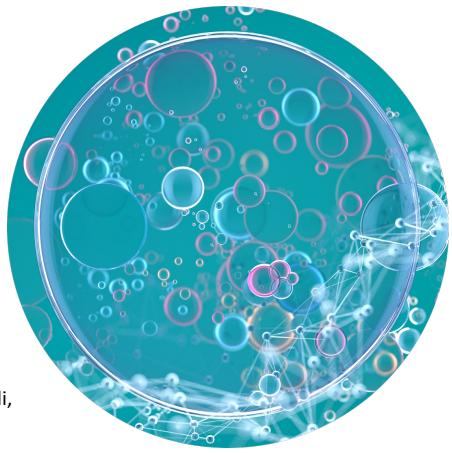
TN-1387

Stability indicating HPLC Method for the Determination of Semaglutide Related Substances and Degradation Products Using Aeris™ Peptide XB-C18



Arunkumar D N[1] , Rajesh Babu Dandamudi, PhD[1] , and Sean Orlowicz [2]

[1] India Phenologix Lab, Phenomenex India, Hitech Defence and Aerospace Park Industrial Area, Mahadeva Kodigehalli, Hobli, Jala Taluka, Bengaluru 562149, India.

[2] Phenomenex Inc., 411 Madrid Ave., Torrance, CA 90501 USA

Introduction

Semaglutide is an agonist of the glucagon-like peptide-1 (GLP-1) receptor, utilized in the management of type 2 diabetes and obesity. Semaglutide functions by increasing insulin secretion, lowering glucagon levels, and delaying gastric emptying. These actions result in better blood sugar regulation and facilitate weight loss. It emulates the effects of the endogenous hormone GLP-1, which is responsible for appetite control and glucose metabolism.

Separating impurities in Semaglutide production presents challenges due to the complex nature of peptide-related impurities. These impurities often include D-amino acid isomers, truncated sequences, oxidized or reduced forms and others arising from deamidation, phosphorylation, acylation and glycosylation. To achieve effective separation of these impurities, a systematic methodology and careful selection of the stationary phase is often necessary. Enhanced retention and selectivity are required to distinguish between the numerous peptide impurities that closely resemble one another. For this purpose, an HPLC method of great separative capability was developed using Aeris™ Peptide XB-C18 column. This column is specifically engineered for peptide-based separations. XB-C18 ligands featuring di-isobutyl side chains yield a uniformly bonded stationary phase with increased ligand spacing relative to conventional C18 phases, allowing for more effective interactions of longer (>20 residues) peptides with the stationary phase. Additionally, the core-shell particle morphology minimizes analyte diffusion resulting in narrower peaks, providing outstanding resolution and peak shape.

In this application note Forced degradation studies were conducted to assess the stability-indicating nature of the method. Semaglutide was subjected to various stress conditions, including:

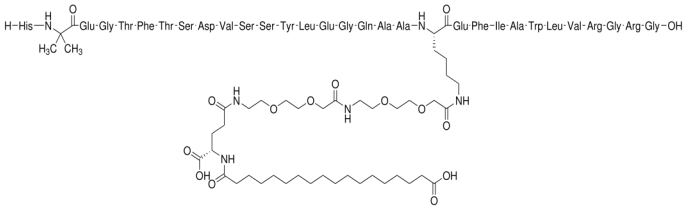
- Acidic Degradation (0.1 mL of 1N HCl at room temperature for 5 hour)
- Basic Degradation (0.1 mL of 0.1N NaOH at room temperature for 4 hour)
- Oxidative degradation (0.1 mL of 1% H_2O_2 at room temperature for 1 hour)
- Thermal degradation (80 °C water bath for 15 hours)

This method effectively separated Semaglutide from its 8 known and as many as 35 unknown degradation impurities, demonstrating specificity and its stability-indicating capability. Peak purity testing using a PDA detector confirmed the homogeneity of the Semaglutide peak in all degradation samples.

Standard and Impurity Standard Preparation

Standard/sample solution: 1.0 mg/mL of Semaglutide in Diluent
Impurity standard: 1.0 mg/mL in Diluent
Spiked sample: 1% of each impurity spiked in Semaglutide standard with respect to 1.0 mg/mL test concentration.

Structure of Semaglutide



List of Impurities:

The standard and impurities were purchased locally from ManoTri Pharma and Simson Pharma.

S.No	Analyte	IUPAC (Sequence)
1	D-Asp (9)-Semaglutide	H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-**D-Asp**-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys (AEEAc-AEEAc-γ-Glu-carboxy heptadecanoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH
2	D-His (1) -Semaglutide	**D-His**-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys (AEEAc-AEEAc-γ-Glu-carboxy heptadecanoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH
3	D-Phe (6) -Semaglutide	H-His-Aib-Glu-Gly-Thr-**D-Phe**-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys (AEEAc-AEEAc-γ-Glu-carboxy heptadecanoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH
4	D-Ser (8) -Semaglutide	H-His-Aib-Glu-Gly-Thr-Phe-Thr-**D-Ser**-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys (AEEAc-AEEAc-γ-Glu-carboxy heptadecanoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH
5	(3-31)-Semaglutide	H-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys (AEEAc-AEEAc-γ-Glu-carboxy heptadecanoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH
6	Des side chain -Semaglutide	H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-**Lys**-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH
7	(3-31)linear -Semaglutide	H-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH
8	D-Ser (11) -Semaglutide	H-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-**D-Ser**-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys (AEEAc-AEEAc-γ-Glu-carboxy heptadecanoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH

LC Conditions for Related Substances

Column: Aeris Peptide XB-C18, 2.6 µm (**Part No:** 00G-4505-E0)
Dimensions: 250 x 4.6 mm
Buffer 11.5 g of Ammonium dihydrogen phosphate in 1000 mL Milli Q water (100 mM)
Mobile Phase: **A**:Prepare 900:100 v/v (Buffer: Acetonitrile) and add 1 mL of 70% perchloric acid.
B: Prepare 600:300:100 v/v/v (Acetonitrile: Methanol: Water) add 1 mL of 70% perchloric acid.
Diluent Acetonitrile and water (50:50 v/v).

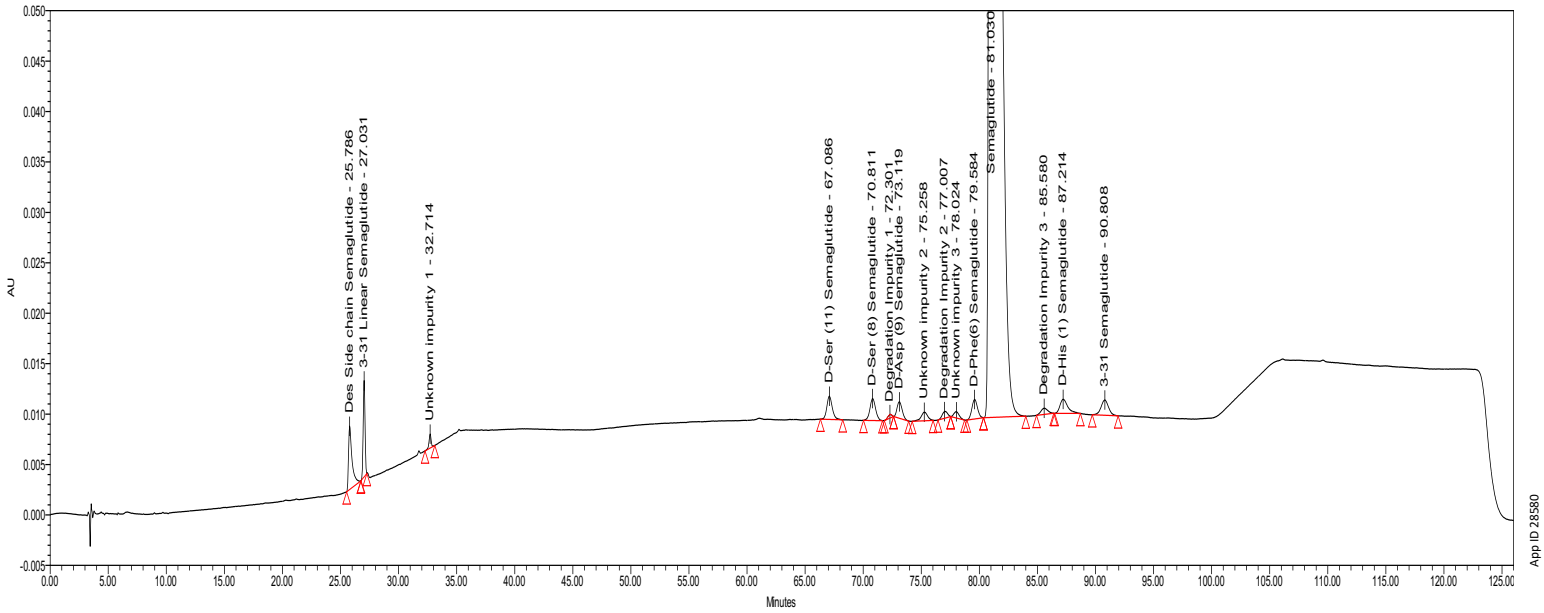
Gradient:

Time (min)	% A	% B
0	50	50
3	50	50
20	45	55
30	34	66
95	32	68
100	25	75
118	25	75
119	50	50
125	50	50

Flow Rate: 0.6 mL/min
Injection Volume: 5 µL
Temperature: 25 °C
LC System: Waters® Arc HPLC with PDA
Detection: UV @ 210 nm

Results and Discussion on Aeris Peptide XB C18, 2.6 µm 250 x 4.6 mm for Related Substances

Figure 1. Chromatogram of Standard spiked with known impurities at 1.0 % level.



	Analyte	Retention Time	Area	% Area	Height	USP Resolution	USP Tailing
1	Des Side chain Semaglutide	25.786	132364	1.16	6297		1.9
2	3-31 Linear Semaglutide	27.031	103660	0.91	9559	3.2	1.0
3	Unknown impurity 1	32.714	17506	0.15	1396	18.6	0.7
4	D-Ser (11) Semaglutide	67.086	75088	0.66	2304	59.2	1.2
5	D-Ser (8) Semaglutide	70.811	74264	0.65	2183	4.3	1.0
6	Degradation Impurity 1	72.301	8341	0.07	365	2.0	0.8
7	D-Asp (9) Semaglutide	73.119	50461	0.44	1638	1.1	1.3
8	Unknown impurity 2	75.258	33417	0.29	849	2.3	0.9
9	Degradation Impurity 2	77.007	21300	0.19	669	1.9	1.0
10	Unknown impurity 3	78.024	19598	0.17	628	1.2	1.3
11	D-Phe(6) Semaglutide	79.584	65330	0.57	1936	1.8	1.1
12	Semaglutide	81.030	10623482	93.32	209534	1.3	2.5
13	Degradation Impurity 3	85.580	25188	0.22	608	3.6	1.1
14	D-His (1) Semaglutide	87.214	65980	0.58	1402	1.4	1.5
15	3-31 Semaglutide	90.808	68189	0.60	1518	3.0	1.0

Figure 2. Semaglutide Standard solution 1 mg/mL for related substance as a sample.

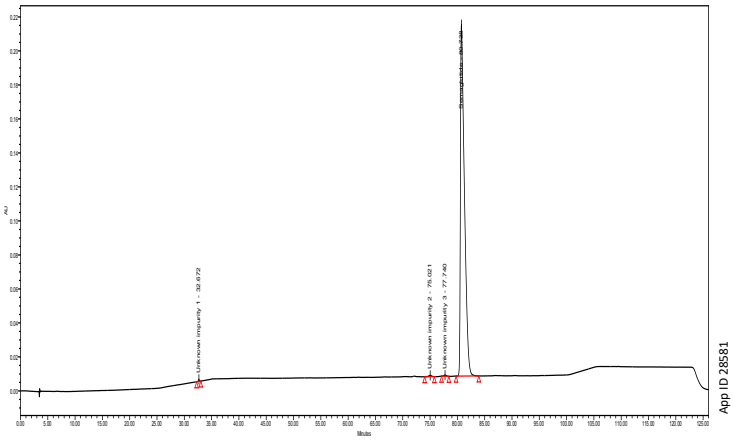
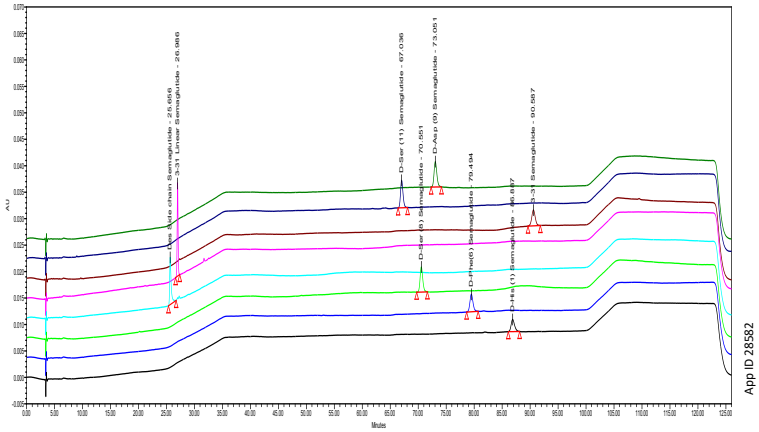
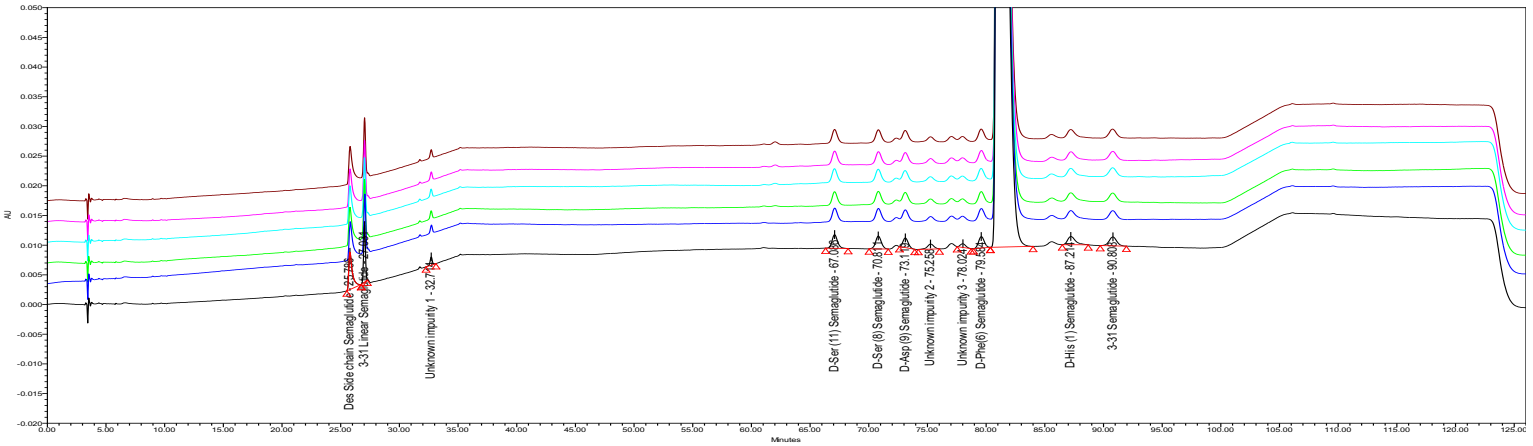


Figure 3. Overlayed chromatogram of individual known impurities



The chromatogram of the Semaglutide standard solution after spiking with known related impurities is shown in **Figure 1 .** As observed, this method provided excellent chromatographic separation of Semaglutide from the eight known impurities spiked, together with three unknown impurities. Three degradation impurities were also observed which may be due to further degradation of the known impurities. The chromatogram of the Semaglutide standard is shown in **Figure 2.** It was observed that the standard contained three unidentified impurities at low concentrations, which were effectively separated from the Semaglutide peak. All identified impurities were well-resolved from the Semaglutide peak, with a resolution of ≥ 1.3, and were confirmed using individual reference standards. An overlay of the standards is presented in **Figure 3**. System suitability was validated by six replicate injections of the sample solution, demonstrating high reproducibility in both retention times and peak areas, as shown in **Figure 4.**

Figure 4. Overlayed chromatogram of six replicate injections of the Standard spiked with known impurities at 1.0 % level.

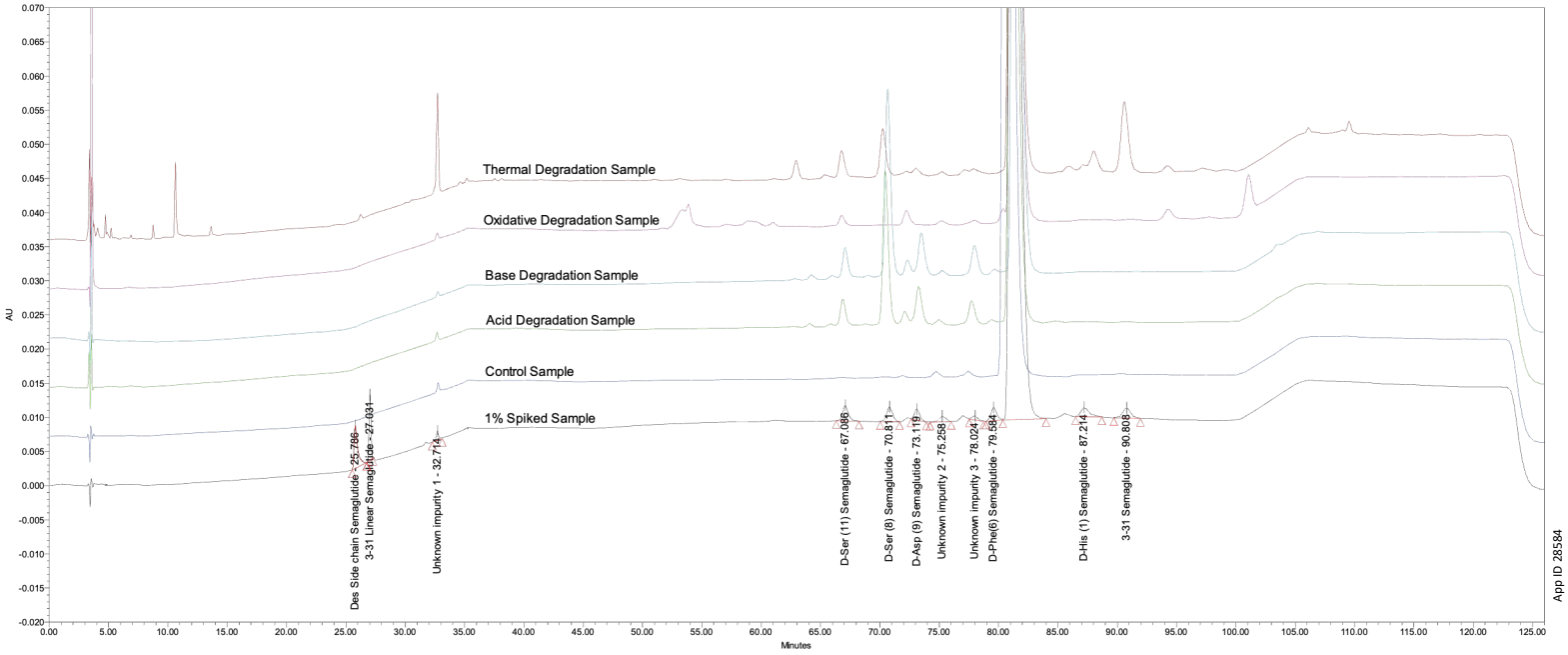


	Des side chain Semaglutide		3-31 Linear Semaglutide		D-Ser (11) Semaglutide		D-Ser (8) Semaglutide		D-Asp(9) Semaglutide		D-Phe(6) Semaglutide		Semaglutide		D-His (1) Semaglutide		3-31 Semaglutide	
	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area	RT	Area
Mean	25.794	132255	27.034	104010	67.087	74437	70.816	74628	73.114	50777	79.580	66687	81.024	10664374	87.214	64569	90.786	65344
SD	0.011	480	0.010	331	0.011	981	0.007	571	0.007	433	0.012	842	0.009	25733	0.010	2199	0.018	1660
%RSD	0.0	0.4	0.0	0.3	0.0	1.3	0.0	0.8	0.0	0.9	0.0	1.3	0.0	0.2	0.0	3.4	0.0	2.5

N=6 Injection

Forced Degradation Studies

Figure 5. Overlayed chromatogram of samples exposed to varied types of forced degradation approaches



Sample Name	Forced Degradation Condition	Percentage of Degradation impurities	Number of degradation peaks
Control sample	-	0.68	3
Acid Degradation Sample	0.1 ml 1N HCl for 5 Hours at room temperature (25 °C)	16.16	14
Base Degradation Sample	0.1 ml 0.1N NaOH for 5 Hours at room temperature (25 °C)	18.61	13
Oxidative Degradation Sample	0.1 ml 1% H_2O_2 for 1 Hour at room temperature (25 °C)	9.36	18
Thermal Degradation Sample	At 80 °C water bath for 15 Hours	13.66	35

The Semaglutide standard (1 mg/mL) was subjected to a series of stress tests, which included acidic degradation (0.1 mL of 1N HCl at room temperature for 5 hours), basic degradation (0.1 mL of 0.1N NaOH at room temperature for 4 hours), oxidative degradation (0.1 mL of 1% H_2O_2 at room temperature for 1 hour), and thermal degradation (80 °C water bath for 15 hours). The outcomes of the chromatographic analyses for each sample after treatment is presented as an overlay chromatogram in F**igure 5**. As expected, the control sample (Semaglutide standard without treatment) contained only 3 unknown impurities detected at low levels. During acid degradation, a total of 14 impurities were identified; in base degradation, 13 impurities were noted whereas in oxidative degradation, 18 degradation impurities were found. Thermal degradation led to the formation of nearly 35 degradation impurities. All degradant peaks were distinctly separated from one another. The degradation impurities resulting from acid and base degradation exhibited similarities, while distinct impurities were generated during thermal and oxidative degradation.

Conclusions

An Aeris Peptide XB-C18 column (250 × 4.6 mm, 2.6 µm) was employed to establish a stability-indicating method for related substances of Semaglutide. The aim of this method is to accurately quantify impurities and degradation products associated with Semaglutide under various stress conditions, by ensuring effective separation of Semaglutide from its impurities. Forced degradation studies were performed to assess the stability and degradation of the drug under different stress conditions, which included acid, base, oxidative (peroxide) and thermal degradations. The findings demonstrate that the developed method is highly effective in resolving both process-related impurities and those generated through forced degradation. Furthermore, the method demonstrates excellent reproducibility, making it a reliable tool for stability-indicating studies of Semaglutide.

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anfrage@phenomenex.com

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

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

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

Czech Republic
t: +420 272 017 077
cz-info@phenomenex.com

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

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

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

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

Hong Kong
t: +852 6012 8162
hkinfo@phenomenex.com

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

Indonesia
t: +62 21 3952 5747
indoinfo@phenomenex.com

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

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

Japan
t: +81 (0) 120-149-262
jpinfo@phenomenex.com

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

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

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

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

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

Poland
t: +48 22 51 02 180
pl-info@phenomenex.com

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

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

Slovakia
t: +420 272 017 077
sk-info@phenomenex.com

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

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

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

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

Thailand
t: +66 (0) 2 566 0287
thaiinfo@phenomenex.com

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

USA
t: +1 (310) 212-0555
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