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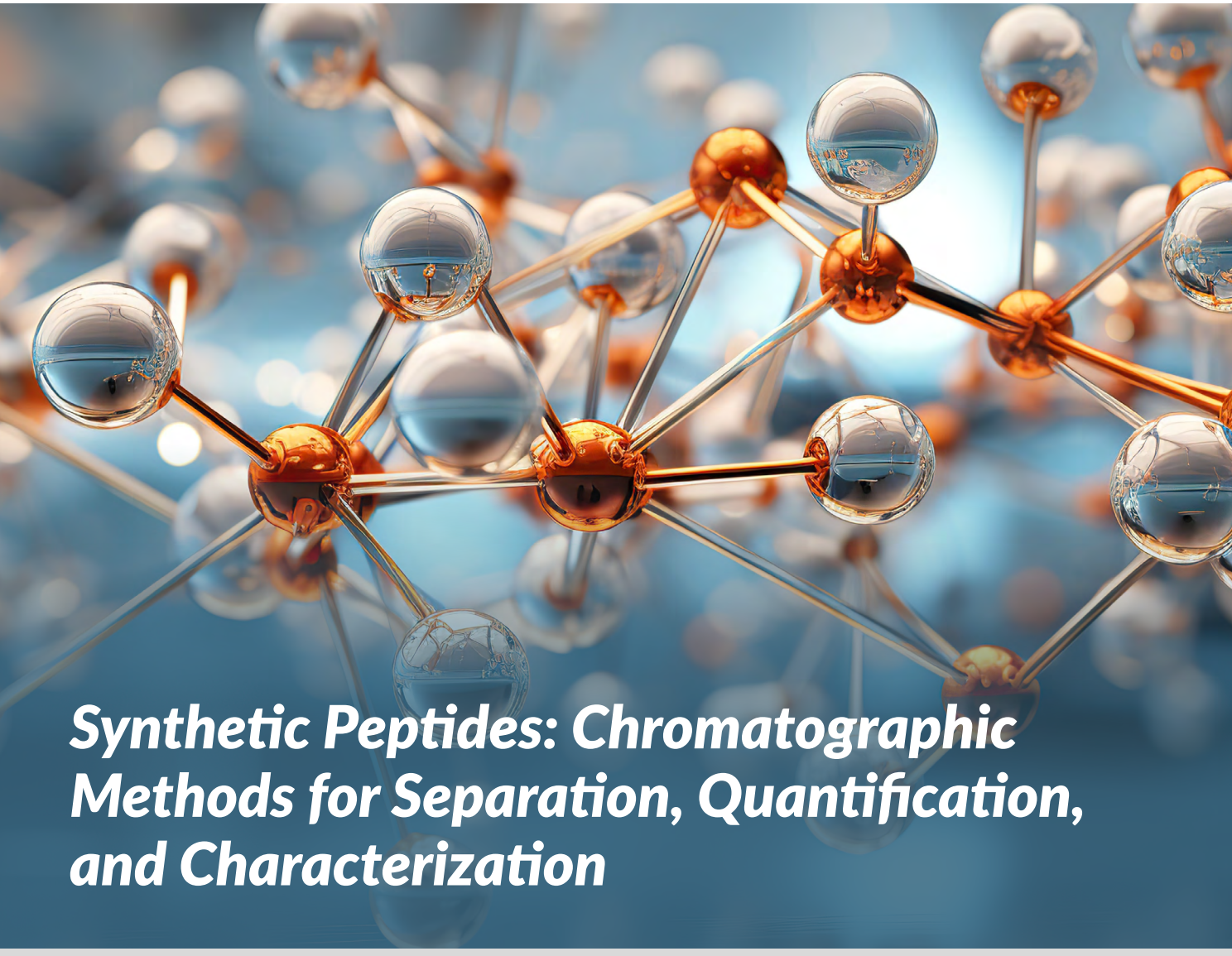

Synthetic Peptides: Chromatographic Methods for Separation, Quantification, and Characterization

- Optimizing Analysis and Purification
- Deamidated Peptide Separation
- Synthetic Peptide Analysis
- Therapeutic Peptide Quantification

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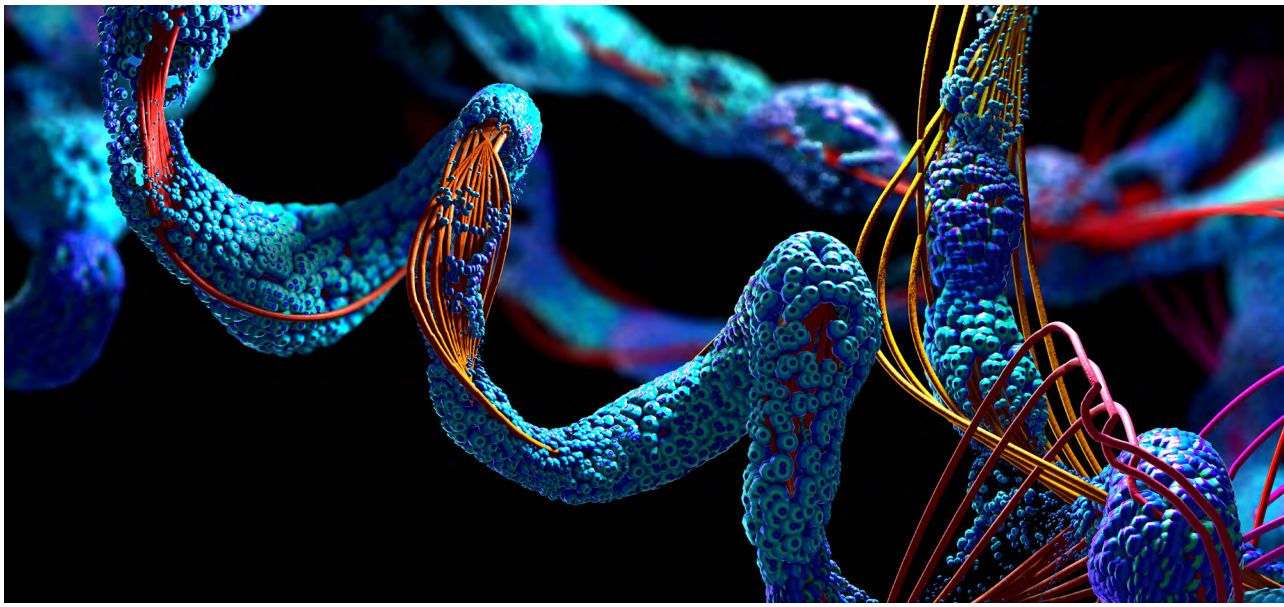
Introduction

Peptides are versatile molecules that have a wide range of applications in biotechnology, medicine, and research. They can be used as drugs, diagnostics, biomarkers, or tools to study cellular processes. However, peptides also pose unique challenges in terms of analysis and purification due to their complexity, diversity, and sensitivity. In this e-book, you will learn about the principles and methods of peptide analysis and purification, covering topics such as peptide synthesis, characterization, separation, identification, and quantification. You will also discover how to use state-of-the-art instruments and columns to achieve optimal results for your peptide projects. The four articles in this e-book will show you how to:

- Optimize the analysis and purification of a synthetic peptide using Agilent PLRP-S columns with different pore sizes
- Separate deamidated peptides with an Agilent AdvanceBio Peptide Plus column using different pH conditions
- Analyze a synthetic peptide and its impurities using mass spectrometry compatible mobile phases with an AdvanceBio Peptide Plus column
- Quantify a therapeutic peptide exenatide in rat plasma using Agilent 1290 Infinity II Bio LC and Agilent 6495 Triple Quadrupole LC/MS systems

Whether you are a novice or an expert in peptide chromatography, this e-book will provide you with valuable insights and practical tips to optimize your peptide analysis and purification workflows.



Optimizing Analysis and Purification of a Synthetic Peptide Using PLRP-S Columns

Reliable columns and media with the scale and pore size for successful purification of synthetic peptides

AUTHORS

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ABSTRACT

Reversed-phase ion-pair chromatography has become increasingly useful for the analysis and purification of synthetic peptides using mobile phases containing trifluoroacetic acid (TFA) as an ion-pair reagent. Scaling up from analytical to preparative high performance liquid chromatography (HPLC) can be costly, time consuming, and often difficult to perform due to differences in chemistries, pH conditions, particle sizes, and column length. This application note describes the analysis of a synthetic peptide and how to scale-up methods using an Agilent PLRP-S analytical HPLC column. The gradient and loading information are applied directly to a larger preparative column packed with an identical material.

INTRODUCTION

Peptide therapeutics are gaining popularity because of developments in biotechnology and bioengineering, including cancer diagnosis and treatment, antibiotic drug development, and new vaccines. Most peptide drugs are produced using solid-phase peptide synthesis (SPPS). The synthesis is performed on a polymeric support or resin, which can easily be filtered from reactions. The synthetic route includes multiple deprotection, activation, and coupling steps. The final peptide sequence is separated from the resin using a cleavage cocktail containing scavengers and other components resulting in the final crude product that is ready for purification. Crude peptides synthesized by solid-phase peptide synthesis are analyzed by HPLC using reversed-phase columns with gradient elution using aqueous acetonitrile (typically containing 0.1% trifluoroacetic acid (TFA) as the ion-pair reagent). Liquid chromatography/mass spectrometry (LC/MS) based peptide analysis is normally used to confirm the structure of the target molecule. However, TFA is not ideal for LC/MS since it causes ion suppression, creating a weaker MS signal. The preferred ion-pair reagent for LC/MS methods is formic acid (FA), a weaker acid than TFA. This work uses human glucagon-like peptide-1 (GLP-1) 7-36 amide, a single polypeptide chain containing 30 amino acids, which has a molecular mass of 3,297.7 Daltons (Da) (FIGURE 1). This application note describes the ability to perform direct scale up from analytical PLRP-S 4.6 × 250 mm, 8 µm columns to larger scale preparative PLRP-S 21.2 × 250 mm, 8 µm columns. Two pore sizes appropriate for the separation of peptides, 100 and 300 Å, were investigated. Agilent PLRP-S is a rigid macroporous styrene divinylbenzene (PS-DVB) HPLC stationary phase with outstanding chemical and physical stability. PLRP-S HPLC media is inherently hydrophobic and does not require a bonded alkyl chain such as C8 or C18 to confer hydrophobicity. The characterization of the final product was confirmed using an Agilent 6545XT AdvanceBio liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF) with an orthogonal AdvanceBio Peptide Mapping column, 2.1 × 100 mm, 2.7 µm.

FIGURE 1. The amino acid sequence of synthetic GLP-1 (7-36) amide.

H-His-Ala-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-Lys-Gly-Arg-NH_2

SAMPLE PREPARATION

Glucagon-like peptide GLP-17-36 amide was synthesized by CS Bio (Menlo Park, CA 94025, USA). The solid supports for the synthesis were provided by Agilent Technologies. The synthesis was achieved using standard side chain protection strategy and coupling conditions (fluorenylmethoxycarbonyl (Fmoc) chemistry).

Analytical equipment

An Agilent 1290 Infinity II LC system was composed of the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117C) with a 10-mm InfinityLab Max-Light Cartridge Cell (G7117-60020)

Preparative equipment

An Agilent 1290 Infinity II preparative LC system was composed of the following modules:

- Agilent 1290 Infinity II preparative binary pump (G7161B)
- Agilent 1260 Infinity II fraction collector (G7157A)
- Agilent 1290 Infinity II preparative column compartment (G7163B)
- Agilent 1260 Infinity II diode array detector (G7165A)

LC/MS equipment

Agilent 1290 Infinity II LC system coupled to the 6545XT AdvanceBio LC/Q-TOF (G6549AA)

Software and data processing

- Agilent OpenLab software suite, version 2.6
- OpenLab ChemStation CDS, version C01.09
- Agilent MassHunter data workstation acquisition, version B10.00
- Agilent MassHunter BioConfirm software, version 10.00

Columns

- **Analytical columns**: Agilent PLRP-S 100 Å, 4.6 × 250 mm, 8 µm (part number PL1512-5800); Agilent PLRP-S 300 Å, 4.6 × 250 mm, 8 µm (part number PL1512-5801)
- **Preparative columns**: Agilent PLRP-S 100 Å, 21.2 × 250 mm, 8 µm; Agilent PLRP-S 300 Å, 21.2 × 250 mm, 8 µm (custom dimension)
- **LC/MS column**: AdvanceBio Peptide Mapping 2.1 × 100 mm, 2.7 µm (part number 655750-902)

Solid support

- AmphiSpheres 40 RAM 0.4 mmol/g 75 to 150 µm (part number PL3867-4764)
- PL-Rink Resin (1% DVB) 0.3 mmol/g 75 to 150 µm

REAGENTS AND CHEMICALS

All reagents were HPLC grade or higher

METHOD CONDITIONS

TABLE 1. Liquid chromatography parameters.

Agilent 1290 Infinity II Analytical LC System	
Parameter	**Value**
Column	Agilent PLRP-S, 4.6 × 250 mm, 8 µm
Thermostat	4 °C
Solvent A	0.1% TFA in water
Solvent B	0.1% TFA in acetonitrile
Gradient	Gradient 1: Time (min) %B 0 to 2 35 2 to 22 35 to 50 22 to 24 50 to 90 24 to 28 90 28 to 30 90 to 35 30 to 36 35 Gradient 2: Time (min) %B 0 to 2 35 2 to 22 35 to 65 22 to 24 65 to 90 24 to 28 90 28 to 30 90 to 35 30 to 36 35
Column Temperature	25 °C
Flow Rate	1.0 mL/min
Injection Volume	5.0 µL
Agilent 1290 Infinity II Preparative LC System	
Column	Agilent PLRP-S, 21.2 × 250 mm, 8 µm
Thermostat	4 °C
Solvent A	0.1% TFA in water
Solvent B	0.1% TFA in acetonitrile
Gradient	Time (min) %B 0 to 2 35 2 to 22 35 to 50 22 to 24 50 to 90 24 to 28 90 28 to 30 90 to 35 30 to 45 35
Column Temperature	Ambient
Flow Rate	21.2 mL/min
Injection Volume	100 µL
Fraction Collection	2.5 mL fractions; time based

TABLE 2. LC/MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Parameter	**Value**
Source	Dual AJS
Polarity	Positive
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Skimmer	65 V
Acquisition Mode	2.5 Hz
Mass Range	100 to 2,100 *m/z*
Acquisition Rate	5 spectra/s
Agilent 1290 Infinity II LC System	
Column	AdvanceBio Peptide Mapping, 2.1 × 100 mm, 2.7 µm
Thermostat	4 °C
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	Time (min) %B 0 to 2 3 2 to 23 3 to 47 23 to 25 47 to 50 25 to 26 50 to 97 26 to 27 97 to 3 27 to 30 3* *isocratic (postrun)
Column Temperature	55 °C
Flow Rate	0.3 mL/min
Injection Volume	20 µL

RESULTS AND DISCUSSION

Two resins were used for the synthesis of the target GLP-1 7-36 amide peptide. The first, AmphiSpheres 40 RAM 0.4 mmol/g 75 to 150 µm, contains a polyethylene glycol chain to improve the performance of the resin for difficult peptides. The second, PL-Rink resin (1% DVB) 0.3 mmol/g 75 to 150 µm is lower loading and suitable for the synthesis of longer peptide chains. The synthesis was performed under identical conditions and resulted in two crude peptides: peptide 1A (from the AmphiSpheres resin) and peptide 1B (from the PL-Rink resin). Purification of peptides usually requires a pore size of 100 or 300 Å. This pore size maximizes the loading capacity while minimizing restricted access or exclusion of larger molecules and retaining the desired mass transfer to achieve best separation. Analytical chromatography of the crude peptides is necessary as a starting point to confirm the presence of the desired molecule and understand the elution characteristics. Initially the elution profile of the two samples was unknown, so different gradient methods were screened (FIGURES 2A, 2B, 3A, AND 3B), and the best (35 to 50 %B) was chosen for the preparative runs. For actual method conditions, see TABLE 1.

FIGURE 2. (A) Gradient optimization of peptide 1A on an Agilent PLRP-S 100 Å column. (B) Gradient optimization of peptide 1B on an Agilent PLRP-S 100 Å column.

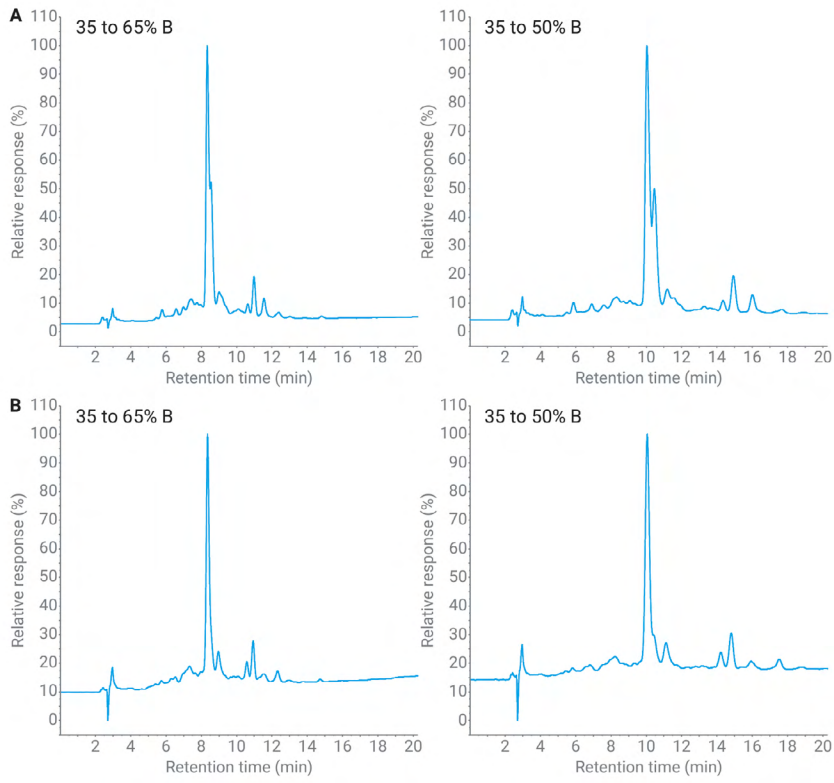


FIGURE 3. (A) Gradient optimization of peptide 1A on an Agilent PLRP-S 300 Å column. (B) Gradient optimization of peptide 1B on an Agilent PLRP-S 300 Å column.

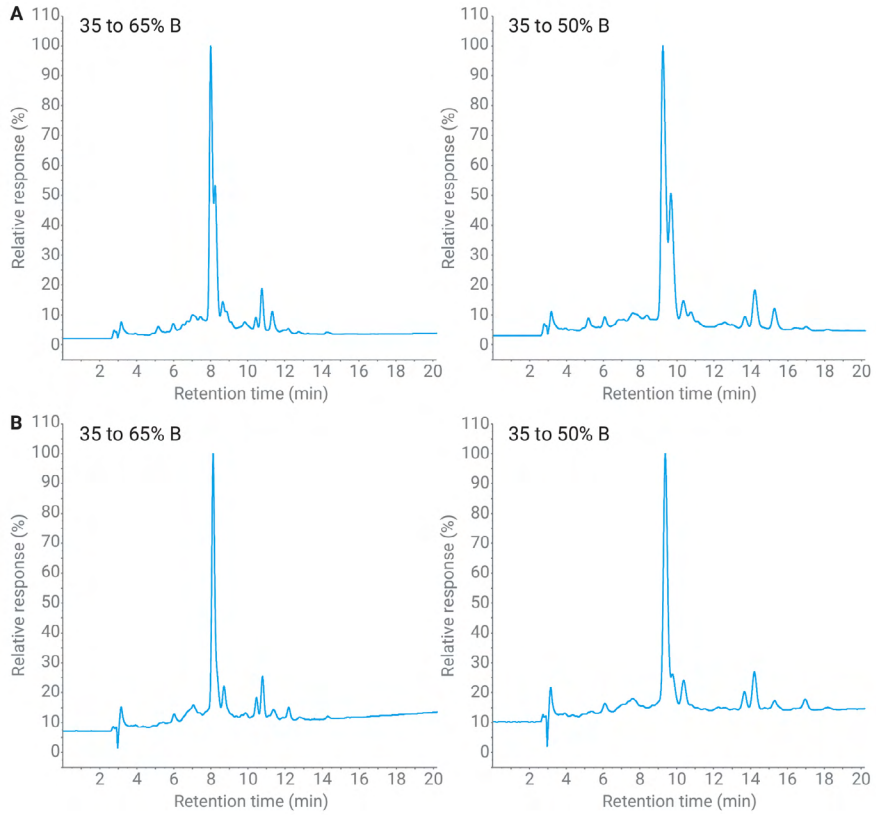
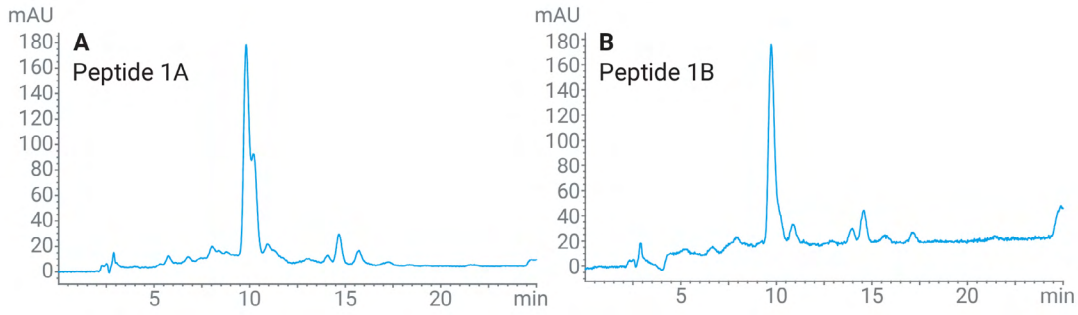


FIGURE 4. Preparative chromatograms of peptide 1A (A), and peptide 1B (B) on an Agilent PLRP-S 100 Å column.



It is evident that peptide 1B (prepared using PL-Rink resin 0.3 mmol/g) resulted in higher crude purity than AmphiSpheres 40 RAM in this example (TABLE 3).

TABLE 3. Crude peptide purity.

	Peptide 1A	Peptide 1B
PLRP-S 100 Å	33.15%	43.19%
PLRP-S 300 Å	41.23%	46.53%

The preparative scale separation was performed by injecting 100 µL of crude peptide at a concentration of 1 mg/mL dissolved in mobile phase A (containing water with 0.1% TFA). A total amount of 1 mg was purified on both PLRP-S 100 Å and PLRP-S 300 Å columns with a 21.2 mm id scale up from 4.6 to 21.2 mm id column dimensions (FIGURES 4 AND 6). The fraction collector was set to collect the full-length product (FLP) using fixed 2.5 mL volume fractions over the time period that the main peak eluted. The product and any closely eluting impurities could easily be identified by reanalyzing the appropriate fractions on the analytical columns (FIGURES 5 AND 7).

Overall purity level is calculated from the peak area percentage of each fraction (TABLE 4).

FIGURE 5. (A) Peptide 1A on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right). (B) Peptide 1B on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right).

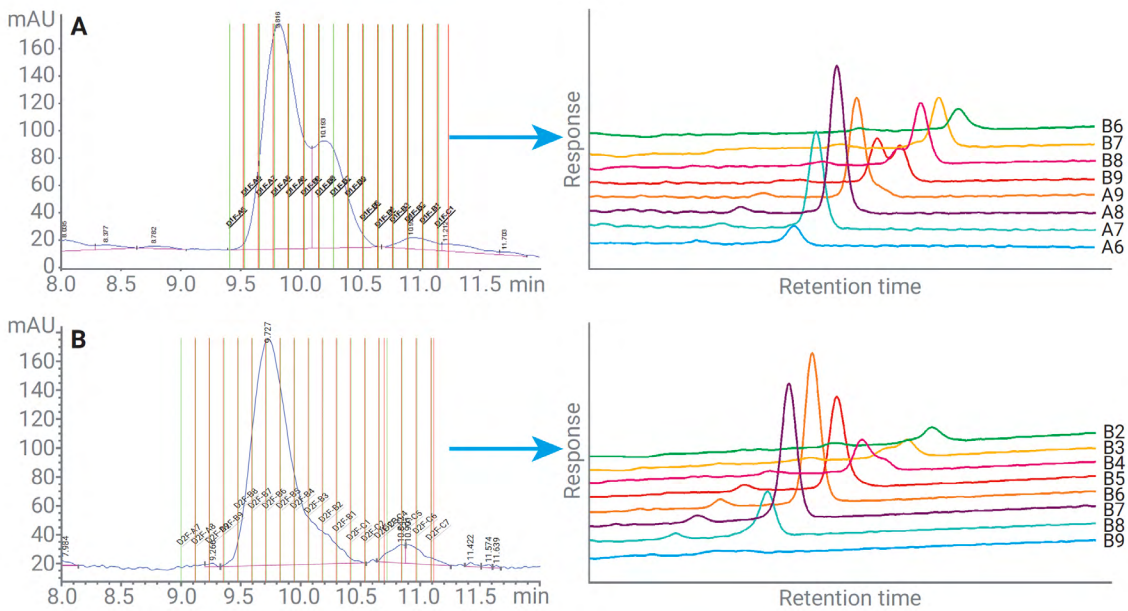


FIGURE 6. Preparative chromatograms of peptide 1A (A), and peptide 1B (B) on an Agilent PLRP-S 300 Å column.

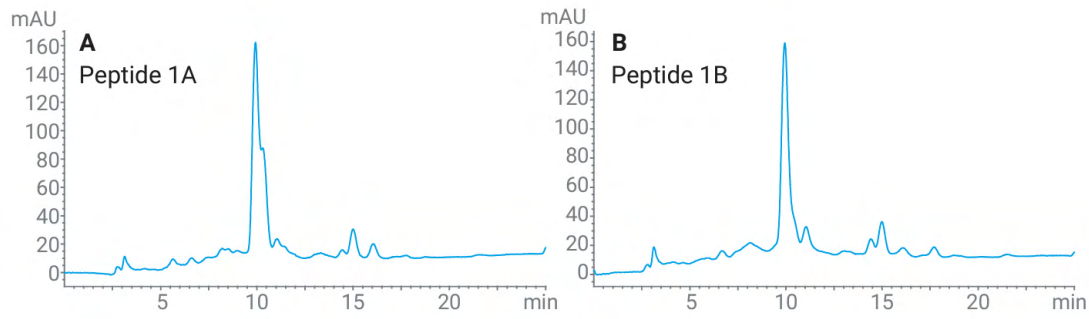
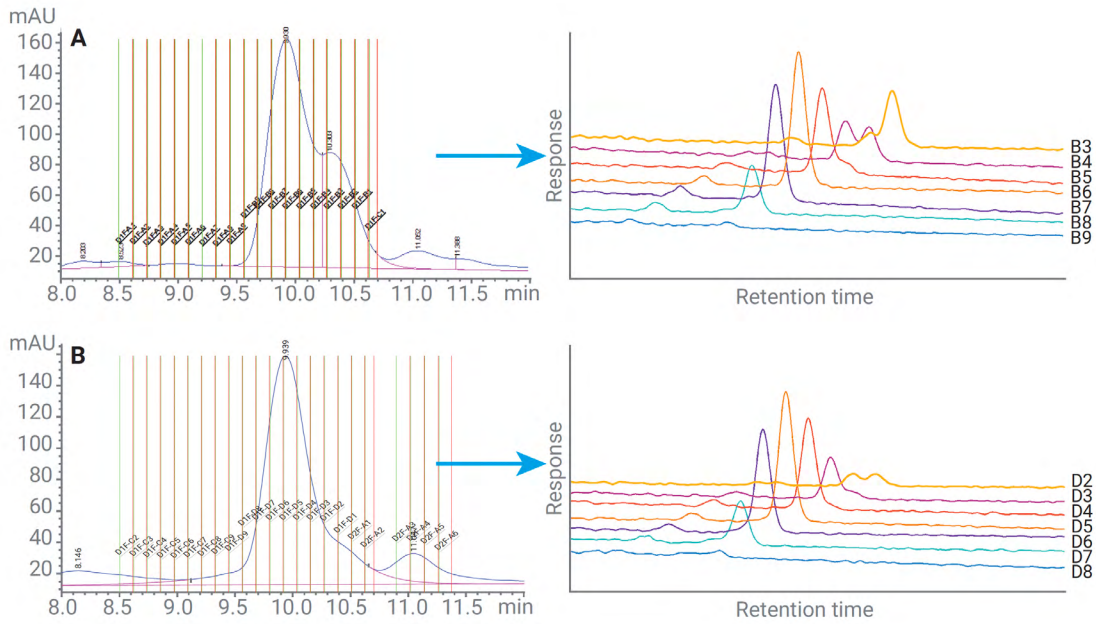


FIGURE 7. (A) Peptide 1A on an Agilent PLRP-S 300 Å column showing fraction reanalysis (right). (B) Peptide 1B on an Agilent PLRP-S 300 Å column showing fraction reanalysis (right).



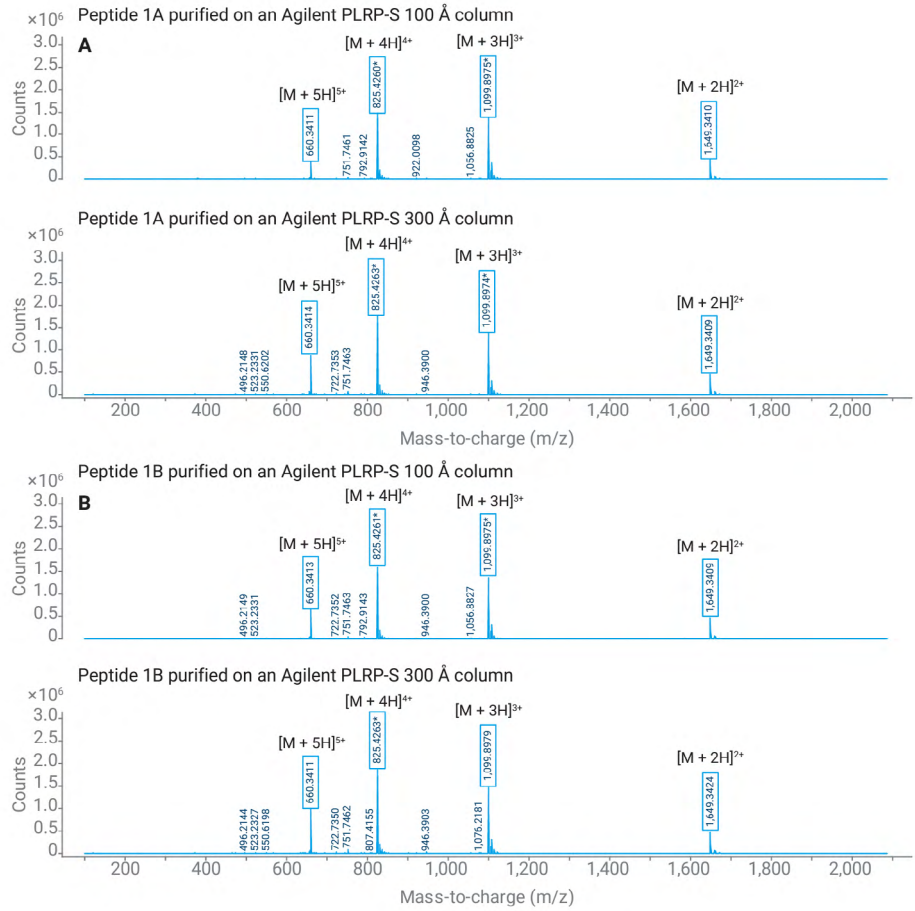
LC/MS analysis of the main component purified was performed on an AdvanceBio Peptide Mapping column to confirm its identity. Synthetic peptide samples can often contain a high number of different molecular weights, impurities, missing amino acids in the sequence, loss of water, sometimes the protecting groups from the synthesis can still be attached to

TABLE 4 Summary of purity and yield from combination of fractions.

Peptide 1A	Area% (Purity)	Overall Yield%
PLRP-S 100 Å (Fractions A6-B9)	89.28	85.59
PLRP-S 300 Å (Fractions B8-B5)	90.26	73.02
Peptide 1B	**Area% (Purity)**	**Overall Yield%**
PLRP-S 100 Å (Fractions B8-B4)	97.81	92.69
PLRP-S 300 Å (Fractions D7-D3)	90.55	90.93

our target molecule in case of unsuccessful cleavage from the solid support. It is therefore critical that analytical methods for synthetic peptides cover a wide range of potential impurities. The major component from the purest fraction gave the expected $[M + 2H]^{2+}$ at 660.34, $[M + 3H]^{3+}$ at 825.42, $[M + 4H]^{4+}$ at 1,099.89, and $[M + 5H]^{5+}$ at 1,649.34. The results correspond to the full-length amino acids sequence of (GLP-1) 7-36 amide of 3,297.7 Da (FIGURE 8). For method conditions and instrument parameters, see TABLE 2.

FIGURE 8. Mass spectral results of purified peptides analyzed by LC/MS on an Agilent AdvanceBio Peptide Mapping column (for method conditions, see Table 2).



CONCLUSION

This application note demonstrates that crude peptide purity may depend on synthesis conditions, including choice of solid phase resin. However, Agilent PLRP-S columns are an ideal choice for ion-pair reversed-phase purification of synthetic peptides. The smaller pore size 100 Å particles with higher available surface area can potentially offer higher purification capacity. Wider pore sizes can lead to better mass transfer and sharper peaks for larger species. By coupling the Agilent PLRP-S preparative HPLC columns to the Agilent 1290 Infinity II preparative LC system, you can achieve efficient separations. Finally, for the LC/MS method, an Agilent AdvanceBio Peptide Mapping column was successfully used with formic acid as a mobile phase modifier to confirm the identity of the molecule.

Separation of Deamidated Peptides with an Agilent AdvanceBio Peptide Plus Column

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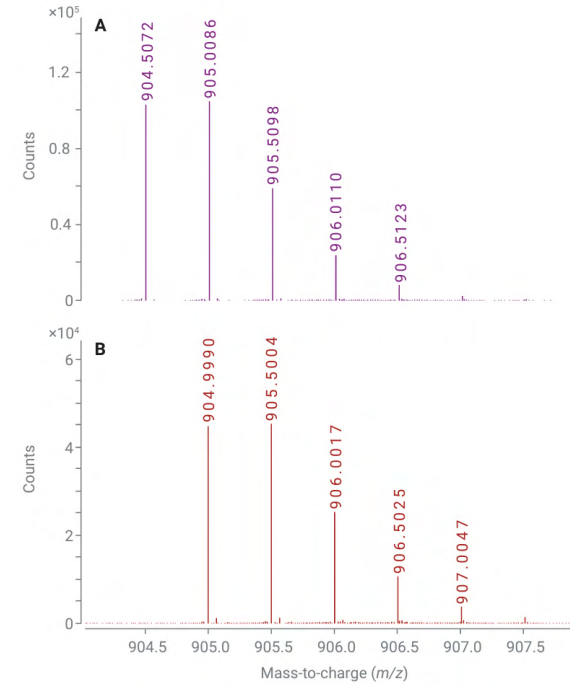
ABSTRACT

Deamidations of glutamine and asparagine are amongst the most common degradations affecting proteins. However, analysis of deamidation by mass spectrometry is challenging due to the small mass shift of less than 1 Da versus the unmodified form. Site-specific deamidation is often determined by protease digestion followed by LC/MS analysis, but even this approach can fail when the unmodified and deamidated forms are not chromatographically resolved. Fortunately, a charged surface C18 column dramatically improves the resolution of deamidated peptides from their unmodified variants. Furthermore, mobile phase optimization can provide additional control over the resolution of these analytes.

INTRODUCTION

Analysis of protein deamidation is important for multiple stages of biopharmaceutical drug development and production. From an analytical perspective, deamidation eliminates an amide group and introduces a new carboxylic acidic group, potentially enabling analysis by charge-based methods such as IEX and isoelectric focusing.[1] However, these techniques do not always resolve deamidated products and cannot confirm deamidation at a given site within the protein. Therefore, many analysts perform a protease digest of the sample and analyze the resultant peptides by reversed-phase LC/MS. Deamidation of asparagines or glutamines is identified by a mass increment of 0.9840 Da versus the unmodified form of the peptide. In some cases, peptides containing these degraded sites are well-separated, and relative quantification can be performed based on the relative signal intensity of the different forms.[2] However, deamidated peptides sometimes coelute with their nondeamidated forms since conversion of asparagine/glutamine to their corresponding carboxylic acids does not result in a large change in hydrophobicity at low pH.[3] Such coelutions result in an overlap of the deamidated peptide signal with the highly abundant 13C isotopes of the unmodified form, as illustrated in FIGURE 1. This can impact the quantitation of deamidation, and in some cases, may even prevent detection of the deamidated variant. This application note demonstrates that a charged surface C18 column greatly enhances selectivity for deamidated variants of peptides versus their unmodified forms when compared to a traditional C18 column. This increases confidence in the ability to detect and quantify deamidation at the peptide level. Based on Agilent superficially porous Poroshell technology, Agilent AdvanceBio Peptide Plus columns feature a hybrid, endcapped C18 stationary phase on a 120 Å pore size, 2.7 µm particle modified to have a positively charged surface. This provides alternative selectivity compared to traditional C18 columns.

FIGURE 1. Mass spectrum of VVSVLTVLHQDWLNGK (A) and a deamidated variant of that peptide (B), showing the overlap between mass spectra.



EXPERIMENTAL

Materials

The mAb sample was expressed and purified from chinese hamster ovary cells. The sample was digested by trypsin, adjusted to a pH of approximately 11 using reagent-grade ammonium hydroxide (Sigma-Aldrich), and incubated for 4 hours at 60 °C to accelerate deamidation. LC/MS-grade formic acid (part number 533002) and acetonitrile (part number 900667) were also purchased from Sigma-Aldrich.

Instrumentation

LC system

An Agilent 1290 Infinity II LC system with the following configuration was used:

- Agilent 1290 Infinity II binary pump (G4220A)
- Agilent 1290 Infinity II autosampler (G4226A)
- Agilent 1290 Infinity II thermostatted column compartment (G1316C)

MS System

Agilent 6546 LC/Q-TOF

Data Processing

LC/MS data were processed by Agilent MassHunter BioConfirm software (version 10.0 SP1) and MassHunter Qualitative Analysis software (version 10.0).

LC/MS Conditions

Parameter	Agilent 1290 Infinity II LC
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, (p/n 695775-949) Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, (p/n 653750-902)
Column Temperature	60 °C
Mobile Phase	A) 0.1 % formic acid in water B) 0.1 % formic acid in acetonitrile
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0 3 2 3 40 40 50.5 100 53 3
Post Time	7 minutes
Injection Volume	3 µL

Parameter	Agilent 1290 Infinity II LC
Source	Agilent Jet Stream
GasTemperature	323 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	35 psi
Sheath Gas Flow	275 °C
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	*m/z* 300 to 1,700
MS Scan Rate (spectra/s)	5
Acquisition Mode	Positive, extended dynamic range (2 GHz) Centroid data format

RESULTS AND DISCUSSION

Five peptides in the mAb digest showing partial deamidation were identified, shown in TABLE 1. These peptides were used to investigate how the choice of column and mobile phase affects the separation of the unmodified peptide from its deamidated variants.

TABLE 1. Five peptides in the mAb digest showing partial deamidation were identified.

Peptide	Sequence (Nondeamidated Form)	*m/z* of $[M+2H]^{2+}$
A	NQVSLTCLVK	581.8103
B	FNWYVDGVEVHNAK	839.4047
C	VVSVLTVLHQDWLNGK	904.5071
D	NTAYLQMNSLR	655.8300
E	GLEWVGYIDPSNGETTYNQK	1136.0323

Column Type

FIGURE 2 shows the separation of peptides and their deamidated variants on two different C18 columns in a typical LC/MS analysis method using a formic acid-modified water/acetonitrile gradient.

The AdvanceBio Peptide Mapping column uses an endcapped C18 silica based on 2.7 µm superficially porous particles with a 120 Å pore size. On this column, deamidated variants typically eluted slightly later than the unmodified form. At least two deamidated variants are detected in each case, likely representing conversion of asparagine into aspartate and isoaspartate. However, in the case of peptide D, one deamidated variant elutes before the unmodified form, while for peptides C and E, a deamidated variant coelutes with the unmodified form. These findings demonstrate that a standard C18 column will not resolve deamidated variants from their unmodified forms in a significant minority of cases.

Meanwhile, the AdvanceBio Peptide Plus column incorporates a positively charged surface on the same type of particle with similar C18 functionalization and endcapping. On this column, all deamidated variants were well resolved from the unmodified form. Furthermore, all deamidated variants of all five peptides eluted later than their modified forms.

A likely explanation for the difference in behavior between the two columns starts with the observation that the positively charged C18 phase is less retentive for peptides in general versus the standard C18 phase. This reduced retention may result from ionic interactions with peptides since they generally carry a positive charge in the presence of 0.1% formic acid.[4] This retention-reducing effect is stronger for highly basic peptides, and becomes less significant on peptides with greater numbers of acidic amino acids. Since deamidation introduces an additional acidic group, deamidated peptide variants are less basic than their unmodified forms and therefore show greater retention on the charged column.

The enhanced selectivity for deamidated peptide variants versus their unmodified

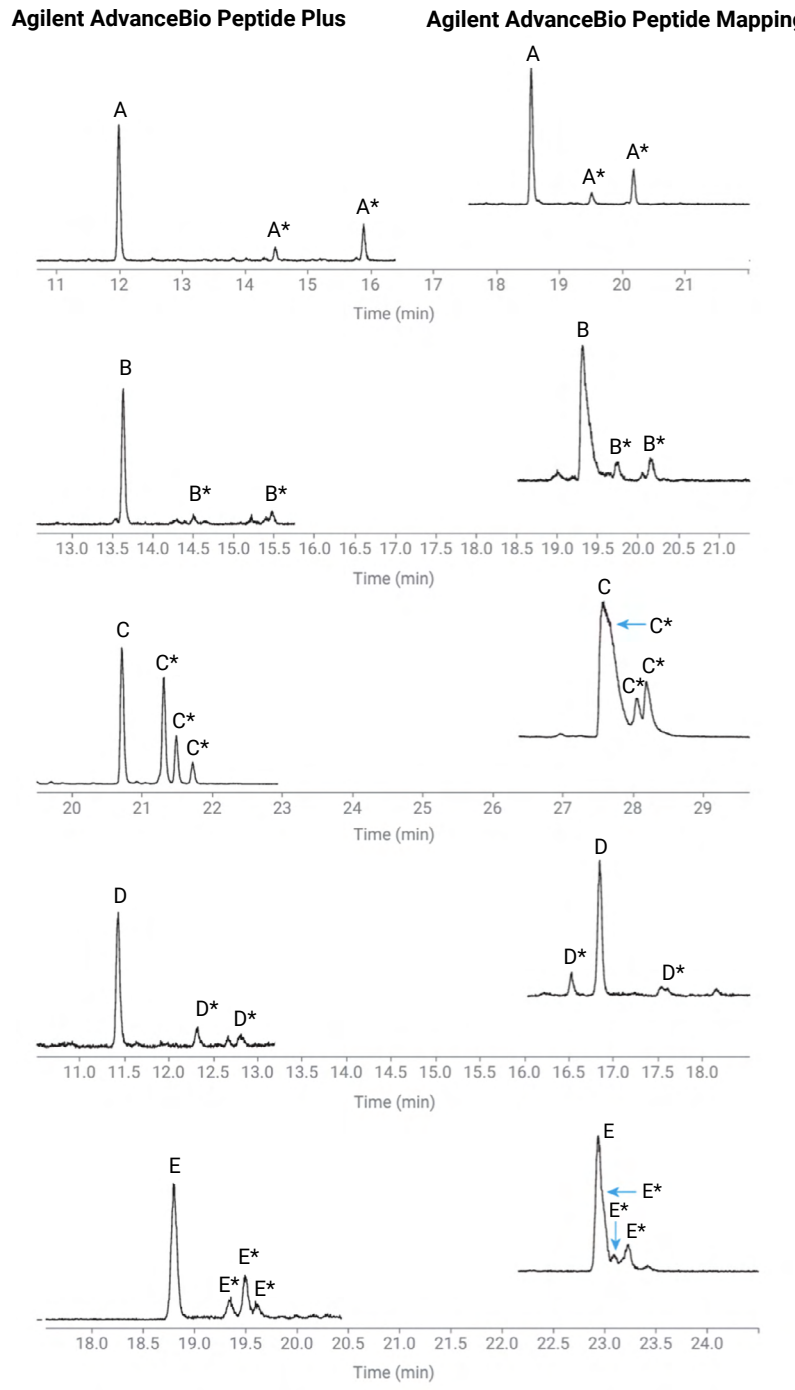
forms greatly reduces the chance of a coelution between these analytes on the charged column, thereby avoiding any challenges that would arise from their overlapping mass spectra.

Mobile Phase

While all five peptides were well-resolved from their deamidated variants on the AdvanceBio Peptide Plus column in 0.1% formic acid, these separations are also greatly affected by mobile phase choice.

FIGURE 3 shows the separation of peptide C from its variants when the aqueous and organic mobile phases are modified with 0.05%, 0.1 or 0.3% formic acid. Dropping the concentration to 0.05% increased selectivity and resolution, while at 0.3% formic acid, resolution was somewhat reduced. A similar pattern is observed for peptide E.

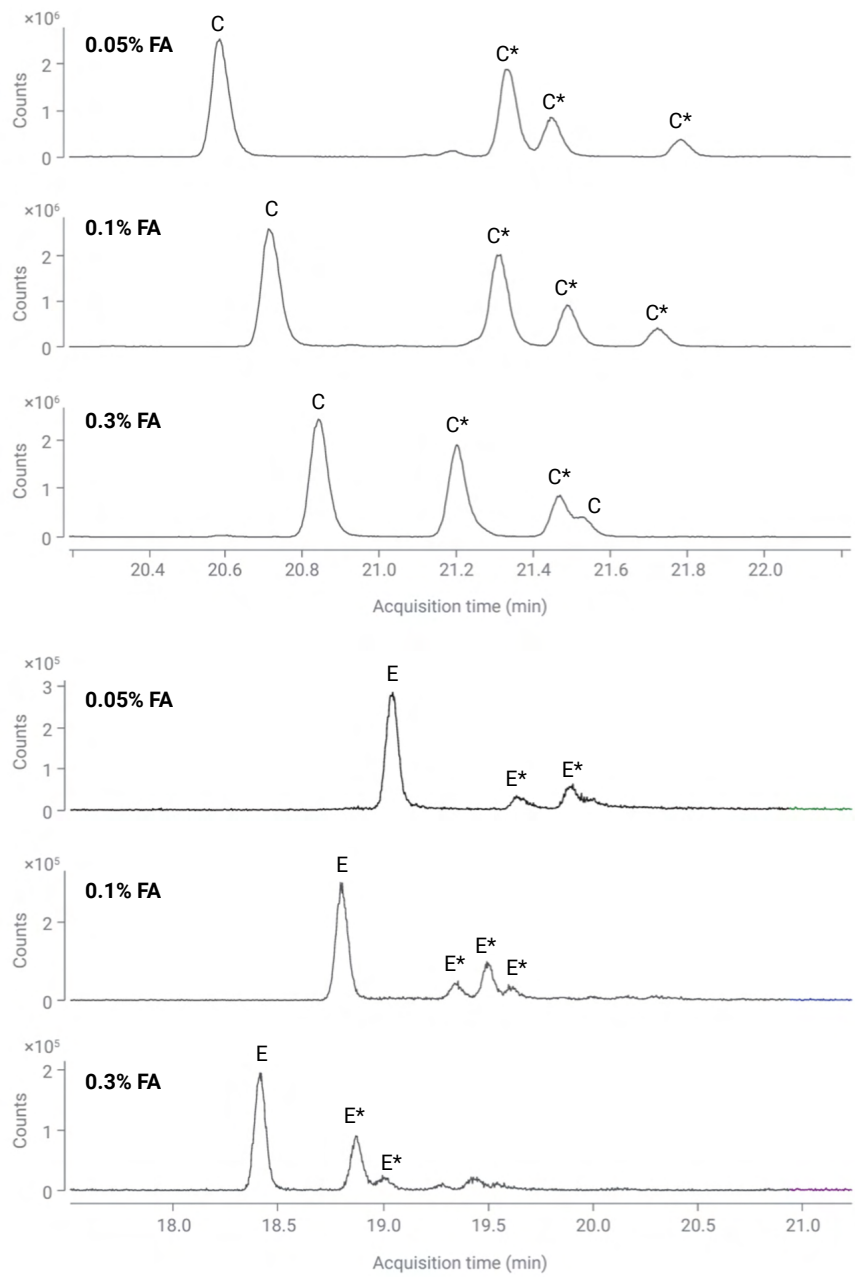
FIGURE 2. Separation of peptides and their deamidated variants (indicated by *) on the Agilent AdvanceBio Peptide Mapping column (elevated and eluting later) and the Agilent AdvanceBio Peptide Plus column (eluting earlier) under the same conditions with 0.1% formic acid mobile phase modifier.



While formic acid is often the favored mobile phase modifier for LC/MS peptide separations, trifluoroacetic acid (TFA) is sometimes used to improve peak shape, even though it has a detrimental effect on ESI-MS sensitivity.[5] TFA lowers mobile phase pH more than formic acid, and therefore suppresses the ionization of the carboxylic acids formed by deamidation. Furthermore, TFA is reported to reduce the impact of ionic interactions by acting as a stronger ion pair reagent than formic acid. These effects may impact the ability of the AdvanceBio Peptide Plus column to separate deamidated variants of peptides from their unmodified form.

FIGURE 4 shows the effect of substituting 0.1% formic acid for 0.1% TFA. Under this condition, selectivity for deamidated variants over the unmodified

FIGURE 3. Separation of peptides on Agilent AdvanceBio Peptide Plus with increasing concentrations of formic acid mobile phase modifier. Selectivity for deamidated peptide variants over their native forms is maximized at lower concentrations.



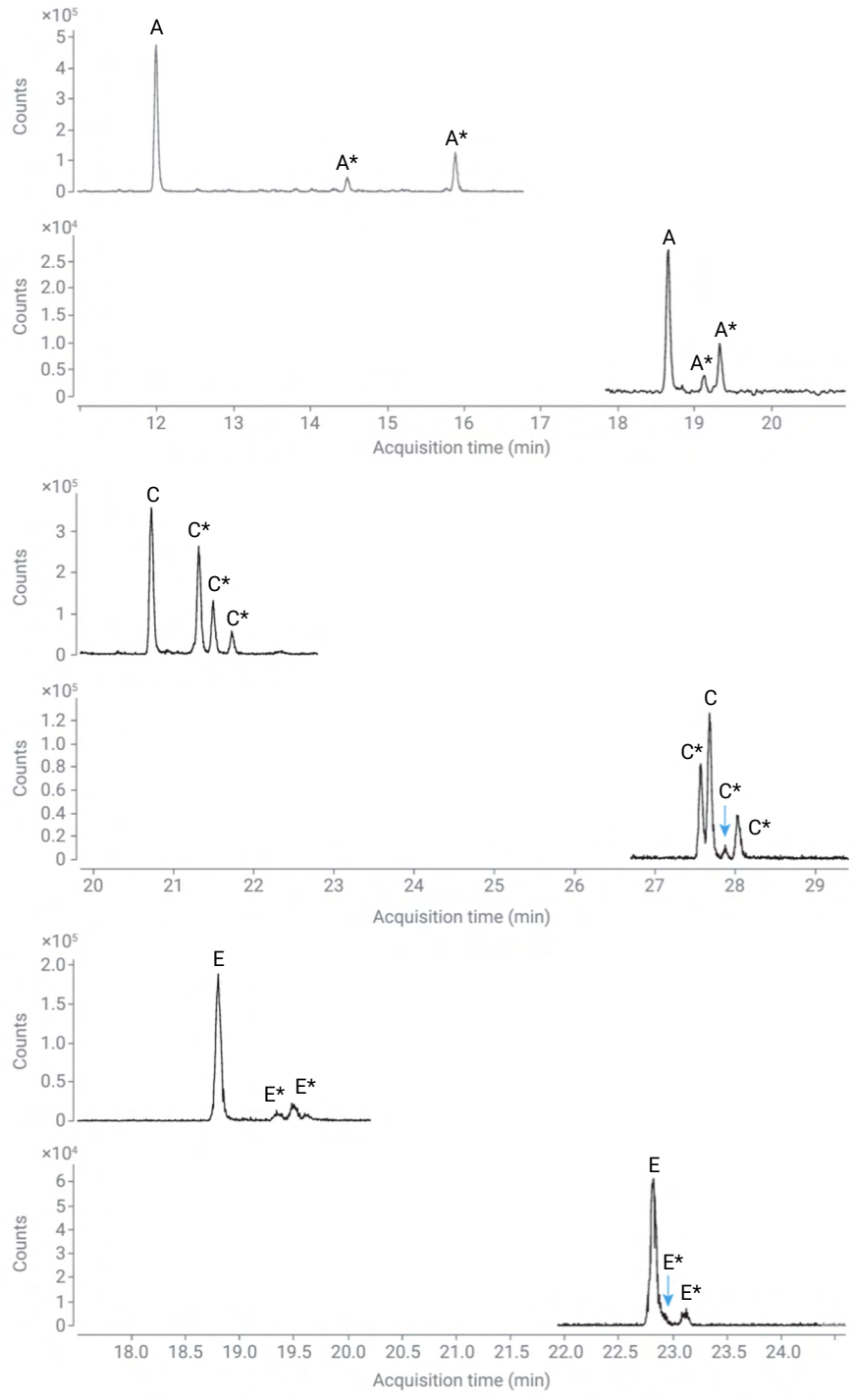
form was significantly reduced. For peptide A, this simply resulted in less baseline between the unmodified form and the variants. However, in the case of peptide C, one of the deamidated variants eluted before the unmodified form. Meanwhile, resolution was lost between the unmodified form of peptide E and one of its deamidated variants.

Overall, the general pattern of deamidated peptides eluting later than the unmodified variant was preserved in the 0.1% TFA condition on AdvanceBio Peptide Plus. However, analysts should be aware that the chances of coeluetion when using TFA are much higher than when using formic acid.

CONCLUSION

The AdvanceBio Peptide Plus column shows greater selectivity for deamidated peptide variants versus their

FIGURE 4. Separation of peptides and their deamidated variants on Agilent AdvanceBio Peptide Plus with 0.1% formic acid mobile phase modifier compared to 0.1% TFA.



unmodified form when compared to a standard C18 column. This dramatically improves confidence that deamidated forms of peptides can be detected and quantified either manually or by automated analysis software by preventing issues with overlapping mass spectra. Selectivity can be altered by increasing or decreasing the concentration of formic acid mobile phase modifier. These findings may be useful to anyone analyzing deamidation of proteins using LC/MS, as described in the application note Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies.[2]

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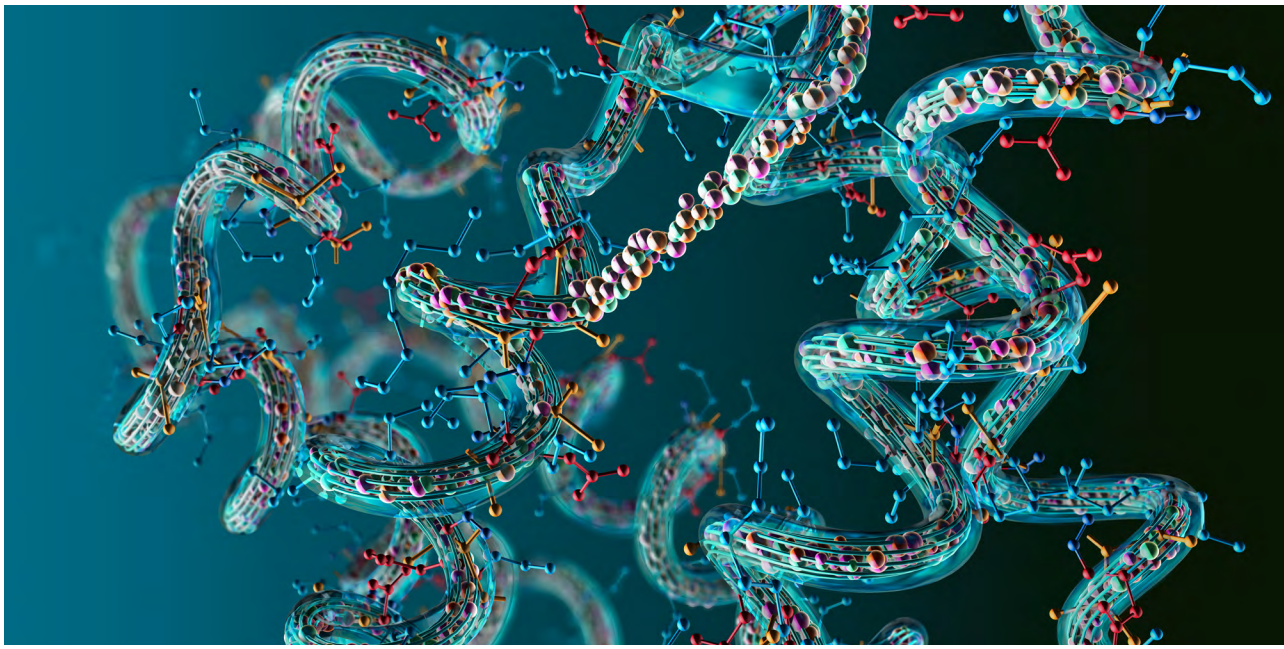

Analysis of a Synthetic Peptide and Its Impurities

Using mass spectrometry compatible mobile phases with an Agilent AdvanceBio Peptide Plus column

AUTHORS

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ABSTRACT

Conventionally, chromatographic peptide separation with UV detection is performed using C18 reversed-phase HPLC columns and mobile phases containing trifluoroacetic acid (TFA) as ion pair reagent. However, although TFA provides improved resolution, it can suppress the mass spectrometry (MS) signal. Formic acid (FA) is a preferred ion pair reagent for MS detection but can result in suboptimal separation with many traditional C18 columns. This application note presents the use of the Agilent AdvanceBio Peptide Plus column to separate synthetic peptide impurities with MS-compatible FA as a mobile phase modifier.

INTRODUCTION

Most peptide drugs are manufactured using solid-phase peptide synthesis. Synthetic peptide-related impurities can come from raw materials, manufacturing processes, or be generated by degradation during manufacturing or storage.[1] Traditionally, peptide separation is achieved using reversed phase columns with trifluoroacetic acid (TFA) as a mobile phase modifier and UV as a detector. However, TFA is not ideal for mass spectrometry (MS) since it can suppress the MS signal.

To identify the impurity peaks in an LC/MS method, formic acid (FA) is a preferred mobile phase modifier but results in suboptimal separation with traditional C18 columns. TFA (pKa ~0.23) can lower the pH to protonate residual (incompletely alkylated or endcapped) silanol sites on the stationary phase surface, leaving no negative charge to interact with positively charged peptides facilitating good peak shape. In addition, TFA anions form an ion pair with positively charged peptides, increasing their hydrophobicity and increasing their retention time. By contrast, FA (pKa ~3.77) is a weaker acid than TFA and cannot lower the pH enough to protonate all the silanol sites so the interaction between the silanols and peptides is not masked completely. This often leads to broader peaks, increased tailing, and overall lower resolution and peak capacity compared with using TFA as a modifier. The Agilent AdvanceBio Peptide Plus stationary phase possesses a hybrid, positively charged surface, and can provide better peak shape and separation with FA as a modifier than traditional C18 columns. This note describes an LC method to separate synthetic peptide impurities using FA as a mobile phase modifier that can be run with either UV or MS detection, therefore making method transfer between LC/UV and LC/MS easier. Both LC/MS and LC/MS/MS are used to positively identify some of the impurities found in the sample, synthetic bivalirudin, FIGURE 1. Bivalirudin is a 20 amino acid synthetic peptide that reversibly inhibits thrombin. Quality control of the synthetic peptide requires the identification and determination of impurities. The amino acid sequence of bivalirudin (FPRPGGGGNGDFEEIPEEYL) has a monoisotopic mass of 2178.9858 Da.

Therefore, using LC/MS can accurately determine the mass of the peptide, but by also using MS/MS analysis it is possible to confirm the sequence through the predicted fragmentation pattern, as shown in TABLE 1. Experimental Reagents and chemicals All reagents were HPLC grade or higher. Sample preparation Aged synthetic peptide bivalirudin trifluoroacetate hydrate was purchased from Selleckchem and reconstituted with 0.1 % FA in water to 1 mg/mL.

FIGURE 1. The amino acid sequence of synthetic bivalirudin.

H-D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH

TABLE 1. The predicted MS/MS fragmentation pattern for bivalirudin.

Seq	No.	b	y	No. (+1)
F	1	148.076	2179.993	20
P	2	245.129	2032.925	19
R	3	401.230	1935.872	18
P	4	498.282	1779.771	17
G	5	555.304	1682.718	16
G	6	612.325	1625.697	15
G	7	669.347	1568.675	14
G	8	726.368	1511.654	13
N	9	840.411	1454.632	12
G	10	897.433	1340.589	11
D	11	1012.460	1283.568	10
F	12	1159.528	1168.541	9
E	13	1288.571	1021.472	8
E	14	1417.613	892.430	7
I	15	1530.697	763.387	6
P	16	1627.750	650.303	5
E	17	1756.793	553.250	4
E	18	1885.835	424.208	3
Y	19	2048.899	295.165	2
L	20	2161.983	132.102	1

Instrumentation

For HPLC experiments, an Agilent 1290 Infinity LC was used comprising:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)
- Agilent 1260 Infinity II diode array detector (DAD) (G7115A)

For LC/MS experiments, the same 1290 Infinity LC configuration was used with an Agilent 6545XT AdvanceBio LC/Q-TOF detector.

Data Processing

LC/UV data was processed using Agilent OpenLab 2.2 CDS. LC/MS data was processed using Agilent MassHunter BioConfirm B.08.00 software. MS/MS spectra were used to confirm the identities of the synthetic peptides and their impurities.

Method Conditions

HPLC Conditions	
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm (p/n 695775-949)
Mobile Phase	A) 0.1 % formic acid in water B) 0.1 % formic acid in acetonitrile
Gradient	0 min: 17% B 2 min: 17% B 22 min: 37% B 24 min: 95% B 26 min: 95% B 26.1 min: 17% B
Post Time	5 min
Flow Rate	0.4 mL/min
Column Temperature	60 °C
Injection Volume	5 µL (UV); 1 µL (MS)

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Source	Dual Agilent Jet Stream
Gas Temperature	350 °C
Drying Gas Flow	10 L/min
Nebulizer Gas	30 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	*m/z* 100 to 1,700 (MS); *m/z* 50 to 1,700 (MS/MS)
MS Scan Rate	8 spectra/s
MS/MS Scan Rate	3 spectra/s
Acquisition Mode	Positive, extended dynamic range (2 GHz)
Collision Energy	$3.6 \times (m/z)/100 - 4.8$

Results and Discussion

FIGURE 2 shows the separation profile of an aged bivalirudin peptide sample using FA as a mobile phase modifier with UV detection. LC/MS/MS is used to identify several major impurity peaks in the profile, as shown in TABLE 2, with very low mass error. Common impurities include deletion sequences (where an individual amino acid is missing), the presence of incompletely removed protecting groups or modifications of the peptide during removal of the protecting groups, loss of water and, in this particular peptide sequence, Asn is prone to deamidation, which could occur during manufacture or upon storage. A total of five peaks were selected to illustrate the techniques used for identification using a combination of LC/MS and LC/MS/MS.

TABLE 2. Peak identification of aged bivalirudin peptide and major impurities.

Peak	Mass (Da)	Peak ID	Target mass (Da)	Mass error (ppm)
1	2,049.9467	Deletion of Glu	2,049.9432	1.71
2	2,178.9894	Product	2,178.9858	1.65
3	2,121.9663	Deletion of Gly	2,121.9644	0.90
4	2,160.9764	Loss of H_2O	2,160.9705	2.73
5	2,179.9742	Deamidation	2,179.9698	2.02

FIGURE 2. LC/UV chromatogram of synthetic bivalirudin. A zoomed baseline region of synthetic bivalirudin is shown.

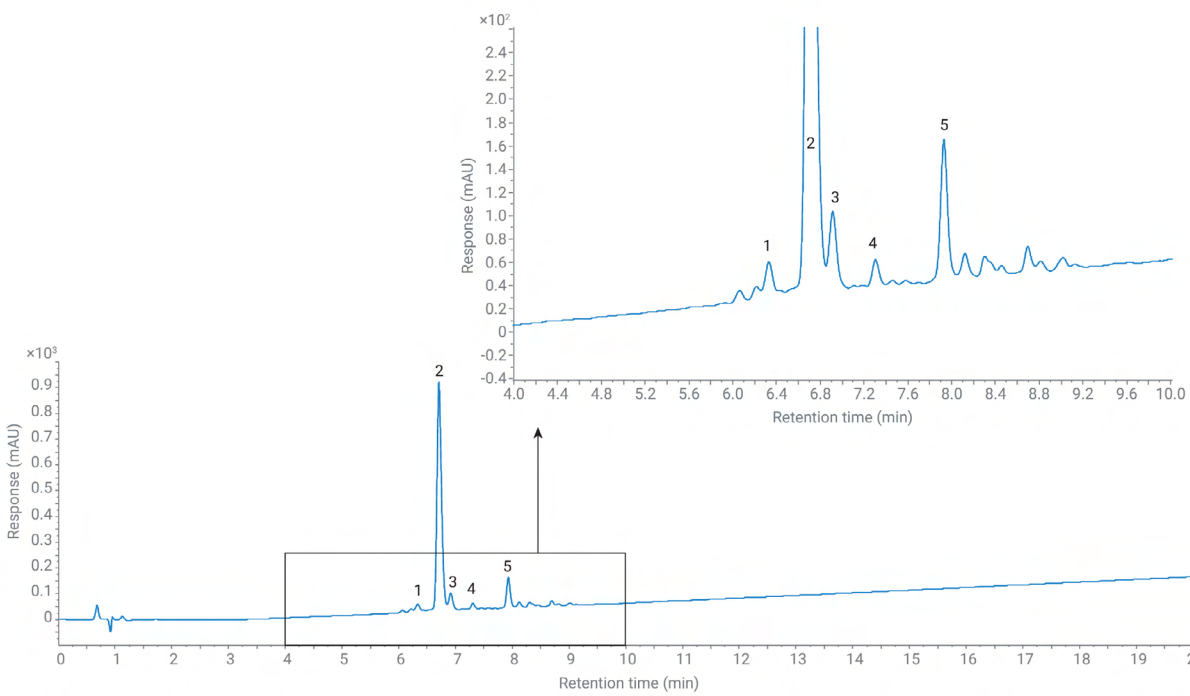
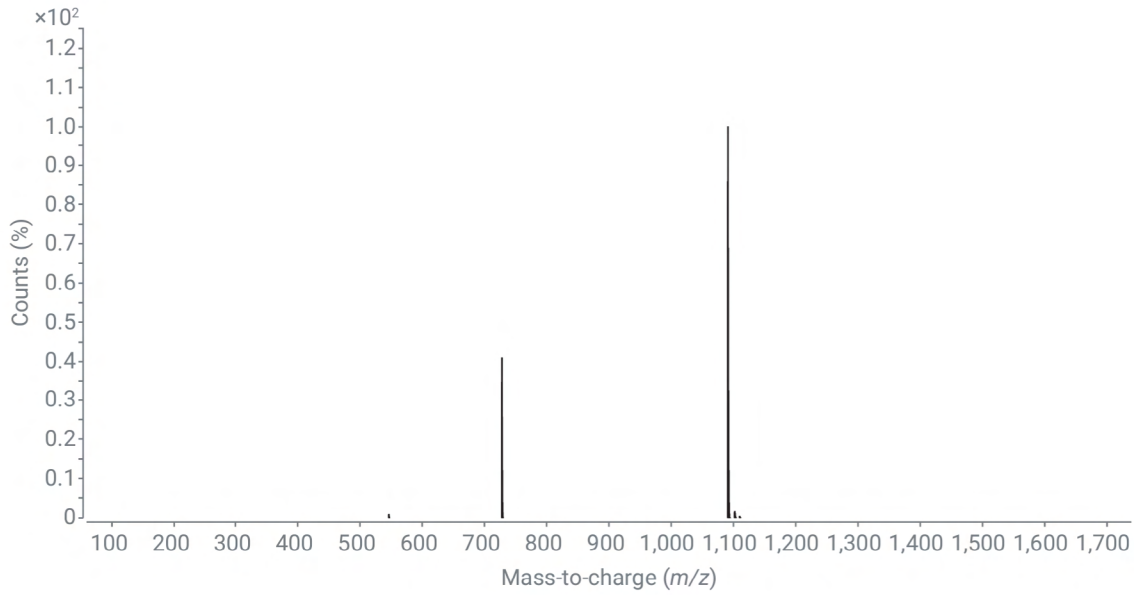


FIGURE 3. MS spectrum of the main product (peak 2)



The major component of the LC/UV chromatogram, Peak 2, gave an MS spectrum shown in Figure 3. This corresponds to [M + 2H]2+ and [M + 3H]3+ m/z which, when deconvoluted, gives the mass of 2178.9894 corresponding to the full-length peptide sequence of bivalirudin, FPRPGGGGNGDFEEIPEEYL. A similar approach is used to identify the earlier eluting impurity, Peak 1. In this case, a similar MS spectrum is obtained (FIGURE 4A), however, the mass of the impurity is 2049.9467 following deconvolution. The mass difference is −129 Da indicative of the loss of glutamic acid. By closer inspection of the LC/MS/MS spectrum, it is possible to identify the position of the missing Glu residue (FIGURE 4B). The BioConfirm software has identified the b15 and y4 fragments for FPRPGGGGNGDFEEIPEYL, a 19 amino acid sequence, indicating that the sequence is missing a glutamic acid at position 17 or 18.

Analysis of impurity peak 3 gives a mass difference of −57 Da, indicative of a missing glycine (FIGURE 5). Impurity peak 4, meanwhile has a mass difference of 18 indicative of dehydration through loss of H2 O (MS spectrum not shown). Finally, analysis of impurity peak 5 gives a mass difference of +1 Da, indicative of deamidation (FIGURE 6A). A closer look at the MS/MS data for this impurity reveals the software has identified that Asn at position 9 (N) has been converted to Asp (D) through deamidation.

FIGURE 4. (A) MS spectrum of impurity, peak 1. (B) MS/MS spectrum of impurity, peak 1.

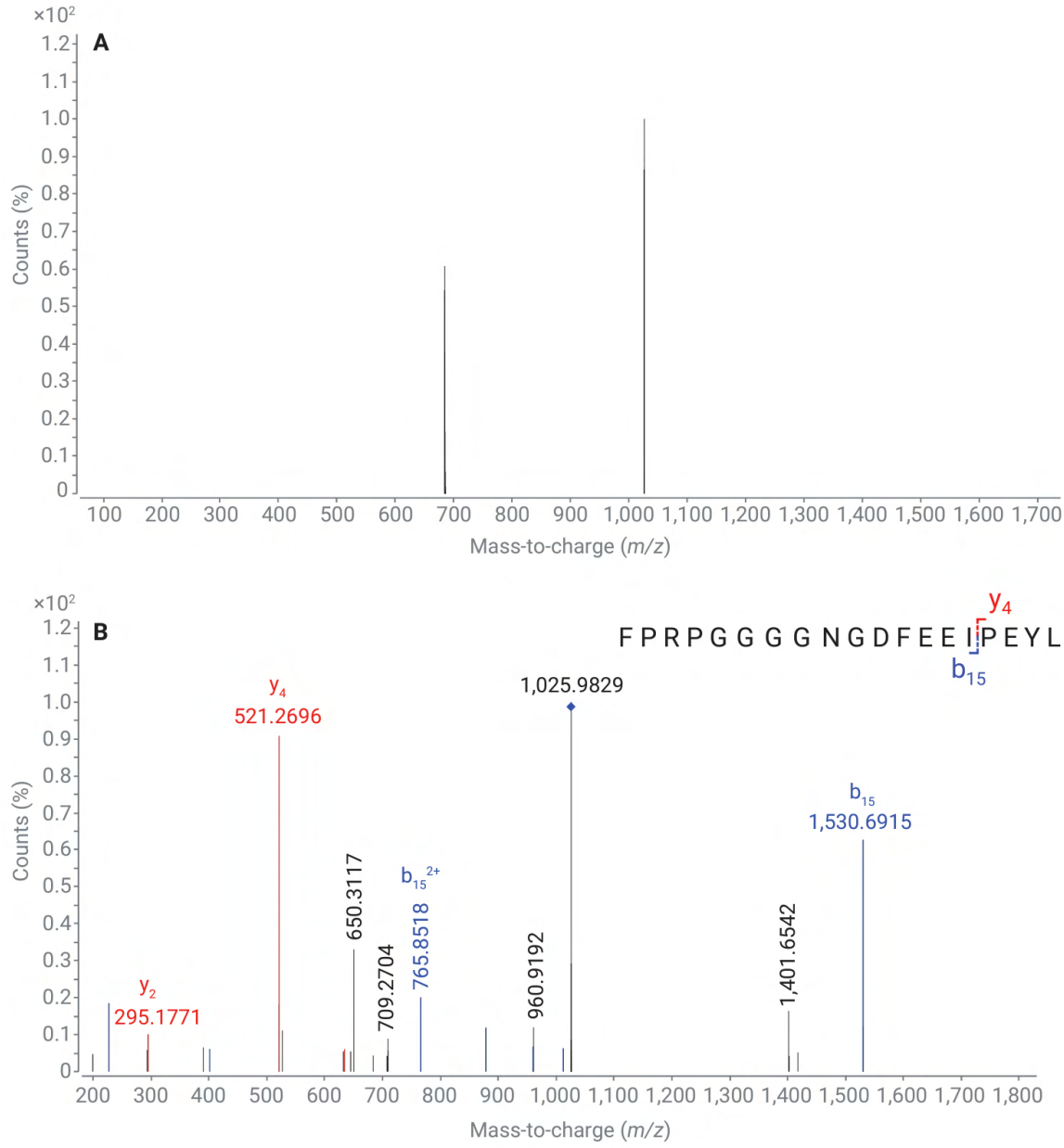


FIGURE 5. MS spectrum of impurity, peak 3

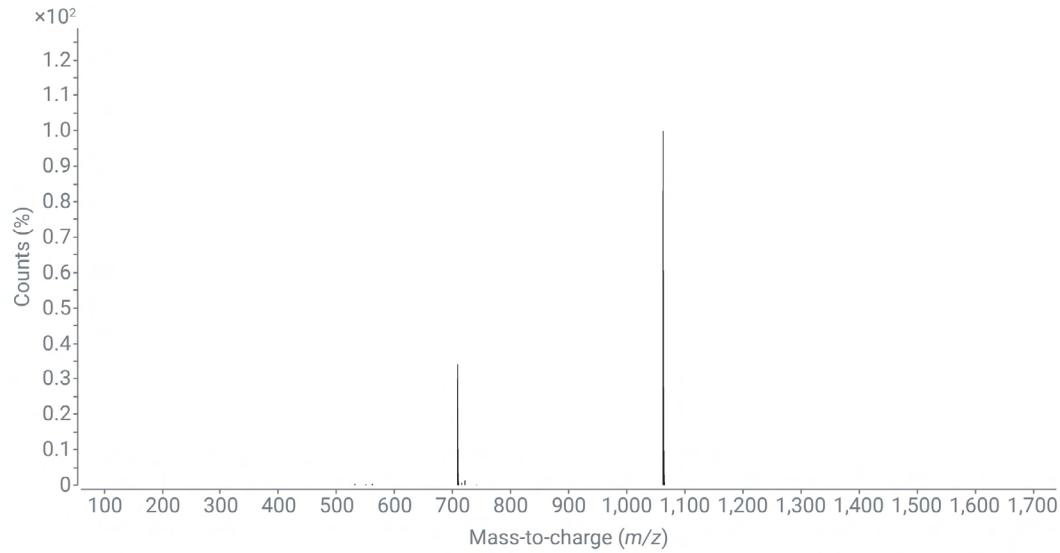
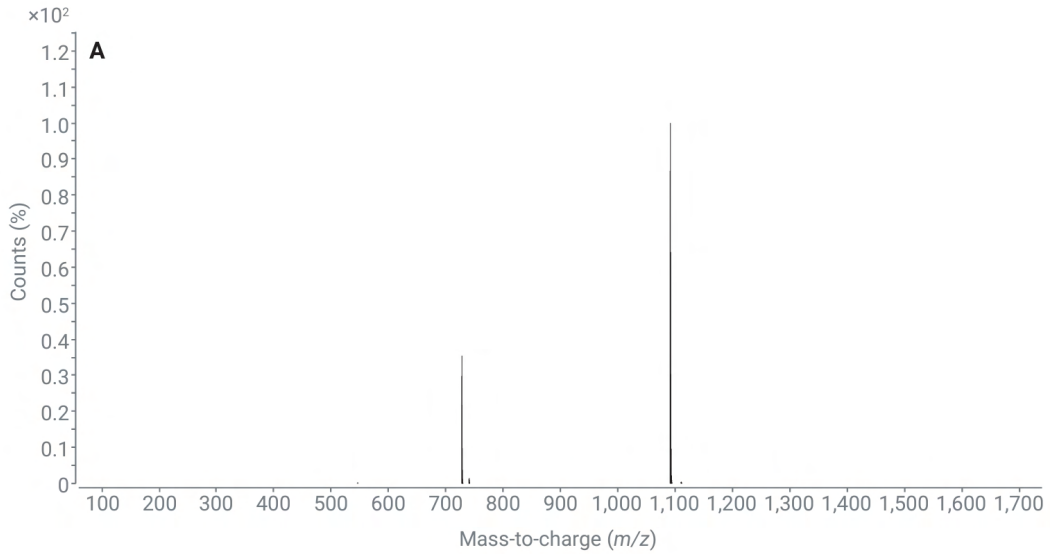
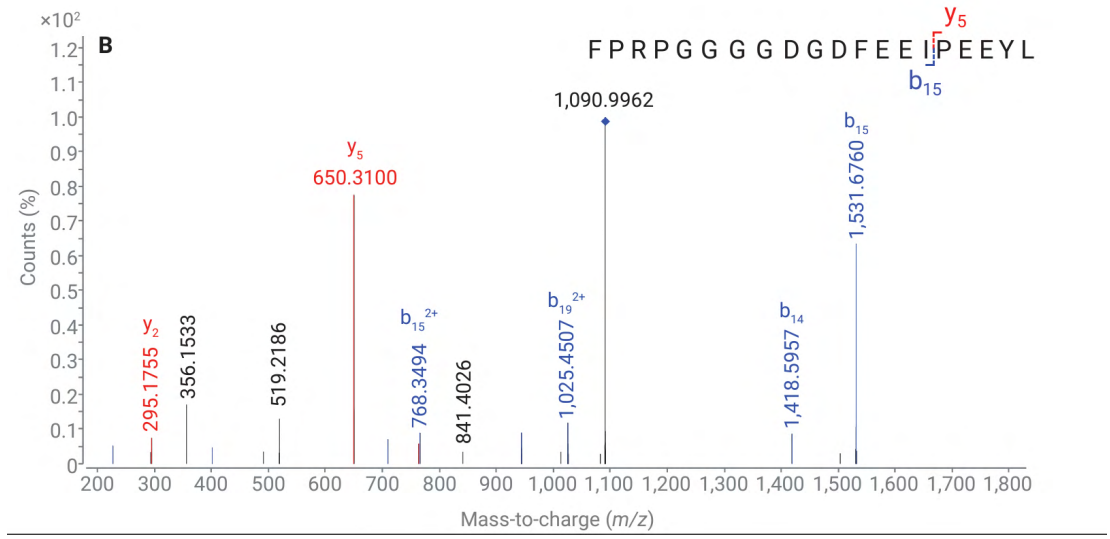


FIGURE 6. (A) MS spectrum of impurity, peak 5.



(Figure 6(B) continued on next page)

FIGURE 6. (B) MS/MS spectrum of impurity, peak 5.



CONCLUSION

In this study, an Agilent AdvanceBio Peptide Plus column was used with formic acid as a mobile phase modifier to analyze a synthetic peptide and its impurities. The method employed can be easily transferred between LC/UV and LC/MS.

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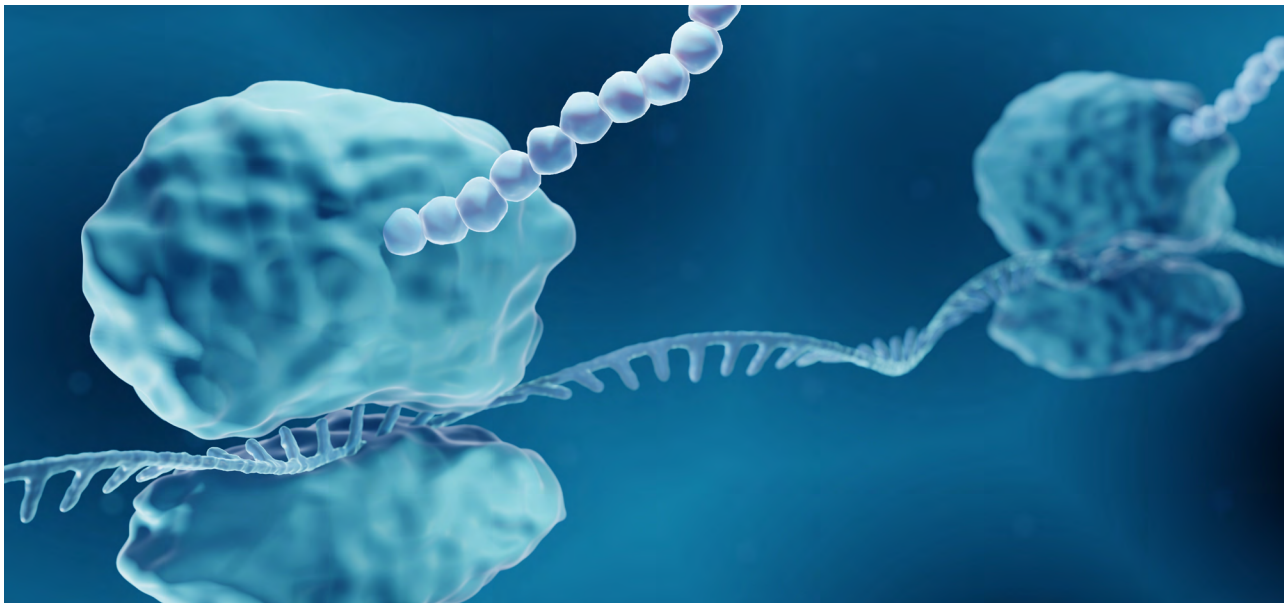


Quantification of Therapeutic Peptide Exenatide in Rat Plasma

Using Agilent 1290 Infinity II Bio LC and Agilent 6495 Triple Quadrupole LC/MS Systems

AUTHOR

Xi Qiu

Agilent Technologies, Inc.

INTRODUCTION

Introduction In recent years, pharmaceutical research and development has shifted focus from small molecule drugs to biologic therapeutics such as large peptide drugs, antibody-drug conjugates, recombinant fusion proteins, monoclonal antibodies (mAb), and oligonucleotide drugs, among others. The traditional analytical method for these large molecules is the ligand binding assay (LBA) due to its high sensitivity, high throughput, low cost, and ease-of-automation. Over the past two decades, liquid chromatography/mass spectrometry (LC/MS) has become an alternative method to analyze these large molecules due to its high specificity, sensitivity, wide dynamic range, and fast method development.[1] At the same time, LC/MS

can avoid cross-reactivity, and has proven to overcome reagent availability compared to traditional LBA.

Exenatide is a large therapeutic peptide approved to treat diabetes mellitus type 2.[2] It is a synthetic version of Exendin-4, a 39 amino acid hormone found in the saliva of the Gila monster reptile with a molecular weight of 4,186.6 Da.[3] Traditionally, the plasma concentration of exenatide was determined by ligand binding assay, which needs time to develop the antibody, and lacks selectivity and specificity. This application note demonstrates an LC/MS method to quantify exenatide in rat plasma using the Agilent Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems (FIGURE 1). The results show that this LC/MS assay is easy, simple, and can be used for exenatide quantitative analysis with excellent sensitivity and reproducibility.

FIGURE 1. Agilent 1290 Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems.

EXPERIMENTAL

Materials and Methods

Exenatide was purchased from MedChemExpress (Monmouth Junction, NJ). Formic acid (FA), was purchased from Sigma-Aldrich (St. Louis, MO). LC/MS-grade acetonitrile was purchased from Agilent Technologies, and 96-well protein LoBind plates were purchased from Eppendorf USA (Hauppauge, NY).

Instrumentation

- Agilent 1290 Infinity II Bio UHPLC system including:
  - Agilent 1290 Infinity II Bio high speed pump (G7132A)
  - Agilent 1290 Infinity II Bio multisampler (G7137A)
  - Agilent 1290 Infinity II thermostat column compartment (G7116A) equipped with a Standard Flow Quick Connect bio heat exchanger (G7116-60071)
- Agilent 6495 triple quadrupole LC/MS system

Sample Preparation

Acetonitrile (600 µL with 1% formic acid) was added to 200 µL of rat plasma aliquots fortified with different concentrations of exenatide. This mixture was vortexed for 5 minutes, then spun down at 16,000 g for 10 minutes. The supernatant was transferred to a 96-well protein LoBind plate, then dried down under nitrogen gas with heating. After drying down, 100 µL of 20% acetonitrile (0.1% FA) was added to reconstitute, and 20 µL was injected into LC/MS for analysis.

LC/MS Analysis

Data acquisition was performed using an Agilent 1290 Infinity II Bio UHPLC coupled to an Agilent 6495 triple quadrupole LC/MS system with Agilent Jet Stream source. Separation was obtained with an Agilent peptide map column (2.1 × 150 mm, 120 Å, 2.7 µm). TABLES 1 AND 2 list the LC and MS parameters used for this workflow.

Positive electrospray ionization of exenatide yielded $[M+5H]^{5+}$ signal at m/z 838.3 as the most intense ion. MRM transitions were optimized and 838.3 → 948.8 was chosen as the quantifier, while 838.3 → 396.3 was chosen as the qualifier with optimal collision energy.

TABLE 1. Liquid chromatography parameters.

LC Conditions	
Column	AdvanceBio Peptide Mapping, 120 Å, 2 × 150 mm, 2.7 µm (p/n 653750-902)
Column Temperature	60 °C
Injection Volume	20 µL
Autosampler Temperature	4 °C
Mobile Phase	A) Water + 0.1 % formic acid B) Acetonitrile + 0.1 % formic acid
Flow Rate	0.5 mL/min
Gradient Program	Time (min) %B 0 10 8 65 8.2 95 9.2 95 9.3 10 12 10
Stop Time	12 minutes

TABLE 2. MS acquisition parameters.

MS Conditions	
Gas Temperature	290 °C
Drying Gas Flow	18 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	1,000 V
High Pressure RF	150 V
Low Pressure RF	80 V

Data Processing

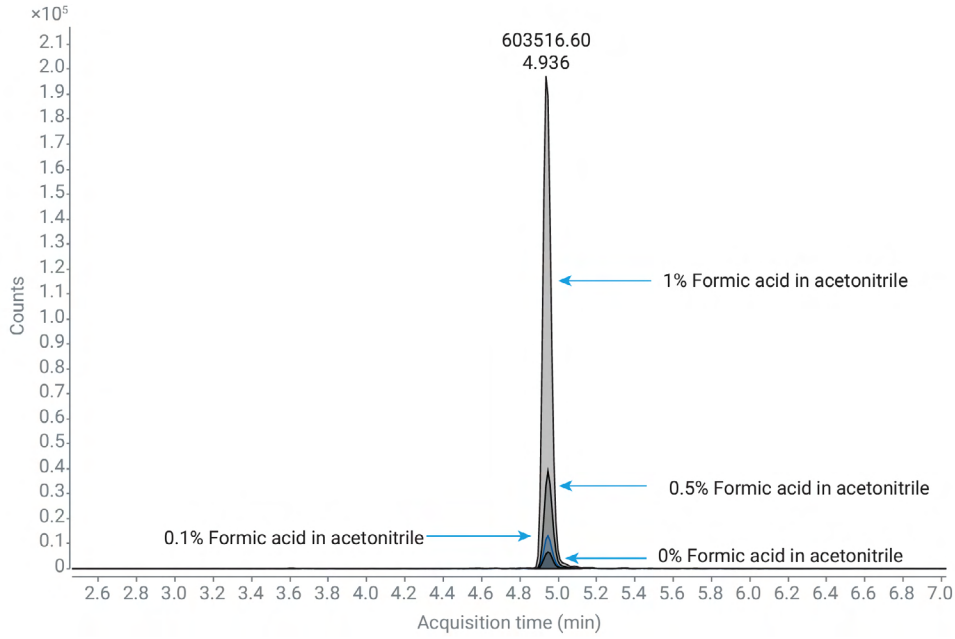
All MS data were processed using Agilent MassHunter Quantitative Analysis software.

RESULTS AND DISCUSSION

Method Optimization for Exenatide Quantitative Analysis

To improve the sensitivity and reproducibility for exenatide quantitative analysis in rat plasma, the sample preparation, LC conditions, and MS conditions were all optimized to achieve best sensitivity and reproducibility. Acetonitrile with 0, 0.1, 0.5, and 1% formic acid were evaluated to precipitate proteins from rat plasma. The data are shown in FIGURE 2. As the extracted ion chromatogram (EIC) of MRM transition showed that 1% formic acid in acetonitrile produced the best analyte response, 1% formic acid in acetonitrile was later used for all sample preparations.

FIGURE 2. Effects of different concentrations of formic acid in acetonitrile for sample preparation.



The UHPLC method was optimized using the peptide map column function, with the temperature set at 60 °C and a flow rate of 0.5 mL/min to achieve the best reproducibility. The column temperature reduced pump pressure and improved the MS sensitivity significantly under these conditions.

Quantitative Analysis of Exenatide from Rat Plasma

Agilent MassHunter Quantitative Analysis software was used to perform quantitative analysis of the calibration curve and quality control samples. With blank rat plasma and an exenatide lower limit of quantification of 0.02 ng/mL (as shown in FIGURES 3A AND 3B), the calibration curve was from 0.02 to 20 ng/mL with quadratic fit and $1/x^2$ weight (as shown in FIGURE 4 and TABLE 3).

The intraday and interday analytical precision and accuracy were determined from three independent preparations, performed over 3 days. The precision and accuracy result of exenatide in rat plasma is shown in TABLE 4. All levels of quality control samples (n = 6) met acceptance criteria of 20% (25% for LLOQ), as recommended by the US Food and Drug Administration. The results demonstrated excellent assay performance using the Infinity II Bio LC and 6495 triple quadrupole LC/MS system for large peptide quantification.

FIGURE 3. (A) EIC of blank rat plasma. (B) EIC of exenatide lowest calibration point.

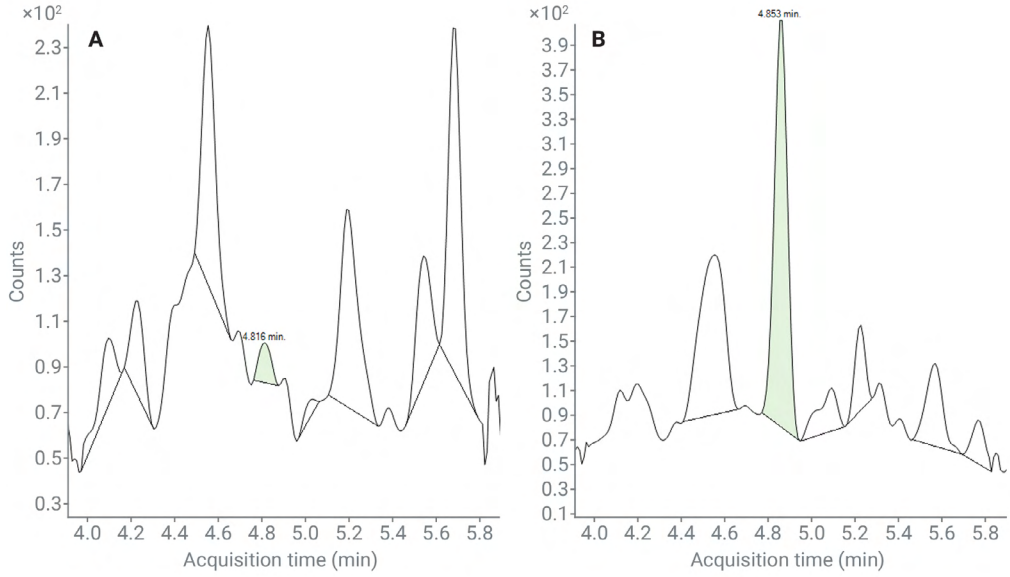


FIGURE 4. Calibration curve of exenatide from 0.02 to 20 ng/mL in rat plasma.

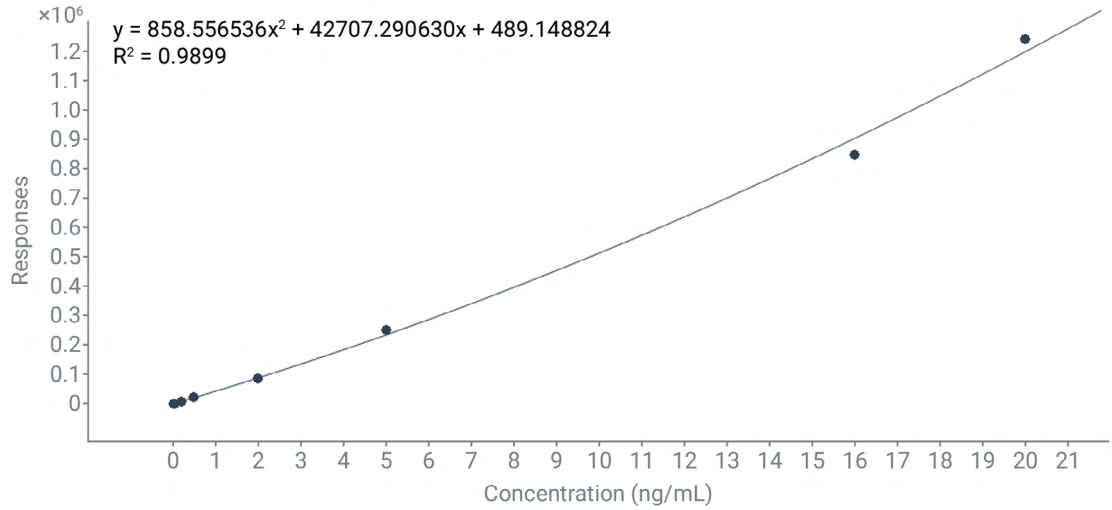


TABLE 3. Exenatide calibration curve performance over three runs.

Calibration (ng/mL)	0.020	0.040	0.200	0.500	2.000	5.000	16.000	20.000
Mean	0.021	0.044	0.201	0.538	1.978	5.315	15.922	19.855
% Bias	5.25	9.00	0.30	7.62	-1.11	6.31	−0.49	−0.72
% CV	3.70	15.57	12.48	5.55	1.37	0.12	6.63	4.90

TABLE 4. Precision and accuracy of quality control samples in mouse plasma (n = 6).

	QC Concentration (ng/mL)	0.02 (LLOQ)	0.06 (Low)	1.00 (Mid)	15.0 (High)
Run 1	Mean	0.0181	0.0649	1.19	15.3
	% Bias	−9.5	8.2	19.0	2.1
	% CV	12.8	12.0	6.7	3.5
Run 2	Mean	0.0225	0.0593	1.04	15.2
	% Bias	12.3	−1.1	3.7	1.6
	% CV	17.0	15.2	4.5	9.8
Run 3	Mean	0.0218	0.0623	1.10	14.8
	% Bias	9.0	3.8	10.0	−1.3
	% CV	14.6	13.7	8.3	8.6
Interday	Mean	0.021	0.0622	1.11	15.1
	% Bias	3.9	3.6	10.9	0.8
	% CV	11.8	4.7	7.7	1.8

CONCLUSION

The Agilent Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems are ideal for large peptide quantitative analysis with excellent assay reproducibility. This application note demonstrates a sensitive LC/MS method for the quantitative analysis of exenatide from rat plasma. This method is simple and fast. In this method, the lower limit of quantification is 0.02 ng/mL from 200 µL of rat plasma, which is equivalent to other solid phase extraction sample preparations. In three qualification runs, intraday and interday QC sample precision and accuracy all met regulatory acceptance criteria, demonstrating excellent assay performance and reproducibility.

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Get tips for optimal chromatographic separation and detection as well as easy selection and ordering information.

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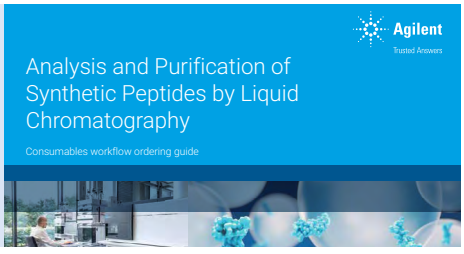

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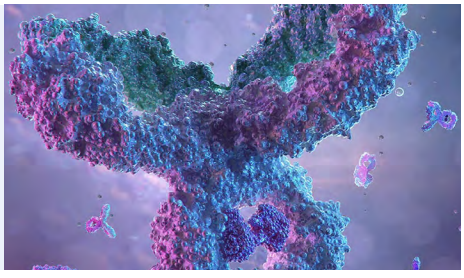

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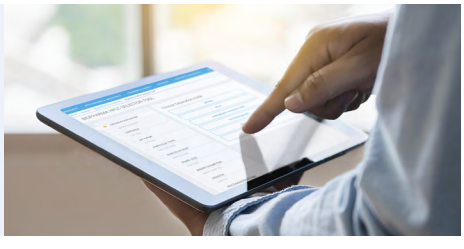

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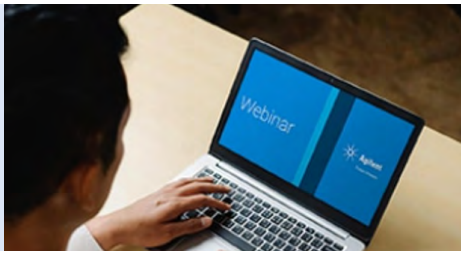

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