1.0 EXECUTIVE SUMMARY

1.1 Study Design

The purpose of this study was to perform an independent laboratory validation (ILV) of Analytical Method GRM030.01A to demonstrate the suitability of this method to determine residues of NOA449280 and SYN503780 in water.

One groundwater sample was used as the representative matrix for this ILV study. One reagent blank sample, control samples in duplicate, fortified samples in quintuplet at the limit of quantification (LOQ) at 0.01 µg/L for NOA449280 and SYN503780 and in quintuplet at 10 times the LOQ (0.1 µg/L) were prepared using the groundwater sample and analysed as according to the procedures specified in Analytical Method GRM030.01A without any modifications.

1.2 Results and Conclusions

Acceptable mean recoveries of between 70% and 110% with a relative standard deviation less than 20% at each fortification level and for each analyte with MRM transitions of NOA449280 (primary transition *m/z* 400.0 → 324.0 and confirmatory transition *m/z* 400.0 → 228.0) and SYN503780 (primary transition *m/z* 278.0 → 202.0 and confirmatory transition *m/z* 278.0 → 146.0) in the water matrix tested.

No significant matrix effect was observed for NOA449280 and SYN503780 in the water tested. Non matrix-matched standards were used for calibration and quantification.

The response of the LC-MS/MS was shown to be linear for NOA449280 and SYN503780 for each transition over a concentration range of 0.05 to 10.0 µg/L for water (equivalent to 2.5 to 500 pg of NOA449280 and SYN503780 injected on to the column, based on a 50 µL injection).

This ILV study was in compliance with OECD guidance document ENV/JM/MONO (2007) 17, EC guidance documents SANCO/3029/99 Rev. 4 and SANCO/825/00 Rev. 8.1, and EPA guideline OPPTS 850.1700.

2.0 INTRODUCTION

This study was designed and conducted to perform an ILV of Analytical Method GRM030.01A (Reference 1) for the determination of residues of NOA449280 and SYN503780 in water, using commercially available instrumentation. The sections of this method necessary to its implementation were translated and referenced at Eurofins|ADME BIOANALYSES under the number AGR/MOA/NOA449280 -5.

This study was conducted in accordance with OECD guidance document ENV/JM/MONO (2007) 17, EC guidance documents SANCO/825/00 Rev. 8.1, and SANCO/3029/99 Rev. 4, and EPA guideline OPPTS 850.7100.

Specifically:

a) To confirm that the method will produce recovery values which are within an acceptable range (i.e. mean recoveries between 70% and 110%, with a relative standard deviation within a run ≤ 20%), for each fortification level and overall. To establish the 95% confidence intervals.

b) To confirm that the limit of quantification (LOQ) of the analytical method is 0.01 µg/L for NOA449280 and SYN503780.

c) To confirm that residue levels of NOA449280 and SYN503780 in control samples are not present at levels above 30% of the LOQ.

d) To investigate the relationship between instrument response and analyte concentration for analyte over concentration ranges typical of those for which the method will be used.

e) To assess suppression or enhancement of instrument response to NOA449280 and SYN503780 in the presence of water matrix

3.0 MATERIALS AND METHODS

3.1 Test and Reference Items

Code Number	NOA449280
Chemical name (IUPAC)	4-Hydroxy-3-[2-(2-methoxy-ethoxymethyl)-6-(trifluoromethyl)-pyridine-3-carbonyl]-bicyclo[3.2.1]oct-3-en-2-one
Molecular formula	$C_{19}H_{20}F_3NO_5$
Molecular mass	399.4 g/mol

Code Number	SYN503780
Chemical name (IUPAC)	2-(2-Methoxy-ethoxymethyl)-6-trifluoromethyl-nicotinic acid
Molecular formula	$C_{11}H_{12}F_3NO_4$
Molecular mass	279.2 g/mol

The test / reference items used for this validation study were the following:

Test / Reference Item	Batch	Purity (%)	Valid until:	Storage Conditions
NOA449280	AMS 1144/1	99.9	30 Apr 2016	+ 20 ± 4°C
SYN503780	KI 6386/18	100	31 Mar 2012	Between 0 and 9°C

The certificates of analysis have been provided by the sponsor. The remaining test / reference items will be stored at Eurofins|ADME BIOANALYSES as long as its quality can be maintained. The structures are shown in Figures 1 and 2.

3.2 Test System

The validation study was carried out using control L08-01322-TR-C-001 water sample supplied by the sponsor. This water type was selected for the ILV as typical of the matrix that is being analysed by Analytical Method GRM030.01A. Details of the water characterisation (performed as part of study S08-01322) are given in Table 1.

3.3 Preparation of Analytical Standard Solutions

200 µg/mL stock solutions of NOA449280 and SYN503780 were prepared separately in acetonitrile.

Fortification solutions of NOA449280 and SYN503780 were prepared at 0.01 µg/mL and 0.001 µg/mL in acetonitrile/ultra pure water (70/30, v/v) from the primary stock solutions.

Calibration standards for analytical determination by LC-MS/MS were prepared from the above mentioned fortification solutions over an appropriate range in acetonitrile/ultra pure water (20/80, v/v), then were ten-fold diluted in acetonitrile/ultra pure water (20/80, v/v) to achieve the target concentration range from 0.05 to 10.0 µg/L.

3.4 Fortification Levels

Recovery samples were prepared by fortifying appropriate amounts of the relevant fortification standards to the groundwater sample to give recovery levels at 0.01 µg/L (LOQ) and at 0.10 µg/L (10 times the LOQ). For each fortification level, five recovery samples were prepared. In addition, two control samples and one reagent blank were prepared for each sample batch. The fortification levels are summarized in Table 2.

3.5 Sample Analysis

The reagent blanks, untreated controls and recovery samples were analysed through the procedures specified in Analytical Method GRM030.01A. The method procedures were followed as written without any modifications. In summary, acidified environmental water samples (10 mL, acidified using 200µL formic acid) were concentrated using solid phase extraction (StrataX, 60mg/3 mL). After elution with methanol, samples were evaporated to dryness and dissolved in acetonitrile/ultra pure water (20/80, v/v) for LC-MS/MS analysis. The LC-MS/MS conditions and method details are summarized in Appendix 1.

Calibration standard solutions containing NOA449280 and SYN503780 at concentrations ranging from 0.05 to 10.0 µg/L (equivalent to 2.5 to 500 pg of analytes injected on to the column, based on a 50 µL injection) was injected along with samples. The detector response (peak areas) for LC-MS/MS was plotted against standard concentration injected for generation of calibration curves using analyst software. The lowest concentration injected was at 50% of the LOQ of the method. The highest concentration injected was equivalent to 100×LOQ .

Each sample set included an appropriate matrix-matched standard, prepared in water matrix. The response obtained from the matrix-matched standard was compared against the response obtained from the standard in acetonitrile/ultra pure water (20/80, v/v) to allow calculation of any matrix effect (either suppression or enhancement of response). The results are presented in Table 9.

3.6 Modifications to the method and potential problems

Analytical Method GRM030.01A was followed as written without any modifications to the method.

3.7 Communication with sponsor

There was no communication between the analytical laboratory and the Sponsor during the ILV.

FIGURE 1: Structure of NOA449280.

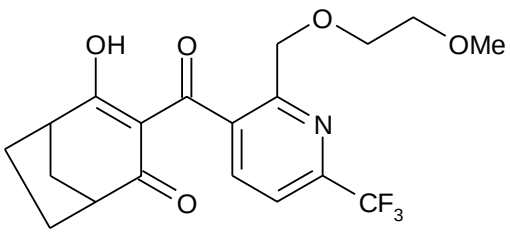
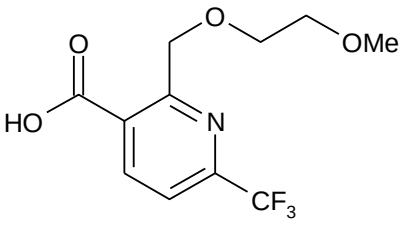


FIGURE 2: Structure of SYN503780.



APPENDIX 1 Analytical Method Description

1. PREPARATION AND USE OF THE STANDARD SOLUTIONS

The stock solutions must be stored in a refrigerator when not in use. The stock, fortification and calibration solutions used for this study were stored between 0 and 9°C and are stable for 6 months protected when from light.

1.1. Stock solution

- Between 2 and 50 mg of NOA449280 and SYN503780 were accurately weighed separately into volumetric brown flasks.
- Adequate volumes of acetonitrile for NOA449280 and SYN503780 were added in order to obtain stock solutions at 200 µg/mL, taking into account the chemical purity. These solutions were sonicated until total dissolution.

1.2. Fortification solutions

Appropriate serial dilutions of NOA449280 and SYN503780 primary stock solutions were performed in acetonitrile/ultra pure water (70/30, v/v) to obtain solutions at 0.01 µg/mL and 0.001 µg/mL.

1.3. Calibration solutions

Appropriate serial dilutions of the fortification solutions were performed in acetonitrile/ultra pure water (80/20, v/v), at the following concentrations.

0.0005 – 0.001 – 0.0025 –0.005 – 0.01 – 0.025 – 0.05 and 0.1 µg/mL

Calibration standards were prepared by ten-fold dilution of the above standards in acetonitrile/ultra pure water (80/20, v/v). The following calibration solutions were obtained:

0.05 – 0.1 – 0.25 –0.5 – 1 – 2.5 – 5 and 10 µg/L

APPENDIX 1 Analytical Method Description (Continued)

2. ANALYTICAL SUPPLIES AND APPARATUS

According to availability and laboratory equipment, analytical supplies from other suppliers and apparatus of different design may be used.

2.1. Apparatus and material

- LC-MS/MS: API 4000
- Pump + autosampler: LC20AD-XR (Shimadzu) + SIL20AC-XR (Shimadzu)
- Column oven CTO-20AC (Shimadzu)
- HPLC column: ACE 5 C18 50x3 mm, 5µm (AIT France)
- Cartridge Strata X (60 mg/3 mL) (Phenomenex, ref. 8B-S100-UBJ)
- Analytical balances (Mettler, Sartorius)
- Ultrasonic bath
- Heating block under nitrogen flow
- General glassware
- pH indicator paper
- Various pipettes

2.2. Reagents

- Acetonitrile
- Acetic Acid
- Formic Acid
- Methanol
- Ultra pure water

APPENDIX 1 Analytical Method Description (Continued)

2.3. Preparation of Reagents

- Acetonitrile / ultra pure water (70/30, v/v)
  Mix 700 mL of acetonitrile with 300 mL of ultra pure water.
  Stopper flask securely and mix thoroughly by shaking.

- Acetonitrile / ultra pure water (20/80, v/v)
  Mix 200 mL of acetonitrile with 800 mL of ultra pure water.
  Stopper flask securely and mix thoroughly by shaking.

- 2% Formic acid in ultra pure water
  Mix 2 mL of concentrated formic acid with 98 mL of ultra pure water.
  Stopper flask securely and mix thoroughly by shaking.

APPENDIX 1 Analytical Method Description (Continued)

3. ANALYTICAL PROCEDURE

3.1. Sample preparation

a) If water samples are received deep frozen they should be allowed to defrost completely at room temperature. Defrosted samples should be shaken thoroughly to ensure sample homogeneity prior to analysis.

b) Transfer 10 mL of the water sample to be analysed into a polypropylene centrifuge tube (15 mL size). Sample fortification is to be carried out at this time.Cap the tubes securely and shake gently to mix.

c) Add concentrated formic acid (200 µL) to each sample. Cap the tubes and shake gently to ensure thorough mixing. Check that the pH is < pH 2 using suitable indicator paper.

3.2. Solid Phase Extraction

a) Take one Phenomenex Strata-X SPE cartridge (60 mg, 3 mL) for each sample to be analysed and place on a suitable vacuum manifold (e.g. IST Vacmaster). Add methanol (2 mL) and allow to percolate through each cartridge under gravity or draw through under vacuum to the level of the top frit at a rate of approximately 1 mL/min, discarding the column eluate. Do not allow the cartridge to become dry. Add ultra pure water (2 mL) to the top of the each cartridge and allow to percolate through under gravity or draw through under vacuum to the level of the top frit at the same rate, again discarding the column eluate. Do not allow the cartridges to become dry.

b) Load water samples from Section 3.1 (c) onto the SPE cartridges and allow to percolate through under gravity or under low vacuum, at a rate of approximately 1-2 mL/min, to the level of the top frit. Do not allow cartridges to become dry. NOA449280 and SYN503780 are retained on the SPE cartridges.

c) On completion of loading, wash the empty sample tubes with ultra pure water containing 2% formic acid (2 mL) and add the rinse to the cartridge. Allow to percolate through under gravity or draw through under vacuum to the level of the top frit at the same rate, again discarding the column eluate. Do not allow the cartridges to become dry.

d) Briefly apply a high vacuum for approximately 5 – 10 seconds to remove excess water from the cartridges but do not dry for extended periods.

e) Place suitable collection tubes (15 mL polypropylene tubes) under each port, as required, in the manifold rack. Elute the cartridges with methanol (3 mL), under gravity or draw through under low vacuum at rate of approximately 1-2 mL/min to the level of the top frit collecting the column eluate. Apply high vacuum for

approximately 5 seconds to collect the excess solvent from the SPE cartridges. NOA449280 and SYN503780 are eluted in this step.

f) Evaporate the samples to dryness under a nitrogen flow using a heating block with the temperature set to 45 °C. This should take approximately 20 minutes.

g) Add acetonitrile (200 µL) and ultrasonicate thoroughly. Add ultra pure water (0.8 mL) and again ultrasonicate thoroughly to ensure the sample is completely dissolved and thoroughly mixed.

h) Transfer an aliquot to a suitable autosampler vial ready for final determination by LC-MS/MS. The final sample concentration is 10 mL/mL.

APPENDIX 1 Analytical Method Description (Continued)

4. PARAMETERS FOR CHROMATOGRAPHIC ANALYSIS

4.1. Operating conditions

The following parameters were used during the study. They may be adapted if alternative equipment is used.

LC-MS/MS:

- Pump + Autosampler: LC20AD-XR, Shimadzu + SIL20AC-XR (Shimadzu)
- Detector: API 4000
- Data Acquisition: Analyst 1.5.1
- Column HPLC: ACE 5 C18 50x3.0 mm 5 µm
- Column temperature: 40 °C
- Retention time: approximately 1.1 minutes for SYN503780
  approximately 1.9 minutes for NOA449280
- Injection volume: 50 µL
- Autosampler temperature: 4°C
- Flow: 1 mL/minute
- Mobile phase: Solvent 1: Acetonitrile
  Solvent 2: 0.2% acetic acid in ultra pure water
- Gradient:

Time (minute)	% Solvent 1	% Solvent 2
0.0	20	80
2.0	80	20
3.0	80	20
3.1	20	80
4.0	20	80

- Ionisation mode: ESI^+ for 1.4 min (for SYN503780)
  ESI^- for 2.1 min (for NOA449280)
- Scan Type: MRM
- Calibration range: 0.05 to 10 µg/L

Analyte	Parent ion (*m/z*)	Daughter ion (*m/z*)	DP (V)	EP (V)	CXP (V)	CE (V)	Dwell (ms)
NOA449280	400.0	324.0	66	10	31	24	150
		228.0	66	10	53	55	150
SYN503780	278.0	202.0	-40	-10	-18	-11	150
		146.0	-40	-10	-26	-1	150

CAD (collision gas)	6	**TEM (°C)**	550
CUR (curtain gas)	17	**RESOLUTION Q1**	Unit
GS1 (ion source gas 1)	60	**RESOLUTION Q3**	Unit
GS2 (ion source gas 2)	60		
IS (ion spray voltage)	-4500 (period 1) & +4500 (period 2)		

4.2. Calibration

Calibration standards were injected before each series of test sample analyses. Confirmatory standards were injected within each series every four samples. The determination coefficient R^2 was found to be higher than 0.990 in all cases.

4.3. Result calculation

The chromatographic system was calibrated using a calibration curve of NOA449280 and SYN503780 external standards. A linear calibration curve was calculated using the method of least squares with 1/x weighting:

$Y = A \times C + B$

Y = detector response (as peak area) for NOA449280 and SYN503780
A = slope of the linear least squares fit of the calibration curve
C = concentration determined from standard curve (µg/L)
B = Y-intercept of the linear least squares fit of the calibration curve.

The concentration determined from standard curve is: $C = (Y-B)/A$

APPENDIX 1 Analytical Method Description (Continued)

The residue of analyte in each test sample is calculated as follows:

$$\text{Residue } (\mu g / L) = \frac{V_f}{V_i} \times \textit{extract concentration } (\mu g / L) \quad \textit{dilution}$$

Vi = Initial extraction volume (10 mL)
V_f = Final volume (1 mL)

where the final volume includes dilution steps, if applicable.

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Recovery } (\%) = \frac{A}{S} \times 100$$

where:

A = concentration of NOA449280 and SYN503780 found in test sample (µg/L).
S = concentration of NOA449280 and SYN503780 added in test sample (µg/L).