Ultra-Sensitive Quantitation of pg/mL Level Exenatide with Trap-and-Elute Micro-Flow LC/MS/MS

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ABSTRACT

LC/MS method has been chosen preferably for the quantitation of Exenatide in biological samples due to the advantages in excellent selectivity, sensitivity, wide analytical and reproducibility. To further extend lower limit of quantitation (LLOQ) to accurately study lower concentration exenatide in plasma samples (<100 pg/mL), a microflow trap-and-elute LC/MS/MS method was developed and demonstrated here with LLOQ at 2 pg/mL and excellent linearity, quantitation accuracy and precision, as well as low system carryover.

INTRODUCTION

Exenatide is an effective medication to treat type 2 diabetes marketed as Byetta and Bydureon. It is a 39-amino-acid peptide, a synthetic version of exendin-4 which is a hormone found in the saliva of the Gila monster. Exenatide displays biological properties similar to human glucagon-like peptide-1 (GLP-1), a regulator of glucose metabolism and insulin secretion. Exenatide bears a 50% amino acid homology to GLP-1 and is more resistant to metabolic degradation thus has a therapeutic advantage with longer pharmacological half-life.

Reported methods to quantify exenatide concentration in plasma include immunoassay (ELISA)[1,2] and liquid chromatography mass spectrometry(LC/MS)[3,4]. Quantitation of exenatide using immunoassay usually suffers from limited analytical range, endogenous interferences, lack of good repeatability and specificity, and requires expensive antibodies. LC/MS method has been chosen preferably due to the advantages in excellent selectivity (MS/MS or high resolution MS/MSHR), sensitivity, wide analytical range (usually greater than 3 orders of magnitudes), and reproducibility. Although the reported sensitivity at 100 pg/mL[3] maybe sufficient to quantify exenatide concentration in plasma with dosage formulated for quick release, such as Byetta, the challenge remains to quantify ultra-low level exenatide in plasma when slow release formulated exenatide is administrated.

Micr flow ultrahigh performance LC (µUHPLC) has gained substantial popularity among the analytical community where ultrahigh sensitivity, high throughput, and operational cost reduction have been constant challenges. Published methods using µUHPLC/MS reported advantages over high flow LC including up to 14 times sensitivity gain5, capabilities to execute fast gradient separations, reduced source contamination and reduced solvent consumption6 as well as waste handling cost.

In this study, an ultra-sensitive trap-and-elute µUHPLC-MRM method was developed and demonstrated its suitability for exenatide quantitation in plasma samples down to 2 pg/mL with excellent linearity, quantitation accuracy and precision.

MATERIALS AND METHODS

Sample Preparation:
Protein precipitation was performed for blank rat plasma by mixing 1 mL of rat plasma, 3 mL of acetonitrile, and 0.4 mL of formic acid. The mixture was vortexed 15 seconds then centrifuged at 4000 rpm for 15 minutes. The supernatant was transferred to another centrifuge tube and stored under -20 ºC.

The stock standards of exenatide and isotope labeled exenatide internal standard were kindly supplied by GlaxoSmithKline. The stock solutions were prepared in 20% acetonitrile solution in water. Both stock solutions were diluted in prepared rat plasma in series to different concentrations from 1 – 100 ng/mL as working solutions. Then the working solutions for both exenatide and internal standard were used to prepare calibration standards in 9 levels from 2 to 5000 pg/mL with each level contains internal standard at 100 pg/mL and 10% prepared blank rat plasma matrix.

µUHPLC-MRM Analysis
Trap-and-elute workflow was performed on an Eksigent ekspert nanoLC 425 system with two 5 – 50 µL/min gradient flow modules. A QTRAP® 6500 mass spectrometer was used in this study and operated in multiple reaction monitoring (MRM) mode for the best sensitivity and selectivity. The parameters and instrument configuration are listed as following:

Chromatographic System Configuration

On the Eksigent ekspert nanoLC 400 system, two gradient pumps were both configured in 5-50 µL/min flow rate range, and two valves including a 6-port 2-position injection valve and a 10-port 2-position switching valve were synchronized to utilize the sample loading, sample transferring, injection and eluting. The detailed flow diagram is illustrated in Figure 1.

Trap-and-Elute LC Conditions

LC System:	ekspert nanoLC 425 µUHPLC with two 5 – 50 µL/min flow modules
Column:	eksigent ChromXP C18CL 0.5 × 50 mm, 3 µm
Trap Column:	C18, 0.5 × 5 mm, 5 µm
Column Temp.:	ambient
Injection:	50 µL (50 µL loop with 60 µL overfill)

Gradient 1: Eluting Pump

Flow Rate:	40 µL
Mobile Phase:	A) water, 0.1% formic acid B) acetonitrile, 0.1% formic acid

Gradient 1:	Time/min	A%	B%
	0	95	5
	0.5	95	5
	2	10	90
	4	10	90
	4.1	95	5
	5.0	95	5

Gradient 2: Loading Pump

Flow Rate:	50 µL
Mobile Phase:	A) water, 0.1% formic acid B) acetonitrile/isopropanol (50/50), 1% trifluoroethanol, 0.1% formic acid

Gradient 2:	*Time/min*	*A%*	*B%*
	0	100	0
	1.5	100	0
	1.6	0	100
	5.5	0	100
	5.6	100	0
	6.0	100	0

Inline filter: micro inline filter with 1 µm pore size titanium frit for 1/32" PEEKsil tubing

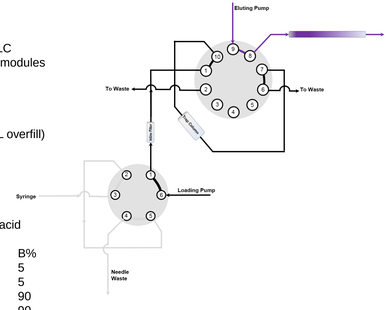


Figure 1. Trap-and-Elute Workflow Flow Diagram

Mass Spectrometry Conditions

System:	QTRAP 6500
Interface:	Microflow Turbo V™ with 50 µm ID electrode
Curtain Gas (CUR):	20
Collision Gas (CAD):	12
Temperature (TEM):	320 ºC
IonSpray Voltage	5200
Ion Source Gas 1 (GS1):	15
Ion Source Gas 2 (GS2):	80
Declustering Potential (DP):	90
Entrance Potential (EP):	8
Scan Type:	Multiple Reaction Monitoring (MRM) Details in Table 1

Table 1. MRM Transitions

Q1 MS	Q3 MS	Time (ms)	ID	CE	CXP
838.2	396.2	70	Exenatide_1	28	24
838.2	299.1	70	Exenatide_2	42	27
844.2	402.2	70	Exenatide_IS	28	15

RESULTS AND DISCUSSION

µUHPLC-MRM Method Development

The scope of this study was to develop an ultrasensitive method that enables the quantitation of ultra-low level exenatide in plasma sample. To achieve that, a large volume injection with trap-and-elute workflow was developed on an Eksigent ekspert nanoLC 400 platform with the best sensitivity at 2 pg/mL. An example of typical result obtained from a plasma sample containing 100 pg/mL exenatide using trap-and-elute workflow is shown in Figure 2.

Excellent specificity was achieved with MS/MS acquisition. Two MRM scans for exenatide were acquired for quantitation and confirmation. As a result, ultra-low level exenatide can be reliably quantitated, as shown in Figure 3, where chromatograms of low level exenatide in diluted plasma matrix at 2, 5, 20, and 50 pg/mL were illustrated.

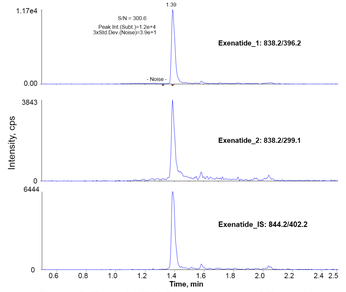


Figure 2. Typical MRM Chromatograms of Exenatide at 100 pg/mL Exenatide in Crashed Plasma Sample

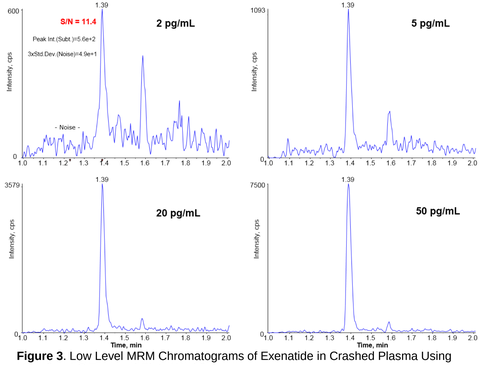


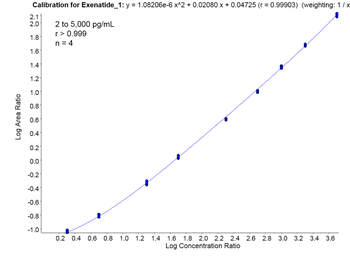
Figure 3. Low Level MRM Chromatograms of Exenatide in Crashed Plasma Using Microflow LC Trap-and-Elute Workflow

A system free of contamination and capability of recovery to zero level background after high concentration analysis is critical for ultra-low level quantitation. Exenatide is a highly hydrophobic large peptide which sticks to sampling path as well as column stationary phase thus an effective cleanup procedure is required to maintain system free of cross contamination between runs. In this study, a strong wash with acetonitrile/isopropanol mixture (50/50, 0.1% formic acid) with 1% trifluoroethanol (TFE) was used to clean needle surface and injected between unknown samples to clean sampling path. No quantifiable peaks were observed in the specific retention time window where exenatide elutes, showing the system free of contamination. With effective wash, the carryover was observed at 0.2%, and zero system background could be reached within 5 injections.

Quantitation Performance

The quantitation performance of this method was evaluated with respect to linearity, accuracy and precision with calibration samples prepared in diluted crashed plasma matrix.

The sensitivity of this method is measured by lower limit of quantitation (LLOQ) defined as the lowest concentration in calibration standards that satisfies both signal to noise (S/N >10) and statistical requirements (precision and accuracy < 20% deviation). The LLOQ was determined at 2 pg/mL. %CV was observed within 7% (n=4) and %Accuracy ranges from 91% to 106%.



CONCLUSIONS

This application note describes a peptide quantitation workflow for ultra-low level quantitation of exenatide using Microflow LC trap-and-elute on Eksigent ekspert nanoLC 425 with sensitive and selective MS/MS detection with QTRAP 6500.

Excellent quantitation performance was achieved with 50 µL injection microflow LC trap-and-elute workflow on Eksigent ekspert nanoLC 425 coupled with QTRAP 6500:

- LLOQ at 2 pg/mL
- 2 pg/mL to 5,000 pg/mL linear range
- %CV < 7%
- Accuracy: 91.6% to 106%
- 10 minutes total run time

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