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SYN550024

SYN550024 – Partial Validation of a Bioanalytical Method for the Determination of SYN550024 in Rat Blood

Method Validation

DATA REQUIREMENT(S): Not Applicable

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COMPLETION DATE: 14 January 2019

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with Good Laboratory Practice (GLP) as required by the United Kingdom Good Laboratory Practice Regulations SI 1999, No. 3106, amended by SI 2004 No.994, which are in accordance with the OECD Principles of GLP, ENV/MC/CHEM (98) 17 and the final report fully and accurately reflects the raw data generated during the conduct of the study.

I Davies — 14 JANUARY 2019

Ian Davies
Study Director
Bioanalysis Department

Date

Performing Laboratory: Sequani Limited, Bromyard Road, Ledbury, Herefordshire, HR8 1LH, United Kingdom

QUALITY ASSURANCE STATEMENT

STUDY PLAN

The study plan for this study has been reviewed and reported to the Study Director:

Date of Review	Date Reported to the Study Director and Management
04 October 2018	05 October 2018

PROCEDURES

Procedures on this study have been inspected and reported to the Study Director and Management:

Date of Inspection	Procedure Inspected	Date Reported to Study Director and Management
16 August 2018	Validation	16 August 2018
07 December 2018	Validation	07 December 2018

In addition, general facilities and systems supporting the above study phases have been inspected and reported to management on at least an annual basis, in accordance with QA Standard Operating Procedures.

QUALITY ASSURANCE STATEMENT (CONTINUED)

REPORT

The draft study report and data have been reviewed and reported to the Study Director and Management:

Report Version	Date of Review	Date Reported to the Study Director and Management
Draft	07-10 December 2018	10 December 2018

The final report has been reviewed by Quality Assurance. As far as can be reasonably established, the methods described and the results presented accurately reflect the raw data generated during the study. The report review was completed on 09 January 2019.

Quality Assurance Unit

14 January 2019

Date

REPORT APPROVAL PAGE

I hereby certify that the work reported in this document was carried out by Sequani Limited and represents a true and faithful account of the study performed.

L. P. La
Sequani Management

14 January 2019
Date

GENERAL INFORMATION

Contributors
The following contributed to this report in the capacities indicated:

On behalf of Sequani Ltd.:

Name	Title
Ian Davies	Study Director
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On behalf of Syngenta Ltd.:

Name	Title
Sarah Whalley	Syngenta Study Manager

Study dates

Study initiation date:	08 October 2018
Experimental start date:	09 October 2018
Experimental termination date:	07 November 2018

Deviations from the guidelines
None

Retention of samples
All stored samples likely to be perishable will be discarded within one month of finalisation of the study report.

Other
The final report and all raw data, relating to this study will be stored in the archives of Sequani Limited for a period of 2 years after which time they will be stored at a location designated by the Sponsor.

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1.0 EXECUTIVE SUMMARY

SYN550024 was recovered from rat blood:water [1:1 (v/v)] using protein precipitation and the processed samples were analysed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). The method was validated over the range 5.00 ng/mL to 6250 ng/mL (blood concentration) by the determination of within-run precision and accuracy, specificity, recovery and matrix effects, the ability to dilute samples into the analytical range, and the determination of stability in biological matrix.

1.1 Results

The maximum within-run accuracy value (mean bias, n=6) for the method was ± 9.50 %.

The maximum within-run precision (n=6) for the method was 12.56 %.

SYN550024 has been demonstrated to be stable in:
Rat blood:water [1:1 (v/v)] stored at room temperature for up to 24 hours.
Rat blood:water [1:1 (v/v)] stored at approximately -80 °C (≤ -70 °C) for up to 29 days.
Rat blood:water [1:1 (v/v)] following 3 freeze-thaw cycles.
Processed sample stored at approximately 4 °C for up to 3 days.
Recovery was ≥ 79.71 % and matrix effects were ≤ ± 6.20 %.
Validation samples prepared at a concentration greater than the method Upper Limit of Quantification (ULQ) could be diluted into the analytical range with acceptable accuracy and precision with dilution factors of up to 100.

All acceptance criteria were therefore met.

1.2 Conclusion

Since all acceptance criteria were met, the method is validated for the determination of SYN550024 in rat blood:water [1:1 (v/v)] over the analytical range 5.00 ng/mL to 6250 ng/mL.

2.0 INTRODUCTION

An analytical method for the determination of SYN550024 in rat blood:water [1:1 (v/v)] over the concentration range 5.00 ng/mL to 6250 ng/mL (blood concentration), has been validated at Sequani Limited in accordance with the definitive study plan. The definitive analytical method, Sequani reference BFI121MS, is detailed in APPENDIX 1. Certificates of analysis for SYN550024 and internal standard (diazepam-D5) are given in APPENDIX 3 and APPENDIX 4, respectively.

3.0 MATERIALS AND METHODS

3.1 Reference Compounds

SYN550024 was delivered to Sequani Limited on 18 July 2018. On arrival, the consignment of SYN550024 was given the Sequani log reference number TI/2018/164. The following table shows details of the compound:

Compound	SYN550024
Supplier	Syngenta
Batch Number	MES 603/1
Appearance	White to off-white solid when refrigerated. Colourless to yellowish liquid at room temperature
Purity	44 % [1]
Re-test Date	31 July 2020
Storage	Refrigerated

[1] Purity based on a water content of 55.9 %. The compound is a potassium salt, however, no salt correction factor was applied.

Diazepam-D5 was used as internal standard for the method. This was delivered to Sequani Limited on 01 October 2018. On arrival, the consignment of diazepam-D5 was given the Sequani log reference number CH/2018/290. Details of this compound are given in the following table:

Compound	Diazepam-D5
Supplier	Sigma-Aldrich
Batch Number	FE07061501
Appearance	Clear liquid
Purity	99.7 %
Expiry Date	31 July 2020
Storage	Frozen, -20 °C

3.2 Experimental Design

The performance of the method for the determination of SYN550024 in rat blood:water [1:1 (v/v)] was validated with regard to the following parameters:

3.2.1 Calibration model

Calibration standards at eight concentration values were analysed, in duplicate, with each analytical run. The peak area ratios of SYN550024 to internal standard were plotted against the theoretical concentration of SYN550024 for each analytical run, and the data subjected to regression analysis (quadratic model), and weighted by a factor of $1/X^2$.

The optimum fit of the data is indicated by the proximity of the correlation coefficient to 1.0 and a value of 0.9850 or greater was considered to be demonstrative of acceptable quality.

For each analytical run to be acceptable, at least 75 % of the back-calculated calibration standard concentrations were required to be within ± 15 % of the theoretical concentration (± 20 % at the Lower Limit of Quantification (LLQ)). Calibration standards not meeting these criteria were excluded from the calibration line.

3.2.2 Precision and accuracy

Validation samples prepared at five concentration levels (5.00 ng/mL, 12.5 ng/mL, 200 ng/mL, 5000 ng/mL, and 6250 ng/mL (VS1, VS2, VS3, VS4, and VS5, respectively)) were analysed in replicates of six on one occasion. The validation sample concentrations were determined from the calibration line and the coefficients of variation (% CV) for each concentration level were calculated within the analytical run (intra-run).

The within-run accuracy was determined by comparing the mean replicate interpolated concentrations for each validation sample with the theoretical (calculated) value. The accuracy was expressed in terms of percentage bias from the theoretical value.

For each analytical run to be acceptable the following criteria were applied:
The precision (% CV) at each concentration had to be ≤ 15 % (≤ 20 % at the LLQ).
The accuracy (% bias) at each concentration had to be ≤ ± 15 % (≤ ± 20 % at the LLQ).

3.2.3 Sensitivity

The validation sample of lowest concentration was used to determine the LLQ of the method. In order to be acceptable, the within-run precision had to be ≤ 20 %, and the accuracy had to be ≤ ± 20 %.

3.2.4 Specificity

The specificity of the method was investigated by the analysis of samples of blank matrix from six independent sources. These chromatograms were compared with calibration

standard chromatograms from the same run for the presence of endogenous interfering peaks. Blank samples of matrix fortified with internal standard were also included in validation runs in order to confirm that no analyte response occurred as a result of use of the internal standard. Any interference was to have been no greater than 20 % of the response of LLQ.

3.2.5 Stability

The stability of SYN550024 was investigated in biological matrix (including long and short term storage and the effect of freeze-thaw cycles), and in processed samples.

3.2.5.1 Short term biological matrix stability

Short term stability of SYN550024 in biological matrix was assessed by the analysis of stability samples at two concentration levels (12.5 ng/mL and 5000 ng/mL), in replicates of six, following storage for 24 hours at room temperature. The concentration data obtained were compared with the corresponding data obtained from the original analysis of the samples following their preparation. SYN550024 was considered stable in rat blood:water [1:1 (v/v)] if the mean response of the stability samples was within 15 % of the mean of the original data.

3.2.5.2 Long term biological matrix stability

Long term stability of SYN550024 in biological matrix was assessed by the analysis of stability samples at two concentration levels (12.5 ng/mL and 5000 ng/mL), in replicates of six, following storage for 29 days at approximately -80 °C (≤ -70 °C). The concentration data obtained were compared with the corresponding data obtained from the original analysis of the samples following their preparation. SYN550024 was considered stable in rat blood:water [1:1 (v/v)] if the mean response of the stability samples was within 15 % of the mean of the original data.

3.2.5.3 Freeze-thaw stability

Freeze-thaw stability of SYN550024 in biological matrix was assessed by the analysis of stability samples at two concentration levels (12.5 ng/mL and 5000 ng/mL), in replicates of six, the samples having been subjected to three freeze-thaw cycles, from approximately -80 °C (≤ -70 °C) to room temperature. The concentration data obtained were compared with the corresponding data obtained from the original analysis of the samples following their preparation. SYN550024 was considered stable in rat blood:water [1:1 (v/v)] following three freeze-thaw cycles if the mean response of the stability samples was within 15 % of the mean of the original data.

3.2.5.4 Processed sample stability

Stability of SYN550024 in processed sample was investigated by the re-injection of a precision and accuracy run (validation sample concentrations 5.00 ng/mL, 12.5 ng/mL,

200 ng/mL, 5000 ng/mL, and 6250 ng/mL). Stability was considered to have been demonstrated if the data met the same criteria as a precision and accuracy run (Section 3.2.2).

3.2.6 Dilution

The effect of diluting a sample was assessed by preparing a validation sample at a concentration above the upper limit of quantification (ULQ) of the method. Six replicates of this sample were diluted into the calibration range with biological matrix prior to analysis. The sample was prepared at five times the ULQ and was diluted into the calibration range using a dilution factor of 100. The mean back-calculated concentration was required to be within 15 % of the theoretical concentration, with precision of ≤ 15 %.

3.2.7 Recovery

Recovery (extraction efficiency) was determined by comparing the responses of validation samples taken through the extraction procedure with those of samples where equivalent amounts of SYN550024 and internal standard were added, post-extraction, to blank matrix. Concentration or dilution steps in the extraction procedure were taken into account when determining the amount of analytes to add post-extraction to the reference samples. Recovery was determined at two concentration levels (12.5 ng/mL and 5000 ng/mL) in replicates of six. An assessment of the recovery of the internal standard was performed, at its working concentration.

3.2.8 Matrix effects

Matrix effects were investigated by comparing the response of samples prepared by adding amounts of SYN550024 and internal standard to extracted blank matrix, with non-matrix aqueous solutions of the analytes of equivalent concentration. Matrix effects were determined at two concentration levels (12.5 ng/mL and 5000 ng/mL) in replicates of six. An assessment of the matrix effects on the internal standard was performed, at its working concentration.

An assessment of matrix effects was also made using six different sources of biological matrix. The six different sources of matrix were extracted and the residues re-dissolved in pure solutions of SYN550024 and internal standard. The coefficient of variation of the peak area ratios of SYN550024 to internal standard was required be ≤ 15 %.

3.2.9 Carryover

Carryover was assessed by inclusion of a double blank sample positioned after each of the highest calibration standards in each analytical run. Carryover (%) was calculated for each analytical run by expressing the mean (n=2) response in the double blank samples as a fraction of the mean (n=2) response of the highest calibrator, and the mean carryover expressed as a proportion of the method LLQ.

4.0 RESULTS AND DISCUSSION

4.1 Calibration Model

(TABLE 1)
A quadratic fit ($1/X^2$) best described the calibration data over the range 5.00 ng/mL to 6250 ng/mL, with acceptable precision and accuracy at the method LLQ and ULQ. The correlation coefficients of the calibration lines throughout the validation were 0.9967 or greater. A representative calibration line is given in FIGURE 1.

The acceptance criteria were met for the calibration model.

4.2 Precision and Accuracy

(TABLE 2)
The within-run precision (% CV) at all validation sample concentrations (VS1- VS5) was ≤ 15 % (≤ 20 % at the LLQ) and therefore acceptable. The maximum within-run precision determined for SYN550024 was 12.56 %.

The accuracy (% bias) at all validation sample concentrations was ≤ ± 15 % (≤ ± 20 % at the LLQ) and therefore acceptable. The maximum within-run accuracy determined for SYN550024 was ± 9.50 %.

The acceptance criteria were met for precision and accuracy.

4.3 Sensitivity

The LLQ of the analytical method was established to be 5.00 ng/mL, given acceptable precision and accuracy at the lowest validation sample concentration.

4.4 Specificity

SRM chromatograms of samples of blank matrix showed no interference at the retention times of SYN550024 or diazepam-D5. The method has therefore been demonstrated to be specific.

SRM chromatograms of a double blank and single blank sample of rat blood:water [1:1 (v/v)] are given in FIGURE 2 and FIGURE 3, respectively. Calibration standards at the LLQ and ULQ are given in FIGURE 4 and FIGURE 5, respectively.

4.5 Stability

4.5.1 Short term biological matrix stability

(TABLE 3)
The mean difference between the stored validation sample data and the reference data was ≤ ± 4.17 % and indicated that SYN550024 is stable in rat blood:water [1:1 (v/v)] when stored at room temperature for up to 24 hours.

4.5.2 Long term biological matrix stability

(TABLE 4)
The mean difference between the stored validation sample data and the reference data was ≤ ± 3.51 % and indicated that SYN550024 is stable in rat blood:water [1:1 (v/v)] when stored at approximately -80 °C (≤ -70 °C) for up to 29 days.

4.5.3 Freeze-thaw stability

(TABLE 5)
The mean difference between the stored validation sample data and the reference data was ≤ ± 2.33 % and indicated that SYN550024 is stable in rat blood:water [1:1 (v/v)] when subjected to up to three freeze-thaw cycles from approximately -80 °C (≤ -70 °C) to room temperature.

4.5.4 Processed sample stability

(TABLE 6)
The precision at all validation sample concentrations was ≤ 15 % (≤ 20 % at the LLQ), and the accuracy was ≤ ± 15 % (≤ ± 20 % at the LLQ) and therefore the data were acceptable. The maximum within-run precision and accuracy determined were 12.97 % and ± 8.00 %, respectively. The data demonstrated that SYN550024 is stable in processed sample for up to 3 days at approximately 4 °C.

4.6 Dilution

(TABLE 7)
The precision and accuracy values determined for the validation sample prepared at five times the method ULQ were 7.69 % and ± 6.72 %, respectively, and demonstrated that samples may be diluted into the analytical range of the method using a dilution factor of up to 100.

4.7 Recovery

(TABLE 8)
The recovery of SYN550024 was determined to be 124.29 % at 12.5 ng/mL and 79.71 % at 5000 ng/mL. The recovery of the internal standard, diazepam-D5, ranged from 75.45 % to 79.51 % at 40.0 ng/mL.

4.8 Matrix Effects

(TABLE 8 and TABLE 9)
The extent to which extracted matrix components influenced SYN550024 analytical response was determined to be 6.20 % at 12.5 ng/mL and 3.37 % at 5000 ng/mL. Matrix effects on the internal standard, diazepam-D5, ranged from -0.12 % to 0.60 % at 40.0 ng/mL.

The coefficients of variation of the peak area ratios of SYN550024 to internal standard in six different sources of biological matrix were ≤ 15 % and therefore acceptable. The % CV was determined to be 9.92 % at 12.5 ng/mL and 1.82 % at 5000 ng/mL.

4.9 Carryover

(TABLE 10)
The mean carryover observed in samples of blank matrix positioned after the highest calibration standards was 0.03 % which corresponded to 26.07 % of the method LLQ.

5.0 DISCUSSION

Since carryover was > 20 % of the LLQ, the structure of analytical runs should be such as to minimise the impact, avoiding expected low concentration samples immediately succeeding expected high concentration samples. In this study, carryover did not appear to adversely affect the precision and accuracy of the validation samples at any level.

6.0 CONCLUSIONS

Since all acceptance criteria were met, the method is validated for the determination of SYN550024 in rat blood:water [1:1 (v/v)] over the analytical range 5.00 ng/mL to 6250 ng/mL.

TABLES SECTION

TABLE 1 Calibration Standard Data for SYN550024

	Calibration Standards and Theoretical Concentrations (ng/mL)								Calibration Curve Parameters			
Analytical Run Reference	**A 5.00**	**B 12.5**	**C 40.0**	**D 100**	**E 400**	**F 1000**	**G 2500**	**H 6250**	**A**	**B**	**C**	**Corr. Coeff.**
BFI0839R002	4.52 5.30	13.8 12.3	49.2a 41.4	103 91.2	425 384	1020 934	2980a 2470	6550 6010	2.37E-08	3.44E-03	3.24E-03	0.9976
BFI0839R003	5.81 4.16	13.2 12.0	41.4 39.2	95.5 98.8	418 418	1020 935	2540 2380	6330 6280	-6.11E-08	3.16E-03	1.36E-03	0.9967
BFI0839R004	5.07 5.29	11.6 10.9	41.8 39.5	104 103	397 384	1030 993	2640 2610	6130 6110	3.14E-09	4.03E-03	1.10E-03	0.9984
BFI0839R002r	4.69 5.12	12.8 13.1	43.4 42.3	93.4 95.9	395 388	970 963	2540 2580	6150 6320	1.60E-08	4.26E-03	2.96E-03	0.9988
BFI0839R007	2.87a 5.24	10.9 12.9	37.1 39.5	97.8 105	377 412	1030 1080	2610 2590	6070 6150	-3.22E-10	2.75E-03	5.65E-03	0.9981
Mean	4.81	12.4	41.5	98.8	400	998	2594	6210				
Std. Dev.	0.824	0.988	3.27	4.81	17.1	46.9	156	160				
CV (%)	17.13	7.97	7.88	4.87	4.28	4.70	6.01	2.58				
Bias (%)	-3.80	-0.80	3.75	-1.20	0.00	-0.20	3.76	-0.64				
n	10	10	10	10	10	10	10	10				

Calibration line equation: $y = Ax^2 + Bx + C$

a: standard excluded from calibration line, bias > ± 15 % (± 20 % at LLQ)

TABLE 2 Accuracy and Precision Data for SYN550024

Analytical Run Reference	VS1 5.00 ng/mL	VS2 12.5 ng/mL	VS3 200 ng/mL	VS4 5000 ng/mL	VS5 6250 ng/mL
BFI0839R002	5.15	11.7	209	5580	6470
	5.36	13.4	227	4280	5620
	4.84	12.0	225	4750	5950
	4.49	12.8	245	4680	6320
	4.87	14.8	205	4800	6200
	4.74	12.9	203	5960	6980
Mean	4.91	12.9	219	5008	6257
Std. Dev.	0.307	1.11	16.3	629	464
Precision (% CV)	6.25	8.60	7.44	12.56	7.42
Accuracy (% bias)	-1.80	3.20	9.50	0.16	0.11

TABLE 3 Short Term Biological Matrix Stability Data for SYN550024

Analytical Run Reference BFI0839R002 BFI0839R003	Reference	Test	Mean difference (%)
Concentration 12.5 ng/mL	11.7 13.4 12.0 12.8 14.8 12.9	12.4 13.2 12.0 13.3 13.3 11.7	
Mean Std. Dev. CV (%) Bias (%)	12.9 1.11 8.60 3.20	12.7 0.712 5.61 1.60	-1.55
Concentration 5000 ng/mL	5580 4280 4750 4680 4800 5960	5210 5470 5050 5780 4850 4940	
Mean Std. Dev. CV (%) Bias (%)	5008 629 12.56 0.16	5217 352 6.75 4.34	4.17

Storage period: 24 hours at room temperature

TABLE 4 Long Term Biological Matrix Stability Data for SYN550024

Analytical Run Reference BFI0839R002 BFI0839R007	Reference	Test	Mean difference (%)
Concentration 12.5 ng/mL	11.7 13.4 12.0 12.8 14.8 12.9	13.8 12.8 12.7 12.8 12.1 11.6	
Mean Std. Dev. CV (%) Bias (%)	12.9 1.11 8.60 3.20	12.6 0.745 5.91 0.80	-2.33
Concentration 5000 ng/mL	5580 4280 4750 4680 4800 5960	4910 4750 4830 4840 4910 4750	
Mean Std. Dev. CV (%) Bias (%)	5008 629 12.56 0.16	4832 71.7 1.48 -3.36	-3.51

Storage period: 29 days at approximately -80 °C

TABLE 5 Freeze-Thaw Stability Data for SYN550024 in Biological Matrix

Analytical Run Reference BFI0839R002 BFI0839R004	Reference	Test	Mean difference (%)
Concentration 12.5 ng/mL	11.7 13.4 12.0 12.8 14.8 12.9	12.6 13.3 13.5 12.0 11.6 12.3	
Mean Std. Dev. CV (%) Bias (%)	12.9 1.11 8.60 3.20	12.6 0.740 5.87 0.80	-2.33
Concentration 5000 ng/mL	5580 4280 4750 4680 4800 5960	4980 5170 5190 5090 5580 4590	
Mean Std. Dev. CV (%) Bias (%)	5008 629 12.56 0.16	5100 322 6.31 2.00	1.84

Samples were subjected to three freeze-thaw cycles from approximately -80 °C to room temperature

TABLE 6 Processed Sample Stability Data for SYN550024

Analytical Run Reference	VS1	VS2	VS3	VS4	VS5
	5.00 ng/mL	**12.5 ng/mL**	**200 ng/mL**	**5000 ng/mL**	**6250 ng/mL**
BFI0839R002r	5.80	11.3	203	5000	6510
	5.16	13.5	224	4220	5730
	5.12	13.6	222	4610	5910
	5.15	12.4	235	4660	5970
	4.84	15.1	206	4810	6220
	5.02	12.6	205	6080	6960
Mean	5.18	13.1	216	4897	6217
Std. Dev.	0.326	1.30	13.0	635	454
Precision (% CV)	6.29	9.92	6.02	12.97	7.30
Accuracy (% bias)	3.60	4.80	8.00	-2.06	-0.53

Storage period: 3 days at approximately 4 °C

TABLE 7 Dilution Data for SYN550024

Analytical Run Reference	31300
BFI0839R004	**ng/mL**
Dilution factor 100	32400
	31500
	35200
	37700
	32100
	31200
Mean	33350
Std. Dev.	2563
Precision (% CV)	7.69
Accuracy (% bias)	6.72

TABLE 8 Recovery and Matrix Effect Data for SYN550024

Analytical Run Reference	**SYN550024**		**Internal standard (diazepam-D5)**	
BFI0839R005	**VS2 level (12.5 ng/mL)**	**VS4 level (5000 ng/mL)**	**VS2 level (40.0 ng/mL)**	**VS4 level (40.0 ng/mL)**
Pure solution	11156.7 11124.8 10591.8 10602.3 10630.6 10529.5	5487724.6 5619566.0 5773161.2 5619514.4 5432611.5 5443414.1	393394.5 400381.4 386269.4 382193.4 383244.8 382964.1	356183.0 362726.0 353305.4 352928.5 349443.2 355606.7
Mean	10773	5562665	388075	355032
Pure solution containing extracted blank matrix	10093.7 9767.0 10121.0 9789.4 10833.9 10024.4	5518344.4 5373580.6 5333428.6 5432810.6 5403155.0 5190957.0	393719.7 384078.4 387058.5 384815.9 382168.6 382597.2	357078.0 353500.2 357422.6 357556.7 357857.0 349394.7
Mean	10105	5375379	385740	355468
Spiked matrix sample taken through the extraction procedure	14686.6 11899.7 12292.2 11743.5 12900.2 11829.9	4034147.7 4370811.9 4379516.5 4247023.0 4299642.1 4376388.0	292283.5 292253.4 282416.9 287404.7 298388.7 293549.3	275477.1 277388.9 284668.2 286468.6 289486.1 282238.6
Mean	12559	4284588	291049	282621
Recovery (Extraction efficiency) (%)	124.29	79.71	75.45	79.51
Matrix effects (%)	6.20	3.37	0.60	-0.12

TABLE 9 Matrix Effect Data in Six Different Batches of Rat Blood:Water [1:1 (v/v)]

Analytical Run Reference	Matrix batch	Peak Area Ratio
BFI0839R005		
Pure solution containing extracted blank matrix (VS2 level)	1	0.0288
	2	0.0304
	3	0.0259
	4	0.0232
	5	0.0247
	6	0.0268
	Mean	0.0266
	Std. Dev.	0.00264
	CV (%)	9.92
Pure solution containing extracted blank matrix (VS4 level)	1	14.8
	2	15.2
	3	14.8
	4	14.5
	5	15.2
	6	15.0
	Mean	14.9
	Std. Dev.	0.271
	CV (%)	1.82

TABLE 10 Carryover Data for SYN550024

Analytical Run Reference	Replicate	Response (peak area)			Carryover (%)	Carryover as proportion of LLQ (%)
		STD A LLQ	STD H ULQ	Double Blank (following STD H)		
BFI0839R002	1	7068.6	7800537.5	1595.9		
	2	7347.0	6657205.2	902.5		
	Mean	7207.8	7228871.4	1249.2	0.02	17.33
BFI0839R003	1	5991.5	5091153.8	2643.0		
	2	4373.9	4975752.5	1133.5		
	Mean	5182.7	5033453.2	1888.3	0.04	36.43
BFI0839R004	1	7245.5	7770478.9	2495.4		
	2	7476.9	7807564.6	2260.6		
	Mean	7361.2	7789021.8	2378.0	0.03	32.30
BFI0839R002r	1	8821.3	9688547.4	2491.6		
	2	9610.4	9776886.2	1402.0		
	Mean	9215.9	9732716.8	1946.8	0.02	21.12
BFI0839R007	1	5990.0	6362995.1	0.0		
	2	8263.9	6297447.1	3302.2		
	Mean	7127.0	6330221.1	1651.1	0.03	23.17
Mean carryover (%)					0.03	26.07

TABLE 11 Analytical Run Summary

Analytical run	Injection Date	Calibration standard acceptance criteria met	Validation (QC) sample acceptance criteria met	Stability acceptance criteria met	Validation Experiment	Run accepted
BFI0839R001	09-Oct-18	Y	N	N/A	Precision and accuracy 1	N
BFI0839R002	10-Oct-18	Y	Y	N/A	Precision and accuracy 1	Y
BFI0839R003	10-Oct-18	Y	Y	Y	Short term matrix stability, specificity	Y
BFI0839R004	12-Oct-18	Y	Y	Y	Freeze-thaw stability, dilution	Y
BFI0839R002r	12-Oct-18	Y	Y	Y	Processed sample stability	Y
BFI0839R005	16-Oct-18	N/A	N/A	N/A	Recovery and matrix effects	Y
BFI0839R006	07-Nov-18	Y	Y	Y	Long term matrix stability	N[1]
BFI0839R007	07-Nov-18	Y	Y	Y	Long term matrix stability	Y

[1] Run rejected since analyte was detected, at a level > 20 % of the LLQ, in two blank matrix samples

FIGURES SECTION

FIGURE 1 Representative Calibration Line for SYN550024

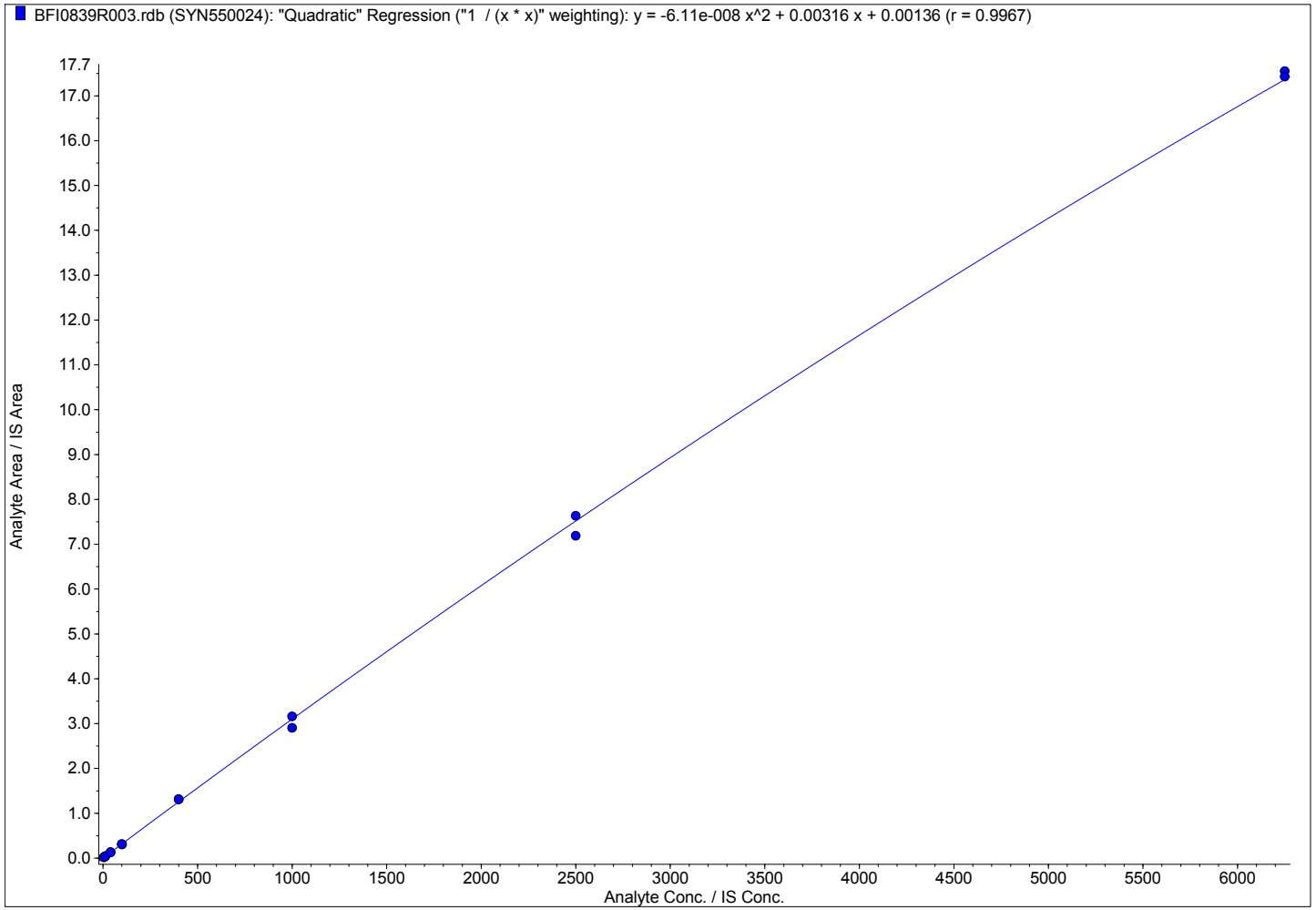


FIGURE 2 SRM Chromatogram of a Double Blank Sample of Rat Blood:water [1:1 (v/v)]

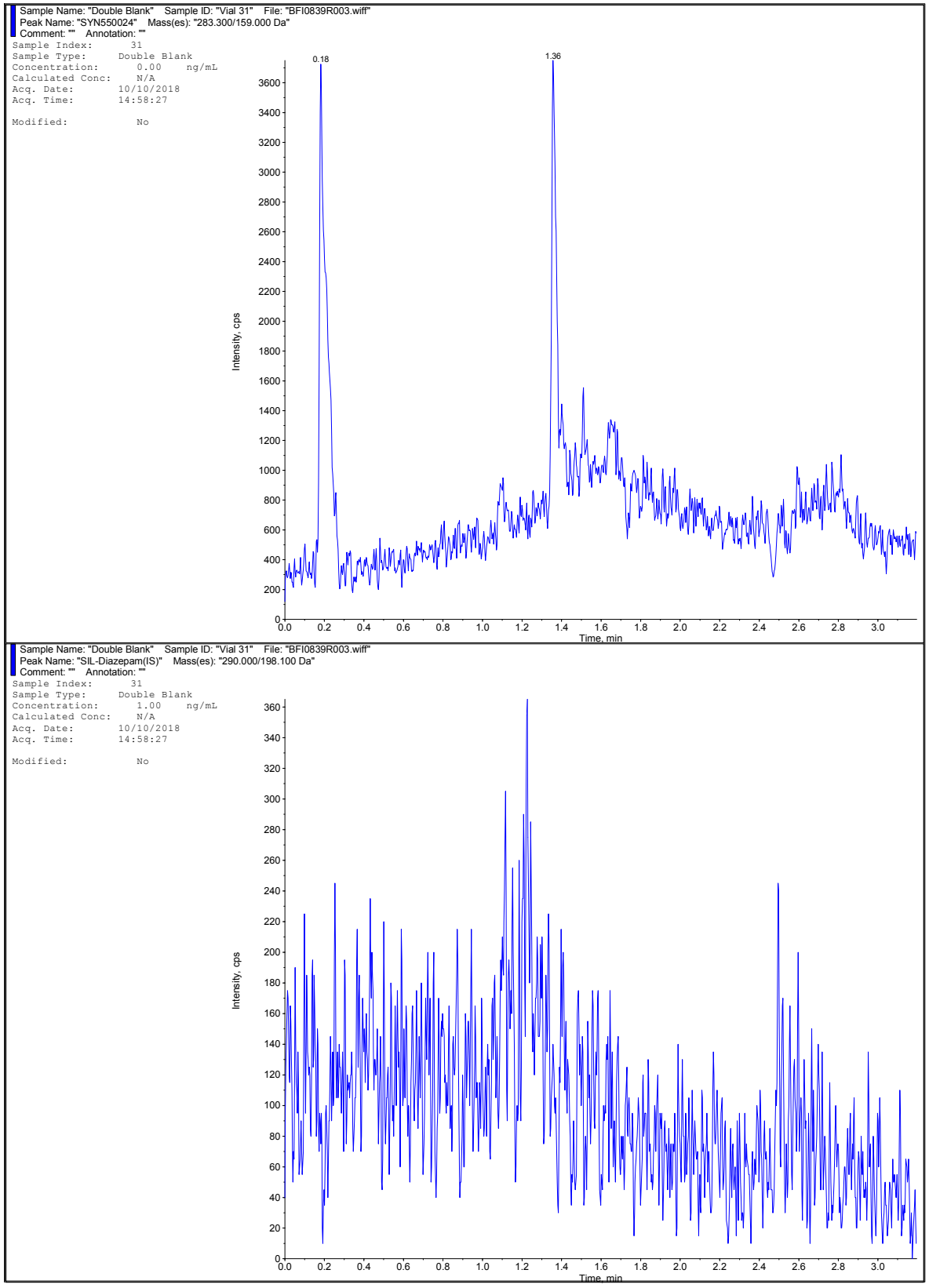


FIGURE 3 SRM Chromatogram of a Single Blank Sample of Rat Blood:water [1:1 (v/v)]

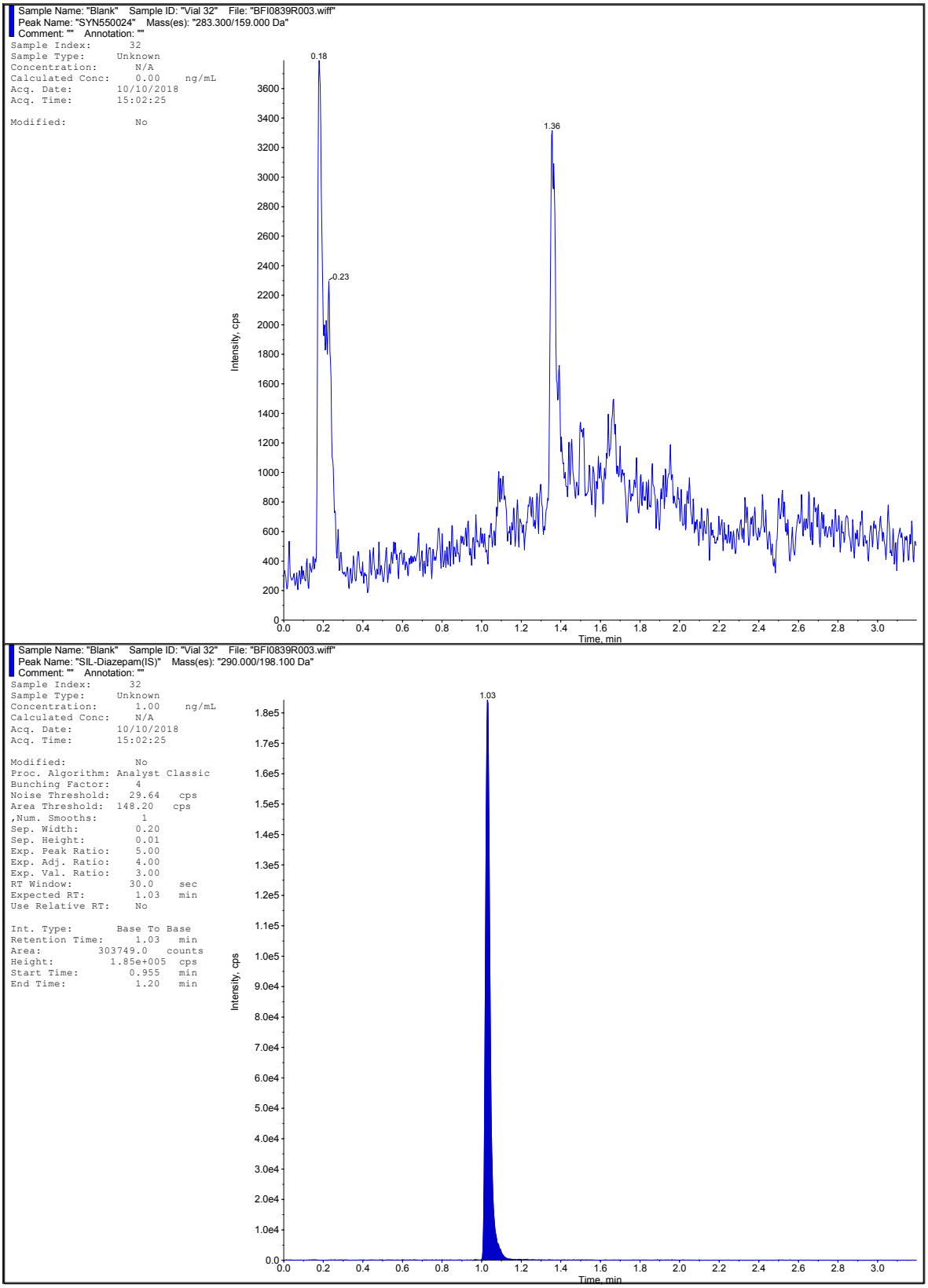


FIGURE 4 SRM Chromatogram of a Calibration Standard at the Lower Limit of Quantification (5.00 ng/mL)

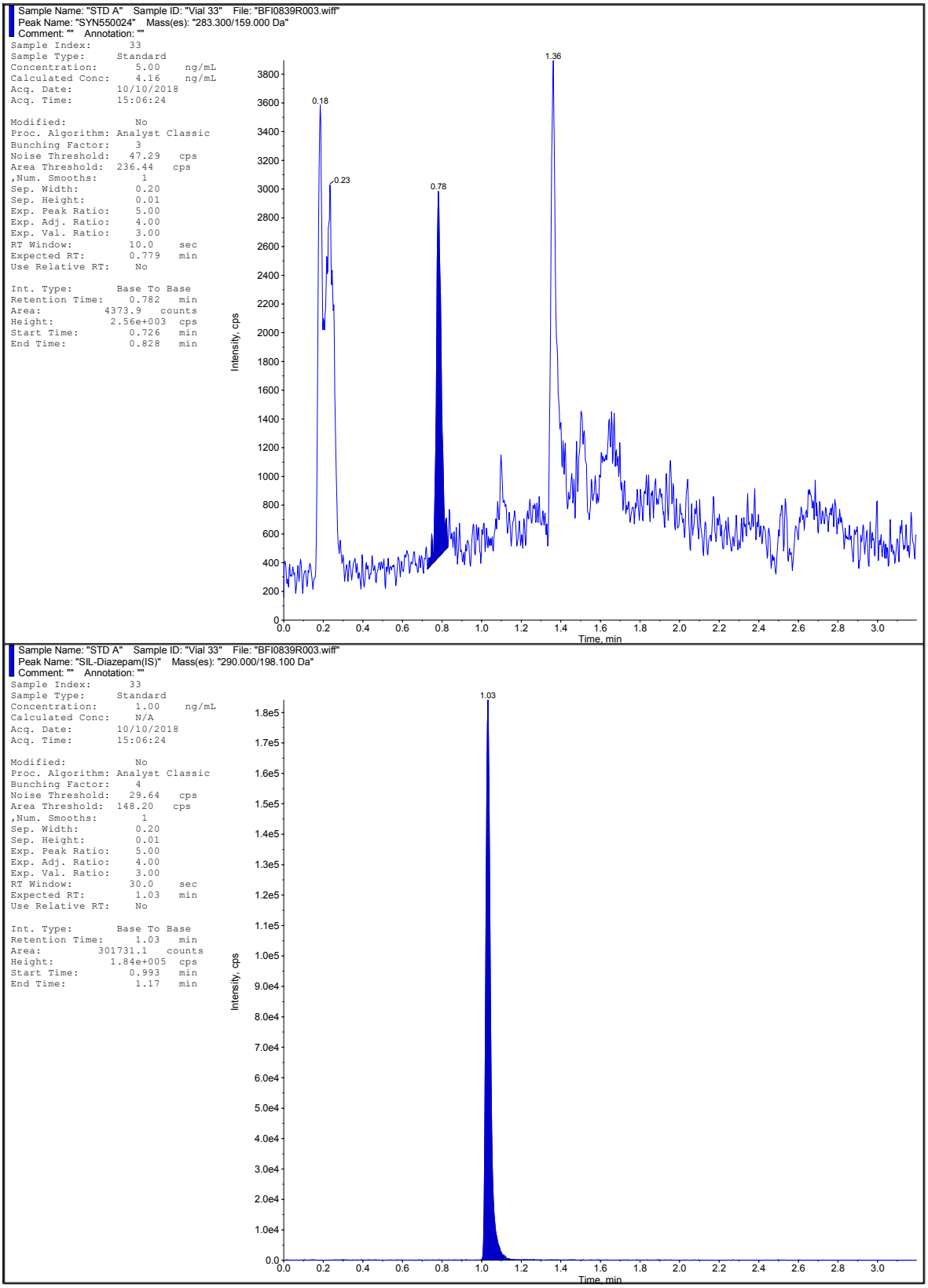
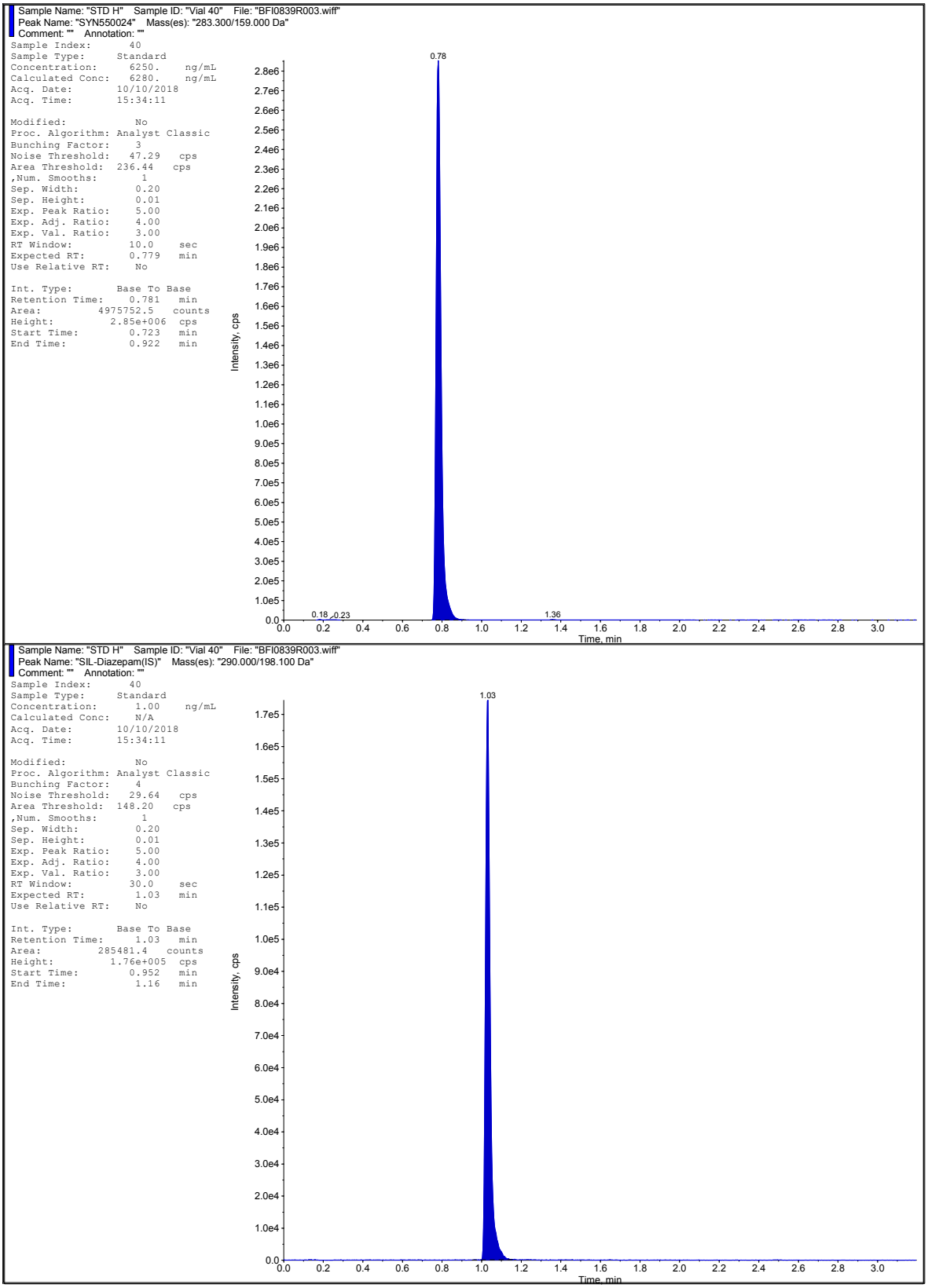


FIGURE 5 SRM Chromatogram of a Calibration Standard at the Upper Limit of Quantification (6250 ng/mL)



APPENDICES SECTION

APPENDIX 1 Analytical Method

Test Item	Matrix		Method No.	Method Type	Revision
SYN550024	Rat blood:water [1:1 (v/v)]		BFI121	MS	002

ANALYTICAL METHOD BFI121MS

Determination of SYN550024 in Rat Blood:Water [1:1 (v/v)] using LC-MS/MS

Revision History

Version	Date Created	Reason for Revision
001	03 Oct 2018	Draft
002	28 Nov 2018	Method validated (BFI0839). Stability information added to sections 5.1.1, 5.2, and 6.3. Information relating to carryover added to section 6.3.

Author: ..... Date 28 NOV 2018

I. Davies

Checked: ..... Date 28 NOV 2018

R. Lane

Study Number.....Signature.....

APPENDIX 1 Analytical Method (Continued)

Test Item	Matrix		Method No.	Method Type	Revision
SYN550024	Rat blood:water [1:1 (v/v)]		BFI121	MS	002

1. PRINCIPLE

SYN550024 (alternative name CSDK387956) is a potassium salt form of CSCY703464, the active metabolite of SYN550023 (alternative name CSCY990728). SYN550024 is determined in rat blood (with EDTA) diluted [1:1 (v/v)] with water by protein precipitation and the processed samples analysed by UPLC analysis with tandem mass spectrometric detection.

2. COSHH ASSESSMENT

Normal precautions are necessary when handling the test item i.e. disposable gloves, safety glasses, and laboratory coats.

3. APPARATUS/GLASSWARE/CONSUMABLES

3.1. APPARATUS

UPLC pump	Agilent *
Autosampler	CTC *
Mass Spectrometer	AB Sciex 6500 QTrap
analytical balance (5 dp)	Sartorius *
analytical balance (3 dp)	Sartorius *
Protein precipitation plates (2 mL reservoir)	Phenomenex*
Pipettes	Gilson *

*Alternative items of equipment may be used.

3.2. GLASSWARE

Grade A volumetric flasks	as required
general laboratory glassware	as required

3.3. CONSUMABLES

pipette tips	as required
tubes	as required

4. REAGENTS / SOLUTIONS

4.1. REAGENTS

Formic acid	analytical grade	Fisher[1]
Acetonitrile	HPLC grade	Fisher[1]
Methanol	HPLC grade	Fisher[1]
Propan-2-ol	HPLC grade	Fisher[1]
UHP water		In house
Dimethyl sulphoxide	analytical grade	Fisher[1]

[1]Equivalent materials from other sources are acceptable for general reagents.

APPENDIX 1 Analytical Method (Continued)

Test Item	Matrix		Method No.	Method Type	Revision
SYN550024	Rat blood:water [1:1 (v/v)]		BFI121	MS	002

4.2. REAGENT SOLUTIONS

4.2.1. 0.1 % (v/v) Formic acid – mobile phase A

1 mL of formic acid and 1000 mL of UHP water are added to a suitable storage vessel and mixed. The solvent is stored at ambient temperature for up to 2 weeks.

4.2.2. Methanol – mobile phase B

1000 mL of methanol are added to a suitable storage vessel. The solvent is stored at ambient temperature for up to 3 months.

4.2.3. Formic acid 0.1 % (v/v) in acetonitrile:propan-2-ol:UHP water [4:3:3 (v/v)] – needle rinse 1

400 mL of acetonitrile, 300 mL of propan-2-ol and 300 mL of UHP water are added to a 1 L measuring cylinder. 1 mL of formic acid is added and the contents mixed. The solution is transferred to a storage vessel and is stored at ambient temperature for up to 3 months.

4.2.4. Methanol – needle rinse 2

500 mL of methanol are added to the auto sampler needle rinse bottle. The solution is stored at ambient temperature for up to 3 months.

4.2.5. 0.1 % (v/v) Formic acid : methanol [65:35 (v/v)]

0.65 mL of formic acid are added to 650 mL of water in a suitable storage vessel and mixed. 350 mL of methanol are added and the solution mixed. The solution is stored at ambient temperature for up to 3 months.

4.2.6. Methanol:DMSO [1:1 (v/v)]

100 mL of methanol are added to 100 mL of DMSO in a suitable vessel and mixed. The solution is stored at ambient temperature for upto 3 months.

APPENDIX 1 Analytical Method (Continued)

Test Item	Matrix		Method No.	Method Type	Revision
SYN550024	Rat blood:water [1:1 (v/v)]		BFI121	MS	002

5. TEST ITEM / REFERENCE STANDARDS

Test Item / Reference Standard	Source
SYN550024 (CSDK387956)	Sponsor
SIL-Diazepam	Sigma Aldrich*

Test items / reference standards are corrected for purity and salt form during weighing.
*Equivalent materials from other sources are acceptable for use as internal standard.

5.1. ANALYTICAL STOCK SOLUTIONS

The quantities and volumes specified are illustrative and may be scaled up or down as required.

5.1.1. SYN550024

Accurately weigh out approximately 10 mg of SYN550024, and dissolve in methanol:DMSO [1:1 (v/v)], with sonication if necessary, and dilute to volume with the same solvent in a 10 mL Grade A volumetric flask to give a solution of approximate concentration 1000 µg/mL. Prepare two solutions, one for preparation of calibration standards (A) and the other for Quality Control samples (B). The amount weighed should be corrected for purity and salt form (unless blood concentrations are reported in terms of SYN550024). These solutions should be stored at approximately 4 °C for up to 32 days.

5.2. DILUTED SOLUTIONS

The duplicate analytical stock solutions (A and B) for SYN550024 should be diluted with DMSO:methanol [1:1 (v/v)] to produce two sets of diluted solutions, each set prepared as indicated in the following table:

Volume taken (µL)	Concentration (µg/mL)	Final volume (mL)	Concentration (µg/mL)
62.5	1000	1	62.5
160	62.5	1	10.0
100	10.0	1	1.00
125	1.00	1	0.125

The volumes specified in the table are illustrative and may be scaled up or down as required where different volumes of diluted solution are required. Diluted solutions have been shown to be stable for up to 5 hours at room temperature.

5.3. INTERNAL STANDARD STOCK SOLUTION

The internal standard SIL-Diazepam is purchased as a 1000 µg/mL solution in methanol. This solution should be stored at approximately -20 °C for long term use.

5.4. INTERNAL STANDARD WORKING SOLUTION

On the day of an analytical run the internal standard stock solution should be diluted with acetonitrile to produce an internal standard working solution, to be used in the sample preparation procedure, as detailed in the following table:

Volume taken (µL)	Concentration (µg/mL)	Final volume (mL)	Concentration (ng/mL)
10.0	1000	1.00	10000
100	10.0	25.0	40.0

The volumes specified in the table are illustrative and may be scaled up or down as required where different volumes of internal standard are required.

6. PROCEDURES

6.1. CALIBRATION STANDARDS

Calibration standards are prepared by aliquotting volumes of the diluted solutions (A) into polypropylene tubes and adding the appropriate volume of rat blood : water [1:1 (v/v)], the blood containing EDTA as anticoagulant. The samples are then mixed briefly. Calibration standards should be aliquotted into polypropylene tubes in appropriate portions and stored at approximately -80°C, if necessary, if stability has been demonstrated. The calibration standards are prepared as indicated in the table below:

Calibration standard	Spiking solution conc. (µg/mL)	Spike volume (µL)	Final volume matrix (mL)	Matrix conc. (ng/mL)	Blood conc. (ng/mL)
A	0.125	4.00	0.200	2.50	5.00
B	0.125	10.0	0.200	6.25	12.5
C	1.00	4.00	0.200	20.0	40.0
D	1.00	10.0	0.200	50.0	100
E	10.0	4.00	0.200	200	400
F	10.0	10.0	0.200	500	1000
G	62.5	4.00	0.200	1250	2500
H	62.5	10.0	0.200	3125	6250

The volumes specified in the tables are illustrative and may be scaled up appropriately where increased volumes of calibration standards are required.

6.2. VALIDATION SAMPLES / QUALITY CONTROL SAMPLES

Validation Samples / Quality Control (QC) samples are prepared by aliquotting volumes of the diluted solutions (B) into polypropylene tubes and adding the appropriate volume of of rat blood : water [1:1 (v/v)], the blood containing EDTA as anticoagulant. The samples are then mixed briefly. Validation / QC samples should be aliquotted into polypropylene tubes in appropriate portions and stored at approximately -80°C. The Validation / QC samples are prepared as indicated in the table below:

APPENDIX 1 Analytical Method (Continued)

Test Item	Matrix		Method No.	Method Type	Revision
SYN550024	Rat blood:water [1:1 (v/v)]		BFI121	MS	002

Validation/QC sample	Spiking solution conc. (µg/mL)	Spike volume (µL)	Final volume matrix (mL)	Matrix conc. (ng/mL)	Blood conc. (ng/mL)
VS1	0.125	8.00	0.400	2.50	5.00
VS2 / QC low	0.125	20.0	0.400	6.25	12.5
VS3 / QC med	10.0	4.00	0.400	100	200
VS4 / QC high	62.5	16.0	0.400	2500	5000
VS5	62.5	20.0	0.400	3125	6250

The volumes specified in the tables are illustrative and may be scaled up or down when different volumes of Validation / QC Samples are required. Validation samples prepared at concentrations greater than 6250 ng/mL (blood concentration) may be prepared directly from the stock analyte solutions if necessary, or from diluted solutions other than those detailed in section 5.2.

6.3. SAMPLE PREPARATION

SYN550024 has been shown to be stable in rat blood : water [1:1 (v/v)] for up to 24 hours at room temperature, up to 29 days when stored frozen at approximately -80 °C, and through 3 freeze-thaw cycles. Processed samples are stable for 3 days within the chilled autosampler compartment (approximately 4 °C). Mean carryover determined in the validation study was > 20 % of the LLQ. Any impact should be minimised by judicious arrangement of study samples, for example, samples with expected low concentrations should not immediately succeed those of expected high concentration (analysis in time profile order is acceptable but avoid late terminal phase samples or controls succeeding t_{max} samples).

Samples are extracted from of rat blood : water [1:1 (v/v)] by protein precipitation according to the procedure given below:

1	Aliquot 100 µL of acetonitrile, into the wells of the protein plate which are intended for the double blank samples.
2	Aliquot 100 µL of working internal standard solution (40.0 ng/mL) into all other wells
3	Aliquot 5 µL of calibration standard, Validation / QC sample, study sample, blank rat blood: water [1:1 (v/v)] into the appropriate wells of the protein plate.
4	Agitate the liquids in the wells by placing the protein plate onto a vortex mixer. Grip the protein plate and mix the samples at a moderate speed for approximately 10 seconds.
5	Place a collection plate or a rack of 1.4 mL micronic tubes within the extraction manifold, and place the protein plate on top of the manifold. Draw the supernatants through the filters into the collection tubes by applying vacuum to the manifold.
6	Add 800 uL of 0.1 % (v/v) formic acid : methanol [65:35 (v/v)] to the tubes, vortex mix for 30 seconds, centrifuge, and inject samples into LC-MS/MS system.

APPENDIX 1 Analytical Method (Continued)

Test Item	Matrix		Method No.	Method Type	Revision
SYN550024	Rat blood:water [1:1 (v/v)]		BFI121	MS	002

6.4. HPLC CONDITIONS

HPLC column	50 x 2.1 mm Kinetex C18, 2.6µm
Column temperature	60 °C
Flow rate	0.7 mL/min
Mobile phase A	0.1% Formic acid
Mobile phase B	Methanol
Needle Rinse 1	Formic acid 0.1 % (v/v) in acetonitrile:propan-2-ol:UHP water [4:3:3 (v/v)]
Needle Rinse 2	Methanol
HPLC mode	Gradient
Typical injection volume	1-15 µL

Gradient programme

Time (min)	Composition (%) A	Composition (%) B
0.00	65	35
1.50	0	100
3.00	0	100
3.01	65	35
3.20	65	35

6.5. MASS SPECTROMETER CONDITIONS

Ionisation mode	Electro spray
Polarity	Positive
Curtain gas (nitrogen)	40*
Gas 1	60*
Gas 2	60*
Collision gas (nitrogen)	5*
Source / auxiliary gas temperature	550 °C
LC effluent split ratio	100 % to mass spectrometer

* The values given are approximate

Mass Spectrometer Analyte Detection Conditions

Analyte	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Typical retention time (min)
SYN550024	283	159	50	0.7
SIL-Diazepam	290	198	50	0.9
SYN550023 *	341	173	50	1.0

Nominal m/z values are shown for precursor and product ions.
* The mass transition for the parent (procide) is retained and only used when specifically required e.g. study plan specifies monitoring or quantification of parent.

7. QUANTIFICATION

The peak area ratios of the calibration standards are plotted against theoretical concentration and a quadratic regression analysis carried out using the proprietary software Analyst® (AB Sciex Ltd). The

calibration line is weighted by a factor $1/X^2$. Validation Sample, Quality Control Sample, and study sample concentrations are interpolated from the calibration line.

8. DEVIATIONS FROM THE METHOD

Any deviations from this method should be documented on the appropriate Analytical Run Front Sheet (SOP BIO.GEN.001).

APPENDIX 2 GLP Compliance Certificate

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

SEQUANI LIMITED
BROMYARD ROAD
LEDBURY
HR8 1LH
UNITED KINGDOM

TEST TYPE(S)

Analytical Chemistry
Mutagenicity
Toxicology

DATE OF INSPECTION: 19/06/2018 – 20/06/2018

DATE OF ISSUE: 21/09/2018

An Inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above named test facility as part of the UK Good Laboratory Practice Compliance Monitoring Programme.

This statement confirms that, on the date of issue, the UK Good Laboratory Practice Monitoring Authority were satisfied that the above named test facility was operating in compliance with the OECD Principles of Good Laboratory Practice.

This statement constitutes a Good Laboratory Practice Instrument (as defined in the UK Good Laboratory Practice Regulations 1999).

Issued by
Dr Andrew J Gray
Head, UK GLP Monitoring Authority

Medicines & Healthcare products Regulatory Agency

MHRA

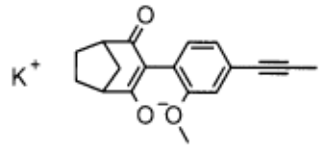
APPENDIX 3 Certificate of Analysis for SYN550024

syngenta

Syngenta Crop Protection AG
GLP Testing Facility WMU
Analytical Development & Product Chemistry
Breitenloh 5
4333 Münchwilen, Switzerland

Certificate of Analysis

SYN550024



MES 603/1 - Purity 44 %

Batch Identification	**MES 603/1**
Other Batch ID	1043058
Product Code	**SYN550024**
Parent	SYN550023
Other Product Code(s)	CSDK387956
ISO Common Name	---
CA Reg. No.	---
CA Index Name	---
IUPAC Name	potassium;3-(2-methoxy-4-prop-1-ynyl-phenyl)-4-oxo-bicyclo[3.2.1]oct-2-en-2-olate
Molecular formula	$C_{18}H_{17}O_3K$
Molecular mass	320.4
Chemical Analysis	
– **Identity of SYN550024***	**confirmed**
– **Content of SYN550024***	**44 % w/w** (estimated error: ± 2 %)
– **Content of water***	**55.9 % w/w**
Methodology used for Characterization / Recertification	NMR, LC, Karl Fischer Titration
Physical Analysis	
– **Appearance***	**yellowish liquid**
Stability:	
– **Storage Temperature**	**< 10 °C**
– **Recertification Date**	**End of July 2020**

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.
This Certificate of Analysis summarizes data which originates either from a single study or from several individual studies. Tests marked with an asterisk (*) have been conducted in compliance with GLP.
Raw data, documentation, study plans, any amendments to study plans and reports pertaining to this/these study/studies are stored under the study number(s) referenced below within the archives of the GLP Testing Facility WMU at Syngenta Crop Protection AG, Switzerland.

Study number of batch characterization: CHMU180409
Study number(s) of batch recertification: ---

Authorization: 24-July-2018

C. Simonin

Dr. Céline Simonin
Analytical Development & Product Chemistry

Page 1 of 1

APPENDIX 4 Certificate of Analysis for Diazepam-D5

Cerilliant
Analytical Reference Standards
a SIGMA-ALDRICH company

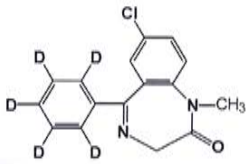
D-910
FE07061501
Revision 00
Page 1 of 9
Product of USA

Certified Reference Material - Certificate of Analysis

Diazepam-D_5, Primary Standard

Cerilliant Quality
ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 15194
ISO 9001
GMP/GLP

Catalog Number:	D-910
Lot:	FE07061501
Expiration:	July 2020
Description:	Diazepam-D_5 in Methanol.
Packaging:	Solution in 2 mL amber USP Type I glass ampoule containing not less than 1 mL of certified solution.
Storage:	Store unopened in freezer (-10 °C to -25 °C).
Shipping:	Ambient. See Stability Section.
Intended Use:	This Certified Reference Material is suitable for the *in vitro* identification, calibration, and quantification of the analyte(s) in analytical and R&D applications. Not suitable for human or animal consumption.
Instructions for Use:	Users should quantitatively transfer desired volume using established good laboratory practices to spike into matrix or to dilute to the desired concentration. Each ampoule is intended for one-time use.
Regulatory:	USDEA Exempt | Canadian TK # 61-399 **Safety: Danger. See Safety Data Sheet**



- Expiration date has been established through real time stability studies.
- Ampoules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette.
- For quantitative applications, the minimum sample size for intended use is 1 µL.
- For MS Applications, we advise laboratories not to mix lots during a single sequence.

Analyte	Certified Concentration Value
Diazepam-D_5	1.000 ± 0.005 mg/mL

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the mass balance purity factor, material density, balance, and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Nominal concentration is calculated based on: the actual measured mass; Mass Balance Purity Factor of the analyte(s); measured mass of the solution; and the density of the pure diluent at 20 °C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics. No adjustment required before use.
- Additional certification information available upon request.

Metrological Traceability

- This standard has been prepared and certified under the ISO Guide 34, ISO/IEC 17025, ISO 9001 and ISO 13485 standards. This standard meets the requirements of a Certified Reference Material and a Primary Standard as defined by ISO and is traceable to the SI and higher order standards through an unbroken chain of comparisons.
- This standard has been gravimetrically prepared using balances that have been fully qualified and calibrated to ISO 17025 requirements. All calibrations utilize NIST traceable weights which are calibrated externally by a qualified ISO 17025 accredited calibration laboratory to NIST standards. Qualification of each balance includes the assignment of a minimum weighing by a qualified and ISO 17025 accredited calibration vendor taking into consideration the balance and installed environmental conditions to ensure compliance with USP tolerances of NMT 0.1% relative error. Balance calibration adjustments are performed weekly utilizing the balance's internal adjustment mechanism. Calibration verifications are performed pre-use. Weigh tapes from the calibration verification are included in the production batch record for thi standard. Production data package available upon request.
- Fill volume is gravimetrically verified throughout the dispensing process using qualified and calibrated balances.
- Concentration is verified against an independently prepared calibration solution gravimetrically prepared.
- Each raw material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of this COA. The density and material Mass Balance Purity Factor is traceable to the SI and higher order reference standards through mass measurement and instrument qualification and calibrations.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS ACCREDITED
REFERENCE MATERIAL PRODUCER

Darron Ellsworth, Quality Assurance Manager

September 11, 2015
Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78665 800-848-7837 / 512-238-9974

Solution Standard Verification

Concentration accuracy and within- and between-bottle homogeneity are analytically verified against an independently prepared calibration solution and to the prior lot.

Solution standard verification demonstrates confirmation that the specified requirements for the Primary Standard have been fulfilled and validated under ISO 13485.

Standard Solution Assay Parameters

Analysis Method: HPLC/UV
Column: Ascentis Express C18, 2.7 µm, 3.0 x 50 mm
Mobile Phase: A::Acetonitrile
B::Water

Gradient Program:

Time (min)	%A	%B
0.0	30	70
4.0	70	30
5.0	70	30
5.1	30	70
7.0	30	70

Flow Rate: 0.8 mL/min
Wavelength: 238 nm

Calibration Curve

Calibration Curve: Linear Regression
Number of Points: 4
Linearity (r): 1.000

Standard Solution	Lot Number	Verified Concentration (mg/mL)		%RSD - Homogeneity	
		Actual Results	Acceptance Criteria	Actual Results	Acceptance Criteria
New Lot	FE07061501	1.008	± 3%	0.5	≤ 3%
Previous Lot	FE10301402	1.019	± 3%	0.3	≤ 3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration solution.
- Within-sample and between-sample homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. Multiple samples pulled from across the lot using a random stratified sampling plan were analyzed to verify homogeneity. % RSD results shown above for the New Lot demonstrate ampoule-to-ampoule homogeneity.

Analyte Certification - Mass Balance Purity Factor

Each analyte is thoroughly identified and characterized using an orthogonal approach. A mass balance purity factor is assigned incorporating chromatographic purity and residual impurities. The mass balance purity factor is utilized to calculate the weighing adjustment necessary to ensure accuracy of the solution standard concentration.

Material Name:	Diazepam-D_5	**Chemical Formula:**	$C_{16}H_8D_5ClN_2O$
Material Lot:	FC10011401	**CAS Number:**	65854-76-4
		Molecular Weight:	289.77

Material Characterization Summary

Analytical Test	Method	Results	
Primary Chromatographic Purity by HPLC/UV Analysis	SP10-0102	99.7%	
Secondary Chromatographic Purity by GC/FID Analysis	SP10-0101	99.8%	
Identity by GC/MS Analysis	SP10-0105	Consistent with Structure	
Identity by LC/MS Analysis	SP10-0107	Consistent with Structure	
Isotopic Purity and Distribution by LC/MS-SIM Analysis	SP10-0107	0.00% D_0 vs D_5	
		0.00% D_0 to D_3	98.40% D_5
		1.59% D_4	
Identity by ^{1}H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure	
Residual Solvent Analysis by GC/FID Headspace	AM1087 [1]	0.24%	
Residual Water Analysis by Karl Fischer Coulometry	AM1346 [1]	0.47%	
Inorganic Content by Microash Analysis	SP10-0135	< 0.2%	
Mass Balance *Purity Factor*		99.04%	

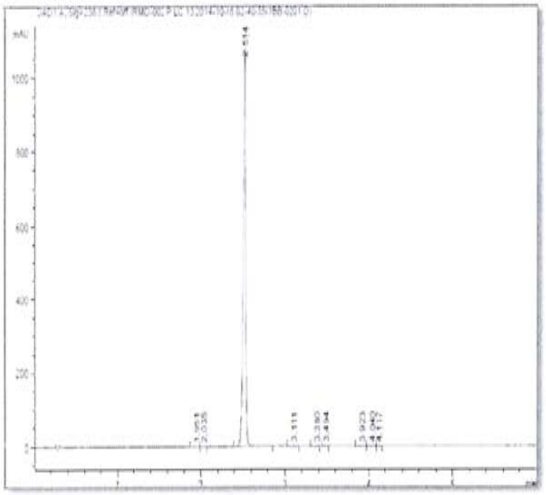
- The primary chromatographic purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other.
- The primary chromatographic purity value is used to calculate the Mass Balance Purity Factor.
- A secondary chromatographic purity method is utilized as a control.
- Mass Balance Purity Factor = [(100 - wt% residual solvent - wt% residual water - wt% residual inorganics) x Chromatographic Purity/100].
- Mass Balance Purity Factor does not include adjustment for chiral and/or isotopic purity.

[1] Validated analytical method

APPENDIX 4 Certificate of Analysis for Diazepam-D5 (Continued)

Spectral and Physical Data

HPLC/UV



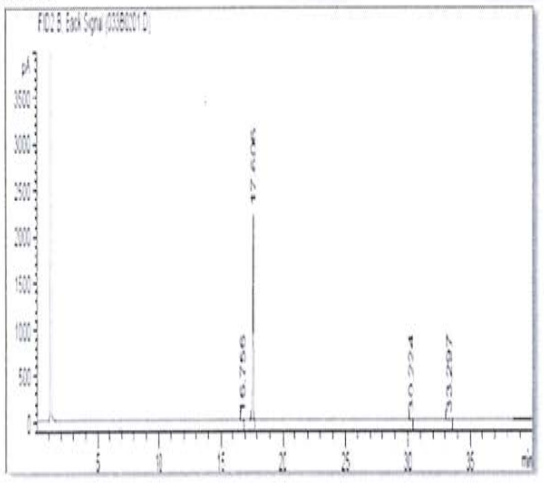
Column:	Ascentis Express C18, 2.7 µm, 3.0 x 50 mm
Mobile Phase:	A::Acetonitrile
	B::Water

Gradient Program:	Time (min)	%A	%B
	0.0	30	70
	4.0	70	30
	5.0	70	30
	5.1	30	70
	7.0	30	70

Flow Rate:	0.8 mL/min
Wavelength:	238 nm
Data File Name:	RMD-002 P LC 13 2014-10-18 07-31-35\1BB-0201.D
Instrument:	LC#13
Sample Name:	FC10011401
Acquired:	October 18, 2014

Peak #	Ret Time	Area	Height	Area %
1	1.66	0.03	0.02	0.00
2	1.92	0.19	0.07	0.01
3	2.02	0.07	0.03	0.00
4	2.61	2167.01	1121.71	99.75
5	3.22	0.05	0.02	0.00
6	3.39	2.54	1.27	0.12
7	3.61	0.04	0.02	0.00
8	3.66	0.05	0.03	0.00
9	4.24	0.78	0.40	0.04
10	4.33	1.72	0.88	0.08
11	4.64	0.06	0.03	0.00

GC/FID



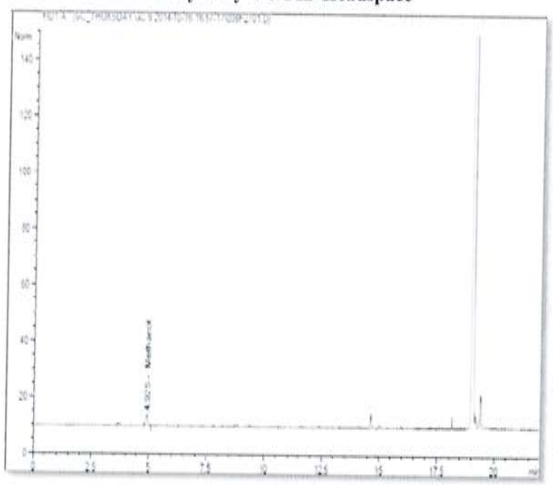
Column:	DB-35ms, 30 m x 0.53 mm ID, 1.0 µm film thickness
Temp Program:	40°C to 200°C at 40°C/min
	200°C to 300°C at 5°C/min hold 16 min
Injector Temp:	Cool-on-Column
Detector Temp:	325°C
Data File Name:	RMD-002 P 2014-10-29 09-34-33 GC 10\033B0201.D
Instrument:	GC#10
Sample Name:	FC10011401
Acquired:	October 29, 2014

Peak #	Ret Time	Area	Height	Area %
1	16.76	13.39	2.89	0.12
2	17.61	11110.80	2217.47	99.77
3	30.22	2.91	0.29	0.03
4	33.30	9.79	0.72	0.09

APPENDIX 4 Certificate of Analysis for Diazepam-D5 (Continued)

Spectral and Physical Data (cont.)

Residual Solvent Analysis by GC/FID Headspace

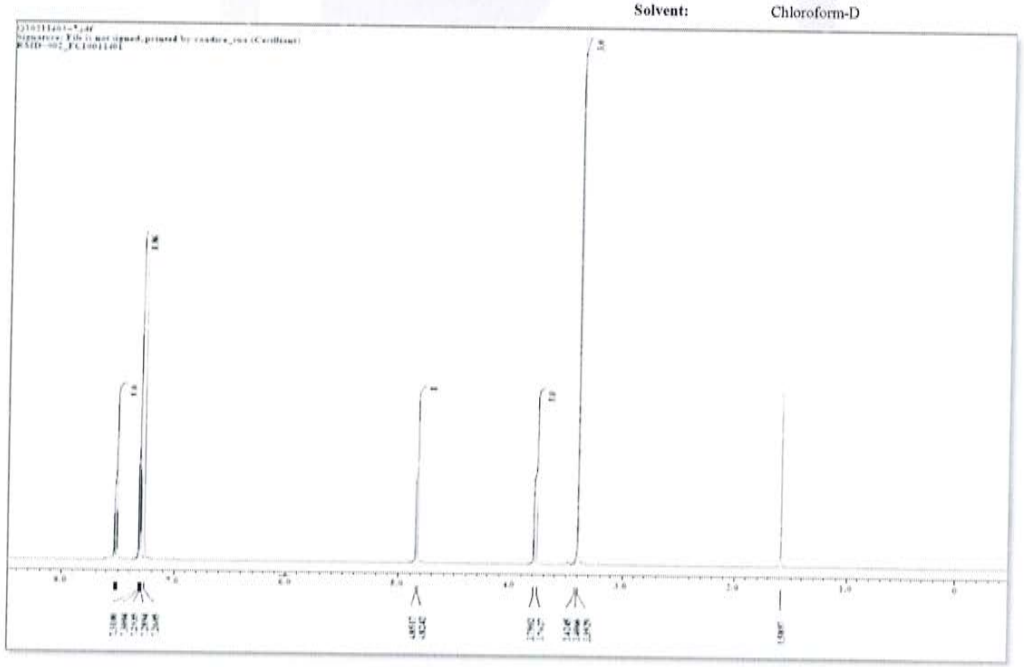


Column:	DB-ALC1 30 m x 0.53 mm, 3 µm film thickness
Temp Program:	40°C (12 min) to 220°C at 40°C/min (5.5 min)
Carrier Gas:	Helium
Flow Rate:	2.0 mL/min
Detector Heater Temp:	250°C
Injector:	Headspace Sampler
HS Oven Temp:	60°C
Vial Equilibration:	10 minutes
Data File Name:	GC 9 2014-10-16 16-57-17\036F2701.D
Instrument:	GC#9
Sample Name:	FC10011401
Acquired:	October 17, 2014

Peak	Compound	Area	Weight %
1	Methanol	25.21	0.24
2	NMP	NA	NA
Total			**0.24**

^{1}H NMR

Instrument: JEOL ECS 400
Solvent: Chloroform-D

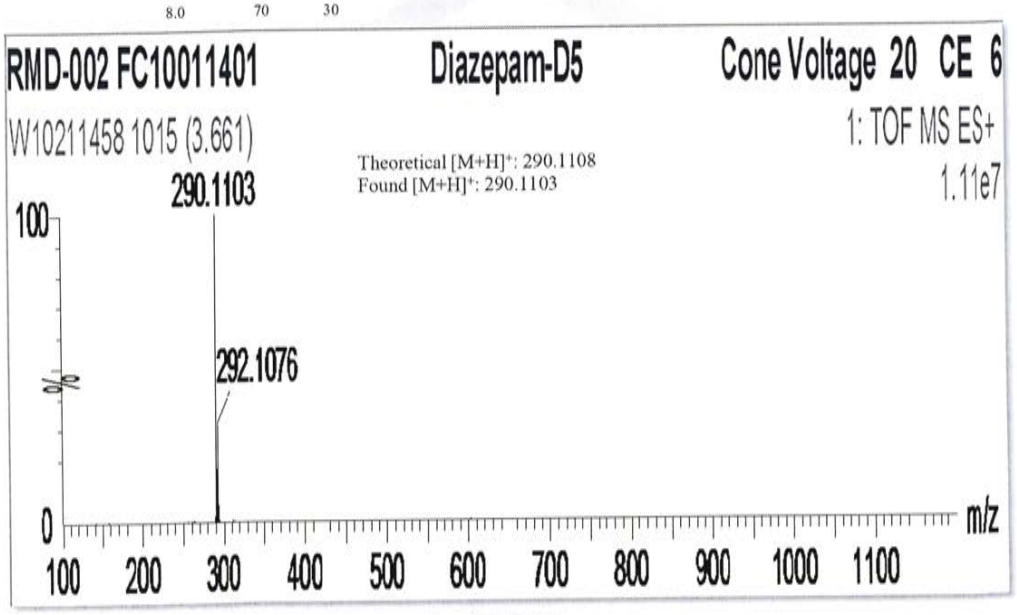


Spectral and Physical Data (cont.)

LC/MS

Column:	Ascentis Express C18, 2.7 µm, 3.0 x 50 mm		
Mobile Phase:	A::0.1% Formic acid in Water		
	B::Acetonitrile		
Gradient	Time (min)	%A	%B
Program:	0.0	70	30
	5.0	40	60
	6.0	40	60
	6.1	70	30
	8.0	70	30

Flow Rate:	0.4 mL/min
Scan Range:	100-1200 amu
Ionization:	Electrospray, Positive Ion
Data File Name:	W10211458
Instrument:	Waters XEVO G2 QTOF
Sample Name:	FC10011401
Acquired:	October 21, 2014

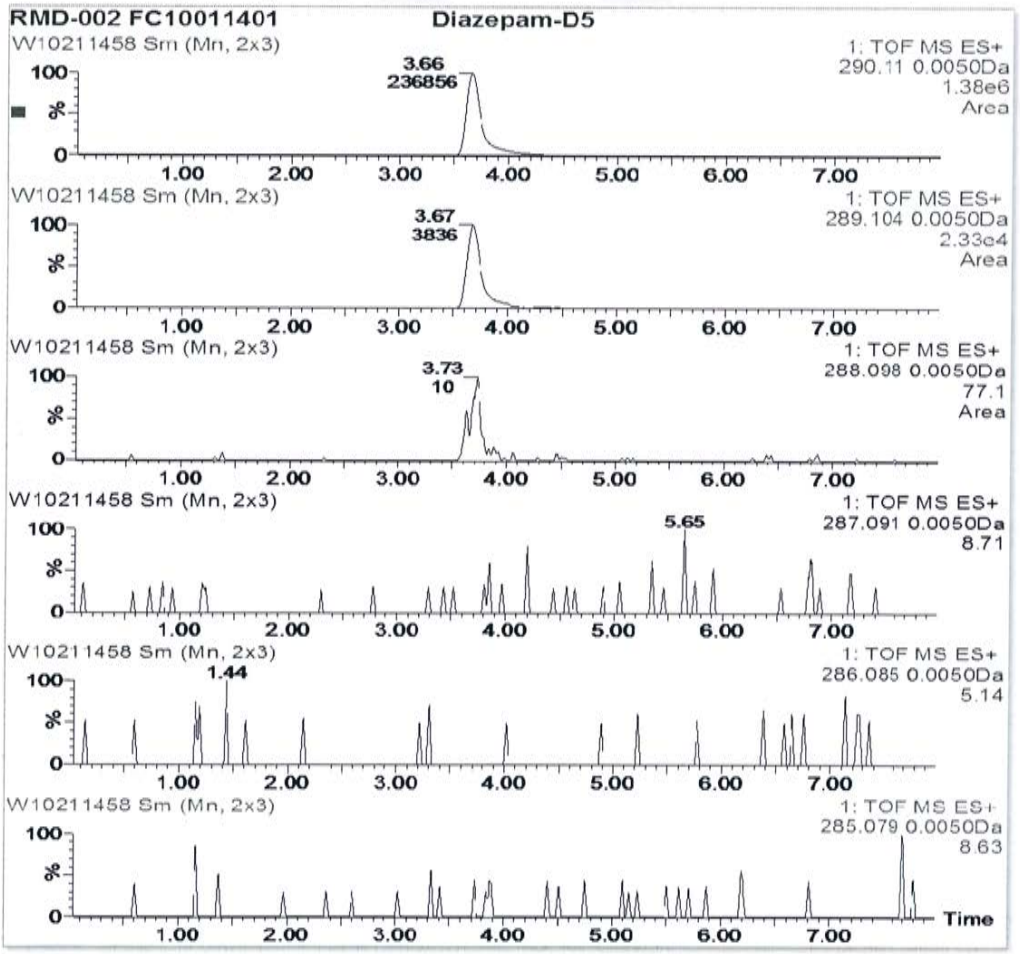


Spectral and Physical Data (cont.)

Isotopic Purity and Distribution by LC/MS

Column:	Ascentis Express C18, 2.7 µm, 3.0 x 50 mm	**Flow Rate:**	0.4 mL/min
Mobile Phase:	A::0.1% Formic acid in Water	**Scan Range:**	285-290 amu
	B::Acetonitrile	**Ionization:**	Electrospray, Positive Ion
Gradient Program:		**Data File Name:**	W10211458
		Instrument:	Waters XEVO G2 QTOF
		Sample Name:	FC10011401
		Acquired:	October 21, 2014

Gradient Program: Time (min)	%A	%B
0.0	70	30
5.0	40	60
6.0	40	60
6.1	70	30
8.0	70	30



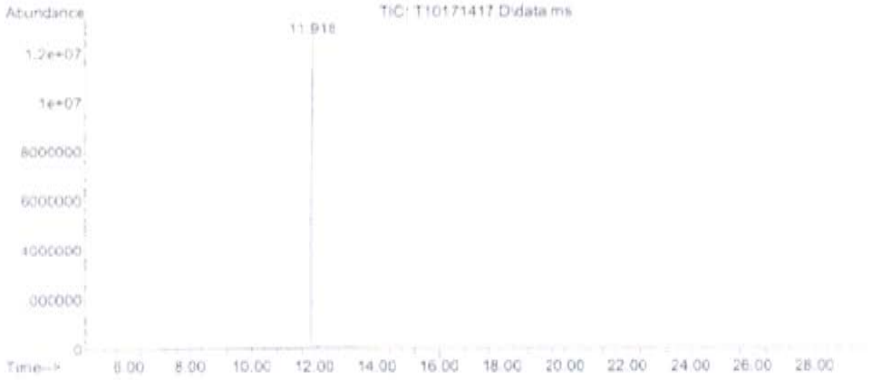
APPENDIX 4 Certificate of Analysis for Diazepam-D5 (Continued)

Spectral and Physical Data (cont.)

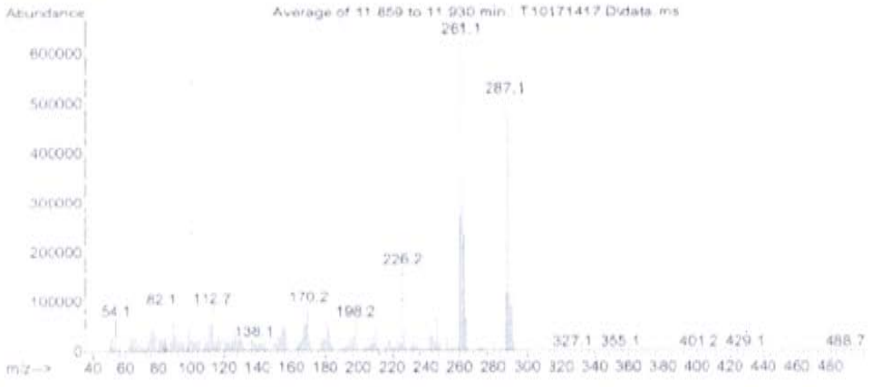
GC/MS

Compound Name : Diazepam-D5
C Number : FC10011401
Instrument : Agilent GCMS
Operator : ECM(ALONG)
Date Reported : Tue Oct 21 15:29:56 2014
Column Type : DB-5ms, 30m x 0.25mm ID, 0.25um film thickness
Temp. Program : 50°C to 200°C@40°C/min, 200°C to 300°C@10°C/min, 26min hold
Injector Temp. : Cool on-column
Carrier Gas : Helium
Flow Rate (mL\min) : 0.80 mL/min
Transfer Line Temp. : 280°C
Scan Range : 50-500

Total Ion Chromatogram



Mass Spectrum



Stability

Short term stability studies have been performed under accelerated conditions for a period of up to four weeks. Short term data is utilized to predict long term stability and to support transport conditions and normal laboratory use. Real-time stability studies are performed at the recommended storage conditions over the life of the product.

Short Term Stability: *A summary of accelerated stability findings for this product is listed below.*		
Storage Condition	**Mean Kinetic Temperature (MKT)**	**Time Period/Result**
Freezer	-15°C	No decrease in purity was noted after four weeks.
Refrigerator	4°C	
Room Temperature	21°C	
40°C	40°C	
Transport/Shipping: *Stability studies support the transport of this product at ambient conditions.*		
Short Term Storage: *Stability data supports short term storage for up to 12 months at Refrigerate conditions.*		
Long Term Stability: *Long term stability has been assessed for Freezer storage (-10 °C to -25 °C) conditions. Stability of a minimum of 60 months has been established through real-time stability studies.*		

COA Revision History

Revision No.	Date	Reason for Revision
00	September 11, 2015	Initial version