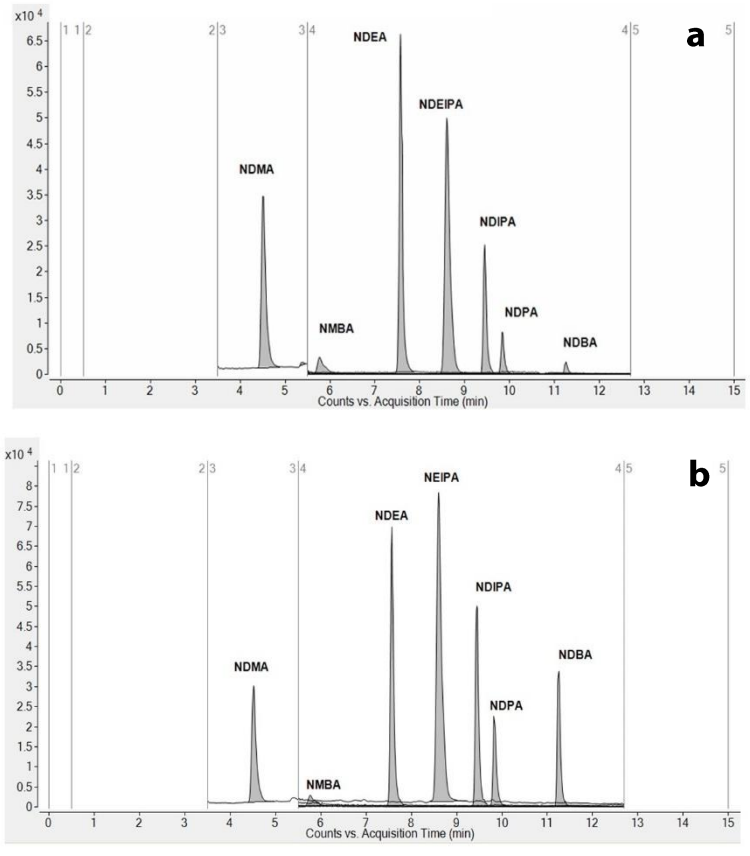

Figure 7: *MRM Extracted Ion Chromatogram spiked with the seven target nitrosamines at 125 ng/g (low spike) for (a) Irbesartan and (b) Metformin drug*

Figure 8: *Bar charts summarising accuracy as percent recovery ± confidence intervals (i.e. 2xStandard Deviation) obtained for both Irbesartan and Metformin drug substances. Dashed black lines show acceptance criteria range*

Accuracy

To demonstrate accuracy, triplicate spiked drug substance samples over a minimum of three concentration levels covering the specified range, were prepared and analysed.

Irbesartan: spike at 125 (low)-1250 (medium)-2500 (high) ppb as ng/g in drug substance (*n*=3 @ each concentration for a total of nine samples)

Metformin Hydrochloride: spike at 125 (low)- 1250 (medium)-2500 (high) ppb as ng/g in drug substance (*n*=3 @ each concentration for a total of nine samples)

Accuracy results were expressed as percent recovery together with confidence interval (CI)

Acceptance Criteria: Recovery 70-130%, RSD% <20%

Figure 8 shows bar charts summarising the accuracy as percent recovery ± confidence intervals (i.e. 2 Standard Deviations) obtained for both Irbesartan and Metformin drug substances.

All analytes gave percent recovery within the acceptance range apart from NDBA spiked in Irbesartan.

Low recoveries could be due to absorbance of the analyte on the suspended drug substance due to its higher lipophilicity compared to the rest of the analytes.

Recovery RSD% for all analytes ranged between 0.2-7.2 % for Irbesartan and 0.9-5.5% for Metformin across all three tested spiked concentrations.

Precision

To demonstrate precision, independent replicates from homogeneously spiked drug substance samples were prepared through the complete analytical procedure from sample preparation to final analysis.

Precision included the following components:

Repeatability (intra-assay): Precision under the same operating conditions over a short interval of time using nine determinations (three replicates of three concentrations)

Intermediate (ruggedness/inter-assay): Within-lab variations of different days and analysts, using nine determinations (three replicates of three concentrations) repeated on a separate day by a different analyst preparing all solvents/reagents fresh.

Irbesartan: spike at 125 (low)-1250 (medium)-2500 (high) ppb as ng/g in drug substance (*n*=3 @ each concentration for a total of nine samples)

Metformin Hydrochloride: spike at 125 (low)- 1250 (medium)-2500 (high) ppb as ng/g in drug substance (*n*=3 @ each concentration for a total of nine samples)

Standard deviation (SD), relative standard deviation (RSD) and CI are reported

Acceptance Criteria: Intra-assay RSD% <20%, Inter-assay RSD% < 25%

Table 5, 6, 7 and 8 summarise Intra-assay and Inter-assay RSD% for the seven target nitrosamines at the three investigated concentration levels for Irbesartan and Metformin, respectively.

Table 5: *Intra-assay RSD% for the seven target nitrosamines at the three investigated concentration levels for Irbesartan.*

Analyte	Low	Medium	High
NDMA	3.6	0.7	0.2
NMBA	6.6	0.8	3.0
NDEA	6.3	2.7	4.1
NEIPA	2.8	5.9	4.2
NDIPA	0.7	1.6	3.3
NDPA	5.5	4.8	2.3
NDBA	18.3	7.4	13.4

Table 6: *Inter-assay RSD% for the seven target nitrosamines at the three investigated concentration levels for Irbesartan*

Analyte	Low	Medium	High
NDMA	16.4	7.3	7.8
NMBA	9.4	5.4	3.7
NDEA	18.5	4.9	1.3
NEIPA	5.6	7.0	6.3
NDIPA	16.0	9.5	3.8
NDPA	17.5	10.8	5.2
NDBA	68.2	20.4	4.3

Table 7: *Intra-assay RSD% for the seven target nitrosamines at the three investigated concentration levels for Metformin*

Analyte	Low	Medium	High
NDMA	1.5	4.5	2.9
NMBA	9.3	5.0	3.6
NDEA	1.6	3.7	4.4
NEIPA	4.1	1.5	2.4
NDIPA	0.9	3.8	1.8
NDPA	2.8	4.4	3.4
NDBA	1.3	4.1	1.8

Table 8: *Inter-assay RSD% for the seven target nitrosamines at the three investigated concentration levels for Metformin*

Analyte	Low	Medium	High
NDMA	6.0	0.4	1.1
NMBA	11.9	4.5	3.9
NDEA	16.3	19	3.8
NEIPA	1.1	2.4	0.8
NDIPA	8.8	1.5	3.7
NDPA	4.9	4.9	2.6
NDBA	3.9	0.9	9.8

RSD% for all target analytes were below 10% for the intra-assay precision and below 20% for the inter-assay precision in both Irbesartan and Metformin, with the only exception of NDBA in Irbesartan.

Specificity

To demonstrate specificity, a spiked drug substance sample -2500 concentration is expressed as ppb (ng/g) of nitrosamines in drug substance- was analysed in MS scan mode and by UV to confirm separation from other components in the sample matrix and to prove elution time range for selected drug substance.

To demonstrate analyte specificity, retention times (RT) and quantifier/qualifier ratios for the MRM data were compared to corresponding reference standard.

Acceptance Criteria: Scan acquisition and UV trace proving elution of drug substance in separate time range from target analytes.

RT of target analytes within 1% of reference standard and Quant/Qual ratio within 30% of reference standard[3]

Figure 9 and Figure 10 show Total Ion Chromatogram, relative MS spectrum and UV trace for the spiked drug substance samples for Irbesartan and Metformin, respectively. Irbesartan drug substance eluted at 10.689 min and Metformin drug substance at 2.185 min.

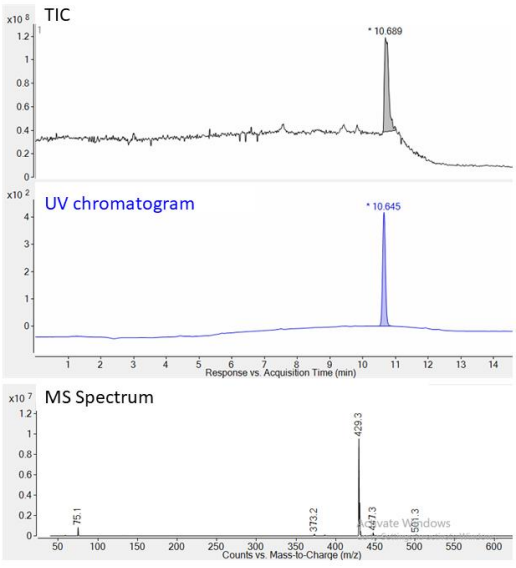

Figure 9: *From the top: Total Ion Chromatogram (TIC), UV trace at 254 nm and MS spectrum for a spiked Irbesartan sample*

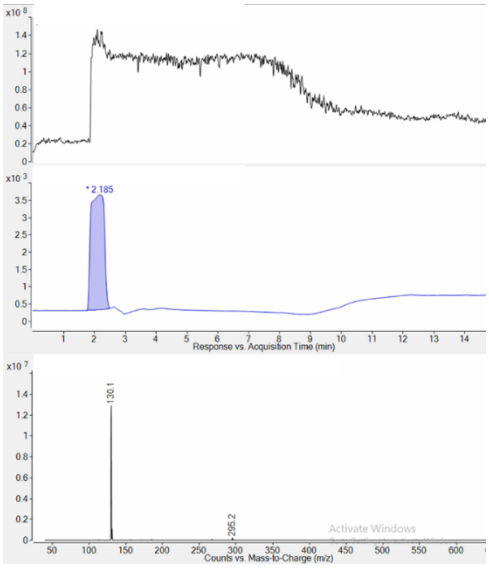

Figure 10: *From the top: Total Ion Chromatogram (TIC), UV trace at 234 nm and MS spectrum for a spiked Metformin sample*

All analytes eluted in a retention time range between 4.5 min and 9.8 min except for NDBA (11.28 min), indicating active ingredients were not coeluting with the target analytes.

Furthermore, all seven target nitrosamines showed agreement of retention time within 1% and Quantifier/Qualifier ratios within 30% of their relative reference standard across the whole dataset.

CONCLUSIONS

This application note describes the optimisation and validation of a fully automated solution for the sample preparation and LCMS/MS analysis of seven target nitrosamines in two drug substances, Irbesartan and Metformin.

All figures of merit evaluated during the validation of this method met acceptance criteria for all analytes in both investigated matrices, except for NDBA, for which further work is being undertaken. The results were presented and discussed with Mark Harrison at AstraZeneca who considered the data acceptable to pass validation for the key nitrosamines.

This optimised and validated analytical solution clearly indicates the benefits of automated sample preparation for complex analyses. In addition to improved accuracy and precision, a fully automated solution reduces analyst interaction with the sample to a minimum. This, alongside the

significant benefit of high throughput unattended operation, minimises analyst exposure to chemicals and limits risks of contamination, which could lead to false positives and therefore results costly delays or erroneous product recalls.

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Colin Towers, Technical Support Chemist Crawford Scientific Ltd
Marcus Chadha, Field Application Specialist Agilent

To discuss implementing this application solution for the analysis of nitrosamines in drug substances contact us and we will be delighted to work with you from conception to method transfer into your laboratory.

We also offer fully validated methods, according to your validation protocol, where required.