Application Note

Biotherapeutics and Biosimilars

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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

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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.

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.

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

Table 1. Liquid chromatography parameters.

LC Conditions	
Column	AdvanceBio Peptide Mapping, 120 Å, 2.1 × 150 mm, 2.7 µm (p/n 653750-902)
Column Temperature	60 °C
Injection Volume	20 µL
Autosampler Temperature	4 °C
Needle Wash	5 seconds in wash port (50/50 water/methanol)
Mobile Phase	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow Rate	0.5 mL/min
Gradient Program	Time %B 0 10 8 65 8.2 95 9.2 95 9.3 10 12 10
Stop Time	12 min

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 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.

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).

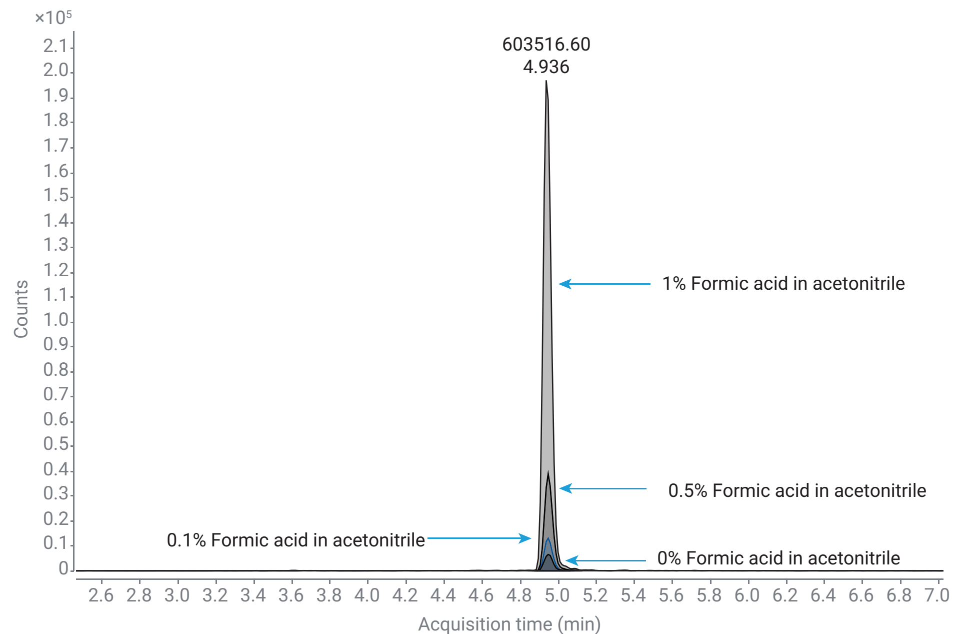

Figure 2. Effects of different concentrations of formic acid in acetonitrile for sample preparation.

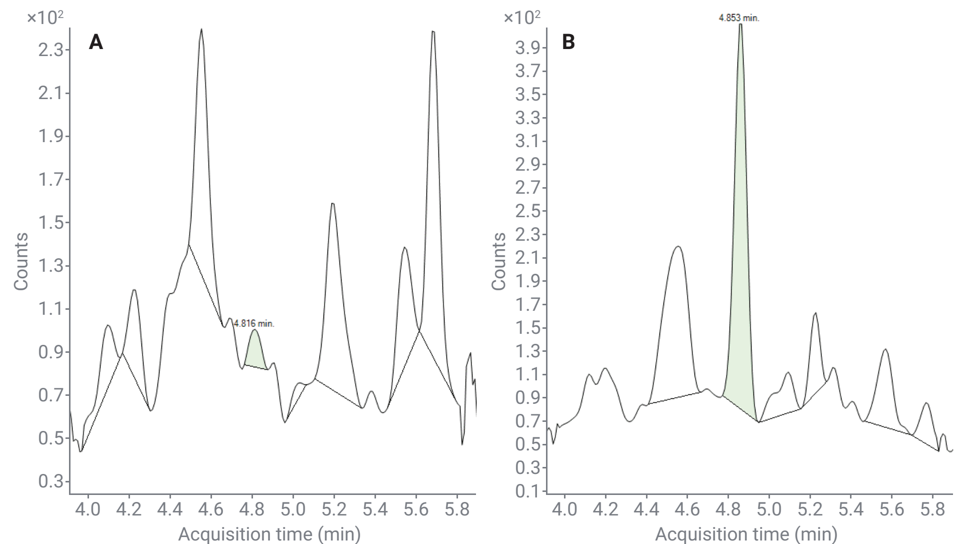

Figure 3. (A) EIC of blank rat plasma. (B) EIC of exenatide lowest calibration point.

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.

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.

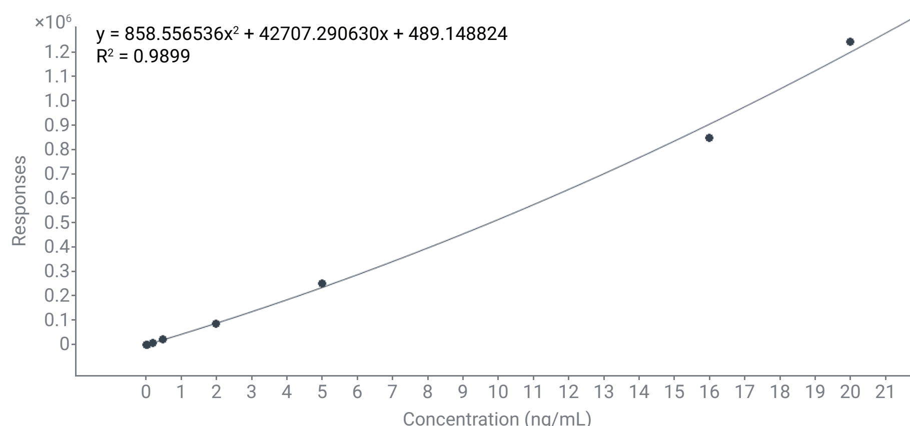

Figure 4. Calibration curve of exenatide from 0.02 to 20 ng/mL in rat plasma.

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

References

1. Jenkins, R. *et al.* Recommendations for Validation of LC–MS/MS Bioanalytical Methods for Protein Biotherapeutics. *AAPS J.* **2015**, *17(1)*, 1–16.
2. Drugs.com AHFS Monographs. https://www.drugs.com/monograph/exenatide.html# (Accessed 2019-03-22).
3. Raufman, J. P. Bioactive Peptides from Lizard Venoms. *Regul. Pept.* **1996**, *61(1)*, 1-18.

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