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A liquid chromatography-mass spectrometry assay for quantification of Exendin[9-39] in human plasma

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Abstract

Exendin[9-39] is a glucagon-like peptide-1 receptor (GLP-R) antagonist and a potential therapeutic drug for treatment of congenital hyperinsulinism by lowering insulin concentration in plasma. A specific and sensitive LC-MS/MS method was validated for quantification of Exendin[9-39] in human plasma. Exendin[9-39] and the stable isopically labeled internal standard eluted at 9.2 min and were analyzed by single reaction monitoring (SRM) of the transitions m/z 842.9 $\rightarrow$ 991.8 and 848.2 $\rightarrow$ 998.8, respectively. The calibration curve was linear in the range 15–1260 ng/mL with a limit of detection of 1.3 ng/mL. The CVs of the standards were 2.7–13.1% within-run and 3.1–13.2% between-run. The matrix effect was >100% and the SPE recovery was 98.4 $\pm$ 12.9 percent. In absence of protease inhibitors, short-term stability at room temperature was only one hour. Accordingly, samples were kept on ice and sample processing was kept below one hour. Human plasma samples from a clinical pilot study in which Exendin [9-39] was administered intravenously were analyzed and concentrations up to 600 ng/mL were reported. Plasma samples from the study were stored at –80°C with internal standard and successfully reanalyzed after twelve months.

Keywords

Exendin (9-39); hyperinsulinism; triple quadrupole mass spectrometry; LC-MS/MS; peptide quantification; GLP-1

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1.Introduction

Congenital hyperinsulinism (HI) is a genetic disorder of pancreatic beta-cell regulation. In HI, excessive insulin production leads to hypoglycemia, especially severe in neonates, which can result in permanent brain damage if not treated early. Current treatments of HI involve inhibitors of insulin secretion such as diazoxide or somatostatin analogues [1, 2]. Children with the most common and severe form of HI, due to inactivating mutations on the beta-cell K_{ATP} channels, are unresponsive to medical therapy and require pancreatectomy.

Exendin[9-39] is an exogenous glucagon-like peptide-1 receptor (GLP-R) antagonist that impairs glucose tolerance in humans and in a variety of animal models. Exendin-[9-39] has been shown effective in lowering plasma insulin concentrations by blocking the GLP-1 induced insulin secretion [3] and is a promising therapeutic agent to prevent hypoglycemia in congenital hyperinsulinism [4, 5]. Exendin[9-39] has a relative molecular mass of 3369.8 g/mol and is a truncated derivate from the C-terminus of Exendin-4, a GLP-1R agonist originally isolated from the saliva of the Gila monster (*Heloderma suspectum*). The synthetic peptide Exendin-4 is commercially known as Exenatide and was approved in 2005 by the FDA for type 2 diabetes treatment. Although GLP-1 half-life in plasma is less than 2 min being rapidly cleared by the action of the serine protease dipeptidyl peptidase-4 (DPP-4) [6], Exendin analogues are not susceptible to DPP-4 cleavage showing longer half-life in plasma [7].

Quantitative analysis of therapeutic drugs and peptides is routinely done in clinical laboratories by immunoassays. Most recently, liquid-chromatography triple quadrupole mass spectrometry is emerging as a selective, sensitive and robust tool alternative[8, 9] for peptide analysis in biological fluids. Liquid chromatography tandem mass spectrometry (LC-MS/MS) has been successfully used for quantification of both endogenous hormone analogs and antagonists (e.g. GIP and GLP-1[10], oxyntomodulin[11], ghrelin[12]) and exogenous bioactive peptides (e.g. enfuvirtide[13], octreotide[14]).

Whereas an enzymatic immunoassay is available for Exendin-4, to our knowledge there is no specific and reliable assay for Exendin[9-39]. In order to study the pharmacokinetic properties of Exendin[9-39] in plasma for therapeutic purposes two conditions need to be met: the method must be specific, and it should use small sample volume. Sample volume is a special concern when patients are infants, from which the amount of blood that can be withdrawn is more limited.

To meet these requirements, we developed a novel quantitative mass spectrometry based assay specific for Exendin[9-39] and that requires only 100 μ L of patient plasma. Based on previous published pharmacokinetic results[3], the linear range of the calibration curve ranging 15.5 to 1260 ng/mL is adequate for determination of Exendin[9-39] concentrations in human plasma. The method was used to quantify Exendin[9-39] concentrations in plasma from subjects participating in a pilot clinical study examining the effect of Exendin[9-39] on fasting glucose regulation [5].

2. Material and methods

2.1. Materials

Exendin[9-39] and stable isotopically labeled ($[^{13}\text{C}_6, ^{15}\text{N}]$ Leu2,12,17)-Exendin[9-39] (Figure 1) were obtained from Bachem. Acetonitrile, water, methanol were Optima LC-MS grade from Fisher Scientific. Formic acid 99+% ampules were purchased from Thermo Scientific and trifluoroacetic acid ampules were purchased from J.T.Baker. Different lots of blank potassium EDTA plasma were obtained from Bioreclamation.

2.2. Preparation of calibration curve and samples

Exendin[9-39] stock solution was prepared in water at a concentration of 2.52 mg/mL and stored in aliquots at -80°C . Similarly, Exendin isotopically labeled internal standard was dissolved in water and stored aliquoted at -80°C at 1 mg/mL. Exendin and its internal standard 20-fold stock solutions for the calibration curve were prepared on the day of the analysis by serial dilution with pooled plasma. Standards for the calibration curve were prepared by addition of 5 μL of the 20-fold stock solution of Exendin[9-39] and 5 μL of 20-fold plasma diluted internal standard solution to 90 μL of plasma. Plasma samples from the pilot clinical study were thawed on ice and 5 μL of the 20-fold internal standard stock were added to 95 μL of sample. Only 25 μL were required for solid-phase extraction (SPE) and the rest was stored at -80°C for analysis on separate occasions. All samples were processed in less than one hour after being thawed to prevent analyte degradation.

2.3. Exendin[9-39] extraction protocol

MCX Oasis microelution plates (Waters) were used for extraction of the analyte and the internal standard. The MCX media was conditioned with methanol and equilibrated with 0.2% formic acid. A 25 μL aliquot of the sample or standard was diluted 1:1 with 0.2% formic acid and loaded to the MCX plate. The sorbent was subsequently washed with 50% methanol in 2% formic acid and Exendin[9-39] was eluted with 150 μL of 5% NH_4OH in methanol. Solvent was evaporated to dryness in a vacuum concentrator and the sample reconstituted in 25 μL of 20% acetonitrile in 0.1 % TFA. Sample vials were vortex mixed and then centrifuged before placing them in the autosampler.

2.4. Chromatography and mass spectrometry

Chromatography and mass spectrometry was performed with a nanoAcquity UPLC system (Waters Corporation) coupled to a Xevo TQ-S triple quadrupole (Waters) equipped with a Trizaic source. The Trizaic nanoTile comprises a trapping column ($180\text{ }\mu\text{m} \times 20\text{ mm}$, 5 μm , Sunfire C18) and an analytical column ($85\text{ }\mu\text{m} \times 100\text{ mm}$, 1.8 μm , HSS T3). Mobile phases A were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). One microliter of sample was injected onto the trap and washed at 20% B for 1.8 min at 8 $\mu\text{L}/\text{min}$. Exendin elution was accomplished with a gradient from 20 to 65% B in 12 min at 0.45 $\mu\text{L}/\text{min}$, followed by a 3 min column wash at 0.7 $\mu\text{L}/\text{min}$ with 85% B. Including column re-equilibration at 20%B, the total run time was 21 min. Under these conditions, Exendin[9-39] retention time was 9.3 min.

LC-MS/MS analysis of Exendin[9-39] on the Xevo TQ-S triple quadrupole mass spectrometer was performed in positive mode. Source parameters were: capillary voltage 3.4 kV and 70 °C source temperature. The transitions used for Exendin[9-39] and the internal standard were m/z 842.9 $\rightarrow$ 991.8 and 848.2 $\rightarrow$ 998.8, respectively. SRM for analyte quantification were generated at cone voltage 40 V and collision energy 18. The cone voltage was set to 50V and collision energy at 22 for transitions 842.9 $\rightarrow$ 396.2 and 848.2 $\rightarrow$ 396.2. These SRMs were monitored to confirm peptide presence. An additional precursor ion scanning function was added to the method to detect the presence of precursors of m/z 184, which corresponds to the choline fragment of phospholipids.

TargetLynx (Waters) was used to process raw data and calculate peak areas for Exendin[9-39] and its internal standard. The smoothing method used was Mean, smoothing iterations were set to 1 and smoothing width was set to 2. The integration window was 2 min. The response, expressed as the ratio of the peak area of the analyte/area internal standard was plotted vs. the concentration in Microsoft Excel and fit by least squares linear regression.

The method was validated following the Guidance for Industry, Bioanalytical Method Validation published by the Food and Drug Administration and available online (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf).

2.5. In vivo study

Exendin[9-39] was administered intravenously for 6 hours to subjects with congenital hyperinsulinism at a rate of 0.02 mg/kg/hr for 2 hours, then 0.06 mg/kg/hr for 2 hours, followed by 0.1 mg/kg/hr for the last 2 hours [5]. Plasma samples for determination of Exendin[9-39] concentration were obtained in EDTA tubes hourly during the infusion and for 3 hours after the infusion was terminated.

3. Results and Discussion

Exendin[9-39], a 31 amino acid peptide amidated at the C-terminus, was analyzed in positive ion mode and the mass spectrum is shown in Figure 1, the most abundant ions being at m/z 843 ($M+4H$)⁴⁺ and m/z 1123 ($M+3H$)³⁺. Similarly, the internal standard spectrum shows abundant ions at m/z 848 ($M+4H$)⁴⁺ and m/z 1130 ($M+3H$)³⁺. The internal standard used for quantification was an analogue of Exendin[9-39] synthesized with stable-isotope ¹³C and ¹⁵N on the three leucines (Leu^{2,13,18}).

The ion at m/z 843 was chosen as a precursor for selected reaction monitoring (SRM) of Exendin[9-39]. The MSMS spectra arising from collision induced dissociation of the labeled and unlabeled precursors ($M+4H$)⁴⁺ are shown in Figure 2. The most abundant product ions observed in the MSMS spectrum at m/z 991.9 corresponded to the ion b_{27}^{3+} and was selected for quantification. The second most abundant ion at m/z 396.19 was assigned to y_4^+ , and used for peptide presence confirmation.

Blank plasma spiked with Exendin[9-39] was used to develop an extraction method. An Oasis sorbent selection plate was used to screen four different chromatographic media. The MCX sorbent yielded the best recovery (data not shown) and the μ Elution plate format was chosen in order to work with small plasma volumes.

3.1. Selectivity

Seven different lots of human EDTA plasma were used to generate blank samples and investigate the presence of endogenous interferences. Matrix blanks were extracted and processed as described in the Materials and Methods section. No interferences in the chosen transition channels were found in the matrices free of the analyte (see supplemental Figure S1). The standards for the calibration curve were prepared with pooled plasma from different lot numbers.

3.2. Linearity

The linear response of the calibration curve was determined by preparation of a set of standards of the following concentration 1.3, 5.2, 15.6, 46.7, 140, 280, 560, 840 and 1260 ng/mL. Supplemental Figure S2 shows that the assay was able to detect as low as 1.3 ng/mL at $S/N > 3$. Although the precision is within 20% at 5.2 ng/mL, the accuracy exceeded the 20% deviation permitted at the lower limit of quantification (LLOQ). Consequently, the LLOQ at $S/N > 10$ is 15 ng/mL with precision and accuracy below 20% deviation. The upper limit of quantification (ULOQ) for the standard curve was 1260 ng/mL. The method was linear in the concentration range between 15–1260 ng/mL.

3.3. Solid-phase extraction recovery

Analyte recovery was calculated as the response of the standard in plasma after SPE extraction divided by the response of an equivalent amount of extracted plasma and post-spiked with the corresponding standard concentration of Exendin[9-39][15]. The percentage of SPE recovery was calculated at seven different concentrations and is shown in Table 1. In average, the % SPE recovery for all seven standard concentrations was 98.45 ± 12.95 , indicating nearly complete recovery.

3.4. Accuracy and precision

Between-run and within-run accuracy and precision are shown in Table 2 and are within acceptable values. Standards for the between-run accuracy and precision assessment were prepared from the stock solutions on three different days. Maximum coefficient of variance (%CV) within-run was 13.1 and between-run 13.2. At the LLQ, the %CV was 13 within-run and 9.6 between run, well between the required 20%.

3.5. Matrix effect and sample stability

Evaluation of the matrix effect was assessed by the post-extraction spike method, proposed by Matuszewski [15]. The post-extraction spike method, compares the response of the analyte spiked to the matrix post-extraction with the response of the analyte dissolved in neat mobile phase. Neat standards were prepared in loading buffer (20% acetonitrile, 0.1% TFA) by serial dilution from the stock solution. Freshly made solutions were placed in the

autosampler at 7 °C and analyzed from least to highest concentration in triplicate to generate a standard curve. Another set of standards were prepared by spiking Exendin[9-39] to post-extracted plasma. The calculated matrix effect for all standards was >100% indicating ionization enhancement for Exendin[9-39] in plasma. Phospholipids can be a source of matrix effects and to evaluate their possible interference, a mass scanning function for precursors of the choline fragment (m/z 184) was included in the method. No co-elution of phospholipids with Exendin[9-39] was observed. The presence of an internal standard prevents a concentration deviation due to matrix effect (Ann Van Eeckhaut et al 2009).

It was noted that the peak areas of Exendin[9-39] prepared in neat solvent decreased between three consecutive injections for all standards. Most likely, Exendin[9-39] is affected by non-specific adsorptive loss of the peptide to surfaces [16]. This effect was less pronounced when the analyte was prepared by serial dilution with plasma (data not shown), suggesting that adsorptive loss is overcome when carrier proteins are present in the sample. The addition of an isotopically labeled internal standard circumvents this potential drawback. No adsorptive loss was observed for the stock solutions with a concentration 1 mg/mL, stored at -80°C for six months.

Short-term stability was evaluated by incubation of a set of standards freshly prepared in k-EDTA plasma at room temperature with no protease inhibitors. Peak area of the samples analyzed at 0, 1, 2 and 3 hours was evaluated and showed that Exendin[9-39] was stable for one hour in plasma at room temperature. It has been reported that Exendin-4 is stable in monkey plasma for 3h on ice but required addition of the protease inhibitor aprotinin to achieve stabilization at room temperature for the same time[17]. Accordingly, standards and samples in our study were kept in an ice bath and the internal standard was added immediately after sample thaw. Care was taken to keep sample processing before SPE below one hour.

Freeze-thaw stability was tested for Exendin[9-39] for different concentrations. The samples were stored for 24 hours at -80 °C and thawed at room temperature. After two additional freeze-thaw cycles the samples were analyzed and compared to freshly prepared samples. An average increase of 25% (data not shown) was observed for peak area of the analyte after three freeze-thaw cycles. When the internal standard was added to the standard prepared in plasma before freeze-thaw stability testing, the average increase of the response was only of 4%. Consequently, when analyzing plasma samples it is advisable to add the internal standard upon sample collection or after a first freeze-thaw cycle, especially if sample reanalysis is required. Long-term stability of Exendin[9-39] in the presence of internal standard was tested by sample reanalysis after twelve months (see Figure 4B) storage at -80 °C.

The residence time in the autosampler for a processed sample was typically 24 hours, during which it was analyzed. Post-preparative stability was assessed by repeated injections of the standards at different time points. The presence of an internal standard ensured that the measured analyte response was stable for an autosampler resident time of at least 68 hours.

3.6. Measurements of Exendin[9-39] in human plasma from a clinical study

The described analytical method was successfully applied for quantification of Exendin[9-39] concentration in human plasma and determination of a pharmacokinetic profile in a pilot clinical study. Exendin[9-39] and the isotopically labeled internal standard eluted at a retention time of 9.3 min (Figure 3). Peak area of the acquired transitions for three standards of the calibration curve and the internal standard are shown in Figure 3 (A, B, C). Figure 3 (D, E, F) shows Exendin[9-39] transitions for the peptide and the internal standard, extracted from human plasma collected at different time points of the study.

Figure 4A shows the concentration of Exendin[9-39] in plasma collected at 0, 60, 120, 180, 240, 300, 360, 420, 480 and 540 min for three research participants in the pilot study. Sample storage stability at -80°C was assessed after twelve months by reanalysis of the plasma aliquots containing the internal standard that had been previously analyzed on day 0.4. A fresh calibration curve was prepared and the plasma samples stored for 12 months at -80°C were thawed in ice and reanalyzed (Figure 4B). The good correlation of concentration values obtained after a 12 month storage period, shows that samples frozen after addition of the internal standard can be reanalyzed with the same outcome.

4. Conclusions

In conclusion, we developed and validated a method for quantification of Exendin[9-39] in human plasma. The assay lower limit of quantification is 15 ng/mL, uses 100 μL of human plasma and is specific for Exendin[9-39]. The method was used to determine levels of Exendin[9-39] in plasma collected during a pilot clinical study. The linearity of the standard curve, over the concentration range of 15–1260 ng/mL, is sufficient to monitor Exendin[9-39] in human plasma at the dosage of the study. Exendin[9-39] stability in plasma is limited to one hour if working at room temperature. Thus, samples should be thawed in ice, rapidly followed by internal standard addition and sample processing should be kept within one hour. Samples stored with internal standard for over 12 months at -80°C were successfully reanalyzed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

(HI)	Hyperinsulinism
(GLP-R)	Glucagon-like peptide-1 receptor
(DPP-4)	Dipeptidyl peptidase-4
(SPE)	Solid-phase extraction
(SRM)	Selected reaction monitoring
(LLOQ)	Lower limit of quantification
(ULOQ)	Upper limit of quantification
(SD)	Standard Deviation.

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- We developed and validated a LC-MS/MS method for quantification of Exendin[9-39] in human plasma
- The assay required only 100 uL of human plasma
- Linearity of the standard curve was 15–1260 ng/mL
- The assay was used to quantify Exendin[9-39] concentration in plasma from subjects participating in a pilot clinical study
- Storage stability at –80C was good after a 12 month sample storage period

Fig. 1. MS spectrum of Exendin[9-39] (top) and the isotopically labeled Exendin[9-39] (bottom), acquired with a Xevo TQ-S mass spectrometer in positive ion mode.

Fig. 2. CID fragmentation spectra and assigned ion fragments of Exendin [9-39] (top) and its internal standard (bottom). The most intense peaks, b_{27}^{3+} and y_4^{4+} were used for Exendin [9-39] quantitation in plasma.

Fig. 3.

Transitions acquired for Exendin[9-39] and its internal standard for three standards of the calibration curve 15.5, 560 and 1260 ng/mL. SRM chromatograms of Exendin[9-39] and its internal standard after extraction from human plasma collected from one patient during the pilot study at times 120, 240 and 360 min.

Fig. 4.

(A) Concentration of Exendin[9-39] in plasma collected from three subjects at time points $t=0, 60, 120, 180, 240, 360, 420, 480$ and 540 min. (B) Sample reanalysis after a twelve month storage period at -80°C .

Table 1

Percentage of SPE recovery for Exendin[9-39]. Recovery was calculated as the response given by the standard spiked in plasma before SPE and divides by the response given by the standard spiked in plasma post SPE.

Nominal concentration (ng/mL)	15.5	46.7	140	280	560	840	1260
% SPE	104	93	96	83	94	95	128

Table 2

Between-run (n=9) and within-run (n=3) precision and accuracy for Exendin[9-39] spiked in plasma at different concentrations.

Nominal concentration (ng/mL)	15.5	46.7	140	280	560	840	1260
Between-run mean	17	49.2	140.4	274.5	553.3	811.5	1262.1
Between-run SD	1.6	6.5	15	26.5	26.2	25.3	46.2
Between-run accuracy (%)	9	5.5	0.3	-2	-1.2	-3.4	0.2
Between-run precision (%CV)	9.6	13.2	10.7	9.7	4.7	3.1	3.7
Within-run mean	17.6	53.2	139.9	252.9	527.9	832.2	1268.6
Within-run SD	2.3	2.9	7.5	6.9	25.5	25.2	61.4
Within-run accuracy (%)	13.4	13.9	-0.04	-9.7	-5.7	-0.9	0.7
Within-run precision (% CV)	13.1	5.4	5.4	2.7	4.8	3	4.8