RESEARCH ARTICLE

Direct Quantitative Analysis of a 20 kDa PEGylated Human Calcitonin Gene Peptide Antagonist in Cynomolgus Monkey Serum Using In-Source CID and UPLC-MS/MS

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Abstract

PEGylation is a successful strategy to improve the pharmacokinetic and pharmaceutical properties of therapeutic peptides. However, quantitative analysis of PEGylated peptides in biomatrix by LC-MS/MS poses significant analytical challenge due to the polydispersity of the polyethylene glycol (PEG), and the multiple charge states observed for both the peptide and PEG moieties. In this report, a novel LC-MS/MS method for direct quantitative analysis of 20 kDa PEGylated CGRP[Cit, Cit] in cynomolgus monkey serum is presented. CGRP[Cit, Cit] is an investigational human calcitonin gene peptide receptor antagonist with amino acid sequence *Ac*-WVTH[Cit]LAGLLS[Cit]SGGVVRKNFVPT DVGPFAF-*NH₂*. In-source collision-induced dissociation (in-source CID) of 20 kDa PEGylated peptide was used to generate CGRP[Cit, Cit] fragment ions, among which the most abundant b_8^+ ion was selected and measured as a surrogate for the 20 kDa PEGylated peptide. A solid phase extraction (SPE) method was used to extract the PEGylated peptides from the biomatrix prior to the UPLC-MS/MS analysis. This method achieved a lower limit of quantitation (LLOQ) of 5.00 ng/mL with a serum sample volume of 100 μL, and was linear over the calibration range of 5.00 to 500 ng/mL in cynomolgus monkey serum. Intraday and interday accuracy and precision from QC samples were within ±15%. This method was successfully applied to a pharmacokinetic study of the 20 kDa PEGylated CGRP [Cit, Cit] in cynomolgus monkeys.

Key words: Therapeutic peptide, PEGylated peptide, CGRP peptide, Quantitation, LC-MS/MS, Peptide bioanalysis

Introduction

Peptides are potentially attractive therapeutic agents due to their high biological activity associated with high target specificity and low toxicity [1, 2]. However, the inherent properties of peptides in their native form often prevent them from being viable therapeutic agents without some modification. In particular, peptides generally have very short circulatory half-lives due to the combined effects of a rapid renal clearance and enzymatic degradation in the blood, liver, or kidneys by endogenous proteases. To overcome these limitations, strategies such as amino acid modifications have been applied to improve peptide stability by rendering the peptides less susceptible to breakdown by endogenous proteases [3–8]. Additional improvements in

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peptide drugability can be obtained through PEGylation [9, 10]. When the size of the attached PEG reaches 20 kDa or above, PEGylation can significantly extend *in vivo* circulatory half-life of the therapeutic peptides [11–13], as well as improve their chemical and physical stability, solubility, and potentially reduce immunogenecity [14–17].

20 kDa PEGylated CGRP[Cit, Cit] is a human calcitonin gene peptide (CGRP) receptor antagonist developed for the treatment of migraine pain [5]. CGRP[Cit, Cit] is a peptide analog of the N-terminal truncated native human CGRP(8–37)-NH$_2$ (Figure 1a), which has demonstrated specific antagonist activity toward CGRP receptors [18]. Amino acid modifications in CGRP[Cit, Cit] (Figure 1b) were designed to improve peptide stability and potency. Specifically, the [Cit, Cit] designation refers to a double citrulline substitution of the two native arginine residues near the N-terminus of human CGRP(8–37)-NH$_2$. The 20 kDa PEGylation was designed to further enhance the pharmacokinetic properties of CGRP[Cit, Cit]. The peptide was PEGylated at the amino group of the side chain of Lys^{25} with a 20 kDa linear PEG and a three-carbon alkyl chain as the linker (Figure 1c).

A sensitive and reliable bioanalytical method was needed to assess the pharmacokinetic properties of PEGylated CGRP[Cit, Cit] as therapeutic agent in the preclinical studies. Thus far, enzyme-linked immunosorbent assay (ELISA) has been the analytical platform of choice for the quantitative measurement of PEGylated peptides in biomatrix utilizing either anti-PEG or anti-peptide antibodies [19–21]. ELISA methods utilizing anti-peptide antibodies could be time-consuming and costly to develop due to the need for specific antibody reagents for each peptide. This is potentially a problem when a large number of peptide analogs need to be evaluated.

While LC-MS/MS has been widely utilized for quantitative bioanalysis of therapeutic peptides and peptide biomarkers [22–24], this methodology faces significant challenges for PEGylated peptides due to the heterogeneity of the PEG moiety and the high molecular weight. When a PEGylated peptide is introduced into an LC-MS/MS system, both the size and charge state distribution of the PEGylated peptide oligomers produce a broad continuous mass spectrum over a wide *m/z* range [25, 26]. This mass spectrum is difficult to interpret and generally not usable for quantitative analysis. As a result, there have been no published reports describing the direct quantitative analysis of PEGylated peptides by LC-MS/MS. Recently, three quantitative LC-MS/MS methods for PEGylated drugs [27–29] have been reported. However, these methods were accomplished by using tryptic digestion prior to LC-MS/MS to generate non-PEGylated signature peptides that could be used as surrogates for the quantitative analysis of the target PEGylated drugs.

Here we report a UPLC-MS/MS method for direct quantitative measurement of 20 kDa PEGylated CGRP[Cit, Cit] in cynomolgus monkey serum. In this method, PEGylated peptides undergo in-source fragmentation in the instrument ionization source after UPLC separation, resulting in unique peptide fragment ions which can be used as surrogates for the quantitative analysis of the PEGylated peptides. To facilitate the development of a reliable UPLC-MS/MS method, 20 kDa PEGylated CGRP[Q, Q] as shown in Figure 1d, was synthesized as internal standard in which the two citrullines in the CGRP[Cit, Cit] were each replaced by glutamine(Q) in order to provide an appropriate mass difference. The resulting method was applied to a pharmacokinetic (PK) study in cynomolgus monkeys.

Experimental

Chemicals and Reagents

CGRP[Cit, Cit], CGRP[Q, Q], and their 20 kDa PEGylated conjugates were prepared at Amgen Inc. (Thousand Oaks, CA, USA) [5]. Acetonitrile, methanol and water (HPLC grade) were obtained from Burdick and Jackson (Muskegon, MI,
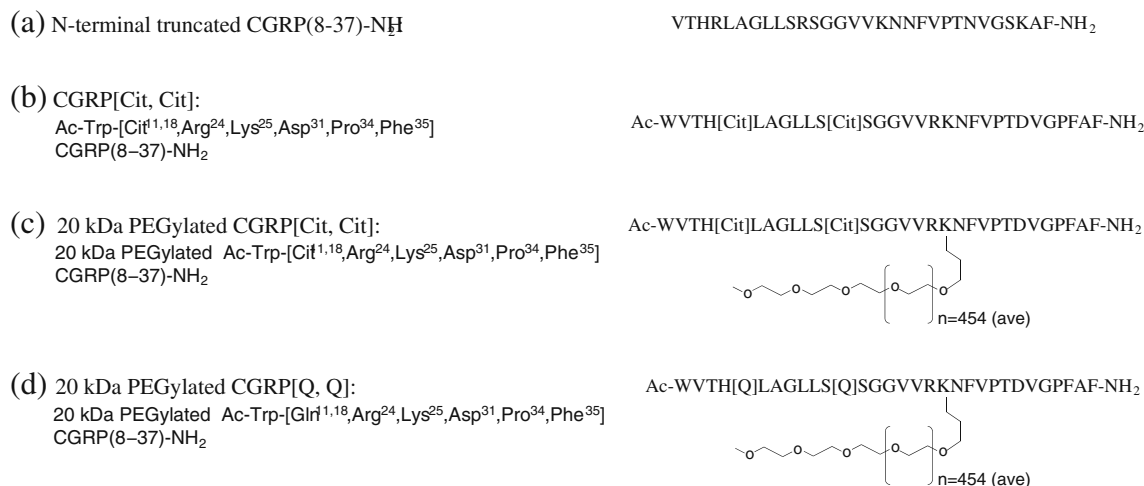
(a) N-terminal truncated CGRP(8-37)-NH$_2$

VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH$_2$

(b) CGRP[Cit, Cit]:
Ac-Trp-[Cit^{1,18},Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]
CGRP(8–37)-NH$_2$

Ac-WVTH[Cit]LAGLLS[Cit]SGGVVRKNFVPTDVGPFAF-NH$_2$

(c) 20 kDa PEGylated CGRP[Cit, Cit]:
20 kDa PEGylated Ac-Trp-[Cit^{1,18},Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]
CGRP(8–37)-NH$_2$

Ac-WVTH[Cit]LAGLLS[Cit]SGGVVRKNFVPTDVGPFAF-NH$_2$

n=454 (ave)

(d) 20 kDa PEGylated CGRP[Q, Q]:
20 kDa PEGylated Ac-Trp-[Gln^{1,18},Arg^{24},Lys^{25},Asp^{31},Pro^{34},Phe^{35}]
CGRP(8–37)-NH$_2$

Ac-WVTH[Q]LAGLLS[Q]SGGVVRKNFVPTDVGPFAF-NH$_2$

n=454 (ave)

Figure 1. Primary structures of: **(a)** N-terminal truncated CGRP(8–37)-NH$_2$; **(b)** CGRP[Cit, Cit]; **(c)** 20 kDa PEGylated CGRP[Cit, Cit]; and **(d)** 20 kDa PEGylated CGRP[Q, Q]

USA). Formic acid (reagent grade) was from Aldrich, Inc. (St. Louis, MO, USA). Ammonium hydroxide (10%–35% NH_3) was from J. T. Baker (Phillipsburg, NJ, USA), Oasis HLB µElution 96-well SPE plates were obtained from Waters Inc. (Milford, MA, USA). Control cynomolgus monkey serum was supplied by Bioreclamation Inc. (East Meadow, NY, USA).

Preparation of Calibration Standards, Quality Control (QC) Samples, and Internal Standard Solution

One hundred µg/mL working solutions of 20 kDa PEGylated CGRP[Cit, Cit] were prepared in methanol/water (50/50, vol/vol) and stored in a refrigerator at 2 to 8 °C prior to use. Serum calibration standards at concentrations of 5.00, 10.0, 25.0, 50.0, 100, 250, and 500 ng/mL were prepared by serial dilution of a freshly prepared standard (5000 ng/mL) in cynomolgus monkey serum. QC samples, at concentrations of 5.00, 15.0, 100, and 400 ng/mL, were prepared by spiking control serum with appropriate dilutions prepared from a 100 µg/mL 20 kDa PEGylated CGRP[Cit, Cit] working solution. The QC samples were then aliquoted into polypropylene tubes and stored frozen at −70 °C. 20 kDa PEGylated CGRP[Q, Q] was used as an internal standard. The internal standard solution was prepared in methanol/water (30/70, vol/vol) at a concentration of 100 ng/mL and stored in a refrigerator at 2 to 8 °C prior to use. The concentration is based on the peptide weight of the PEGylated peptides.

Serum Sample Extraction by Solid Phase Extraction

In order to achieve an LLOQ of 5.00 ng/mL 20 kDa PEGylated CGRP[Cit, Cit], serum samples were prepared using solid phase extraction, which served not only to enrich the PEGylated peptides and remove the endogenous components such as serum proteins and lipids, but also to provide a filtered extract, which is preferred when working with UPLC. Study and QC samples were thawed at room temperature and then vortex-mixed. One hundred µL of each serum sample was aliquoted into the appropriate well of a 96-well plate, followed by the addition of 100 µL of water with 0.1% formic acid and 200 µL internal standard solution. Samples expected to have concentrations above the upper limit of quantitation (ULOQ) were diluted using the blank monkey serum prior to analysis. The samples were then covered and vortex-mixed in preparation for solid phase extraction. An Oasis HLB µElution 96-well SPE plate was conditioned by sequential addition of 400 µL methanol and water under vacuum. The pretreated serum samples were then transferred to the conditioned 96-well SPE plate and drawn through with vacuum. The plate was washed sequentially with 100 µL water, methanol /water/ammonium hydroxide (10/85/5, vol/vol/vol), and finally water again. The final extract was eluted with 50 µL methanol/water (90/10, vol/vol) containing 0.1% formic acid into a 96-well

plate. The 96-well plate was capped with a polypropylene cover (Varian, Lake Forest, CA, USA) and transferred to the autosampler. Samples were then injected onto the UPLC-MS/MS system for analysis. This optimized SPE extraction method using Oasis HLB µElution 96-well SPE plate offers an average SPE extraction recovery greater than 60%.

Chromatography and Mass Spectrometry

The UPLC-MS/MS consisted of an Acquity UPLC system (Waters, Milford, MA, USA) coupled to an API 4000 triple quadrupole mass spectrometer (AB SCIEX, Toronto, Canada) with a Turbo IonSpray ionization source operated in the positive ion mode. UPLC was chosen as the chromatographic platform as it provides highly efficient chromatographic separations combined with reduced run time and increased resolution and sensitivity [30, 31]. The analytical column was an Acquity UPLC BEH C_{18} 2.1 mm×50 mm column with 1.7 µm particle size. A 0.2 µm pre-column filter unit was used to protect the analytical column. The mobile phases were 0.1% formic acid in acetonitrile/water (5/95,
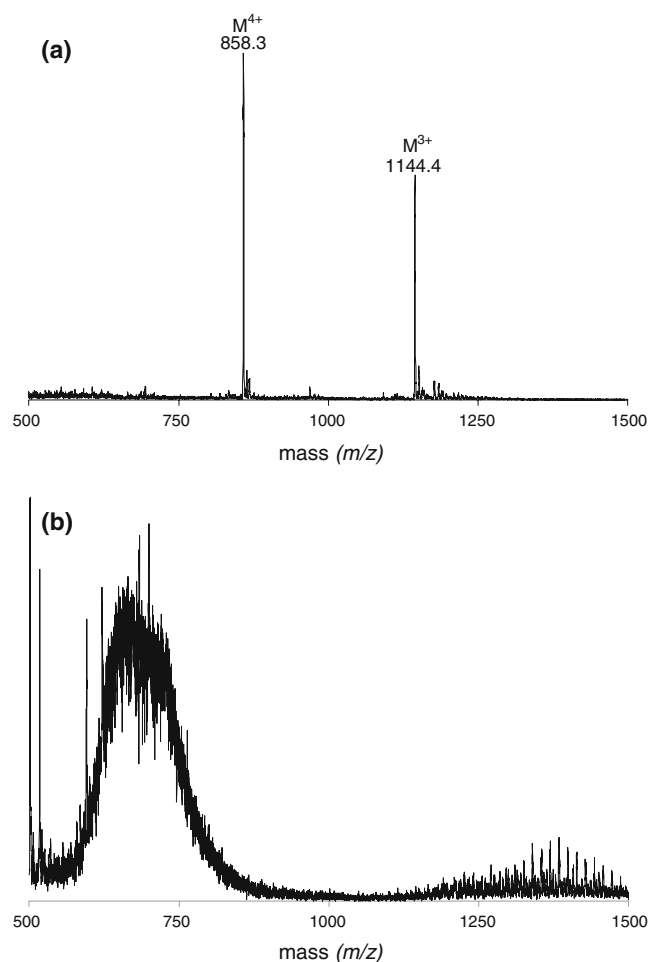

Figure 2. **(a)** ESI-MS spectrum of CGRP[Cit, Cit] at declustering potential=50 V; **(b)** ESI-MS spectrum of 20 kDa PEGylated CGRP[Cit, Cit] at declustering potential=50 V

(a)

DP = 100V

Ac-WVTH[Cit]LAGLLS[Cit]SGGVVRKNFVPTDVGPFAF-CONH$_2$

b$_4$ b$_5$b$_6$b$_7$b$_8$b$_9$

n=454 (ave)

(b)

DP = 180V

b$_8$
964.9

b$_5$
723.5

b$_9$
1077.8

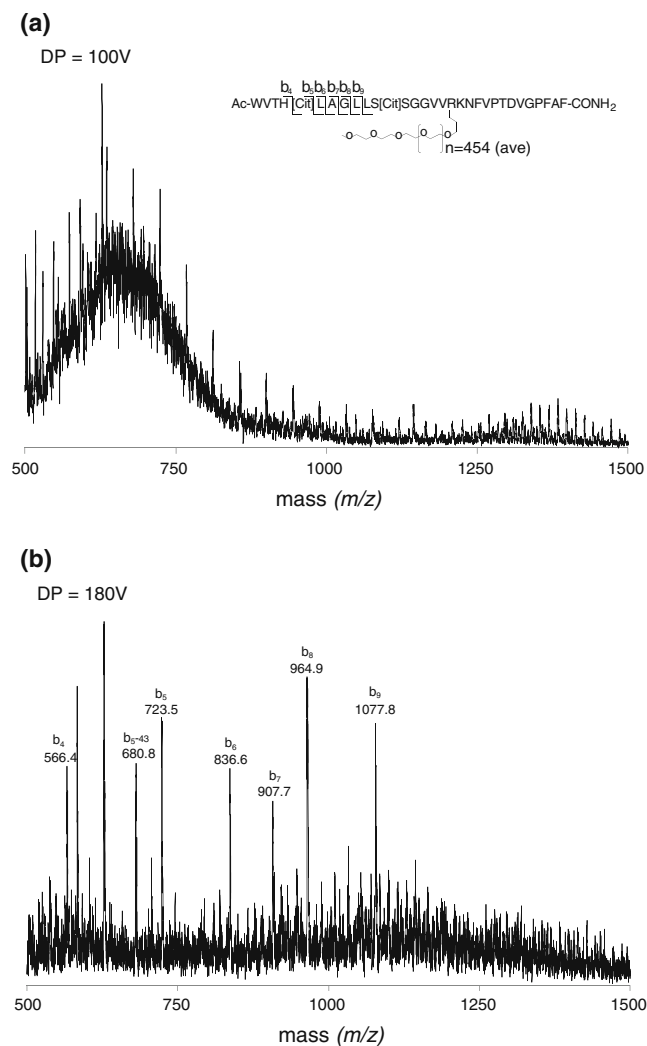b$_4$
566.4

b$_5$-43
680.8

b$_6$
836.6

b$_7$
907.7

Figure 3. In-source CID spectra of 20 kDa PEGylated CGRP [Cit, Cit]. **(a)** Declustering potential=100 V; **(b)** declustering potential=180 V

vol/vol, mobile phase A) and 0.1% formic acid in acetonitrile/water (95/5, vol/vol, mobile phase B). Mobile phase composition was changed linearly from 30% B to 95% B in 1.9 min. After 0.3 minutes the composition was set back to 30% B and left to equilibrate for 0.3 min. Total runtime was 2.5 min. The flow rate was 0.6 mL/min with a column temperature of 70 °C and an injection volume of 10 μL. The autosampler temperature was set at 10 °C. Data was collected and processed using AB SCIEX Analyst software (ver.1.4.1). MRM parameters were optimized by direct infusion of 10 μg/mL PEGylated peptide tuning solutions at 10 μL/min. The ESI spray voltage was set at 5000 V. The source temperature was 500 °C. The curtain gas (CUR) was 30, nebulizer gas setting (GS1) was 40 and the auxiliary gas setting (GS2) was 50 (all arbitrary units). The declustering potential (DP) was optimized and set to 180 V for in-source CID of both analyte and the internal standard. The ion transitions for MS/MS

detection were m/z 964.6→680.6 and m/z 935.6→694.6 for the analyte and the internal standard, respectively.

Assay Performance and Pharmacokinetic Applications

The calibration curve was derived from the peak area ratios (analyte/internal standard) using $1/x^2$ weighted linear least-squares regression of the area ratio *versus* the concentration of the corresponding standard. The regression equation from the calibration standards was used to back-calculate the measured concentration for each standard, QC and unknown sample. Intraday and interday accuracy and precision were assessed from three analytical runs on separate days. Each contained six replicates of each QC concentration at 5.00, 15.0, 100, and 400 ng/mL 20 kDa PEGylated CGRP[Cit, Cit]. The developed method was applied to a pharmacokinetic study to determine the serum concentrations of 20 kDa PEGylated CGRP[Cit, Cit] in cynomolgus monkeys after a single subcutaneous dose of 2 mg/kg. Serum samples were collected at 0.5, 1, 2, 4, 6, 8, 12,
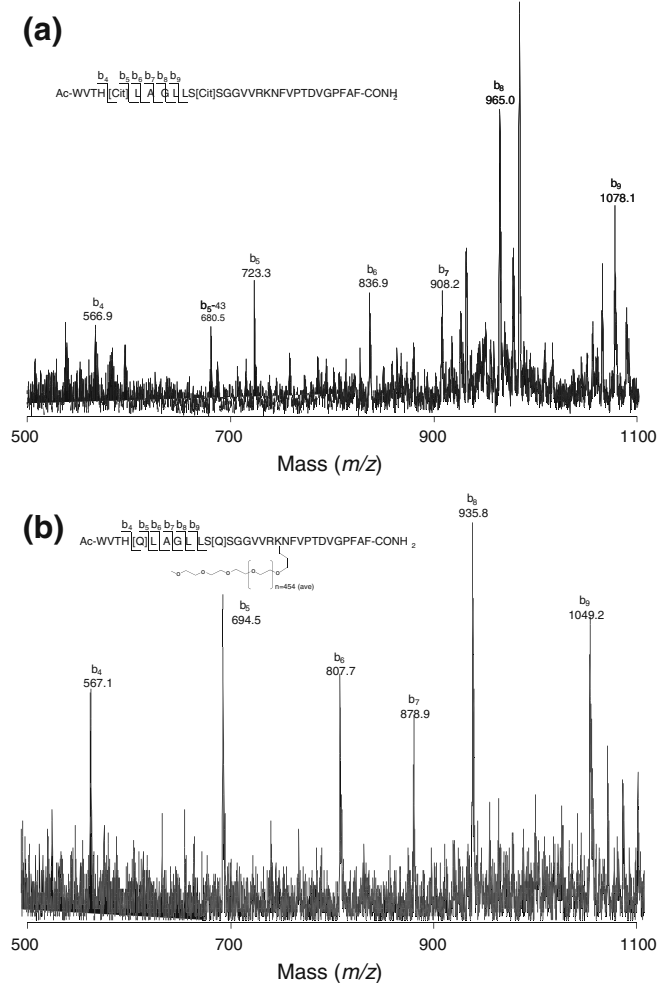
(a)

Ac-WVTH[Cit]LAGLLS[Cit]SGGVVRKNFVPTDVGPFAF-CONH$_2$

b$_4$ b$_5$ b$_6$ b$_7$ b$_8$ b$_9$

b$_8$
965.0

b$_9$
1078.1

b$_5$
723.3

b$_6$
836.9

b$_7$
908.2

b$_4$
566.9

b$_5$-43
680.5

(b)

Ac-WVTH[Q]LAGLLS[Q]SGGVVRKNFVPTDVGPFAF-CONH$_2$

b$_4$ b$_5$b$_6$ b$_7$b$_8$ b$_9$

n=454 (ave)

b$_8$
935.8

b$_5$
694.5

b$_9$
1049.2

b$_4$
567.1

b$_6$
807.7

b$_7$
878.9

Figure 4. In-source CID spectra at declustering potential= 180V. **(a)** Non-PEGylated CGRP[Cit, Cit]; **(b)** 20 kDa PEGylated CGRP[Q, Q]

36, 72, and 96 h after the dose and stored at −70 °C until analysis.

Results and Discussion

ESI-MS and In-Source CID of CGRP[Cit, Cit] and 20 kDa PEGylated CGRP Peptides

Figure 2a shows the mass spectrum of CGRP[Cit, Cit], indicating the peptide molecular ions with charge states of 3+ and 4+ at m/z 1144.4 and m/z 858.3, respectively. When CGRP [Cit, Cit] is conjugated to a 20 kDa linear PEG, the PEG moiety, which accounts for more than 80% of the PEGylated peptide by mass generates a complex mass spectrum, showing a broad continuous profile with inadequate peak resolution in the range of 500–1000 m/z as shown in Figure 2b. This complex mass spectrum is the result of the combined size distribution and charge distribution of the PEGylated peptide. Unlike the spectrum of CGRP[Cit, Cit], the spectrum of the 20 kDa PEGylated CGRP[Cit, Cit] has limited utility for quantitative analysis as no distinct precursor ions can be selected.

Recently a gas-phase dePEGylation method by in-source fragmentation in a Q-TOF mass spectrometer was reported [32]. In-source CID was used to partially truncate the PEG moiety of the PEGylated peptide, so a simplified mass spectrum could be achieved due to the reduced heterogeneity of truncated PEGylated peptide ions, allowing conventional CID MS/MS to be performed for PEGylation site mapping. In-source fragmentation takes place in the atmospheric pressure/ vacuum interface of the mass spectrometer at elevated nozzle/ skimmer potentials, electrospray ionization generated ions are accelerated in the high pressure source region and can acquire sufficient collision energy to induce dissociation. It has been utilized as a valuable tool for structural characterization of intact proteins and peptides [33, 34].

In this study, we have found that fragmentation of 20 kDa PEGylated CGRP[Cit, Cit] using in-source CID can effectively generate abundant CGRP[Cit, Cit] fragment ions. Figure 3a and b show the mass spectra of 20 kDa PEGylated CGRP[Cit, Cit] after applying in-source CID with increasing declustering potentials on a API4000 triple quadrupole mass spectrometer. Compared with the mass spectrum at a non-dissociating voltage (50 V), as shown in Figure 2b, these in-source CID spectra show the gradual disappearance of the PEGylated peptide profile in the m/z range of 500–1000 as the declustering potential increases. At 180 V (Figure 3b), the disappearance of the characteristic broad profile associated with the PEGylated peptide indicates that the polymeric species were completely dissociated and abundant new ion species were observed at m/z 566.4, 723.5, 836.6, 907.8, 964.9, and 1077.8. These ions were definitively identified as the singly charged b_4, b_5, b_6, b_7, b_8, and b_9 fragment ions of CGRP[Cit, Cit] respectively. b_5-43Da ion at m/z 680.8, unique to Cit^5 residue, was observed together with b_5 ion. The in-source CID of 20 kDa PEGylated CGRP

[Cit, Cit] indicated that the fragmentation took place preferentially at the CGRP[Cit, Cit] peptide bonds. The non-PEGylated CGRP[Cit, Cit] was also studied (Figure 4a) under the same in-source CID conditions (declustering
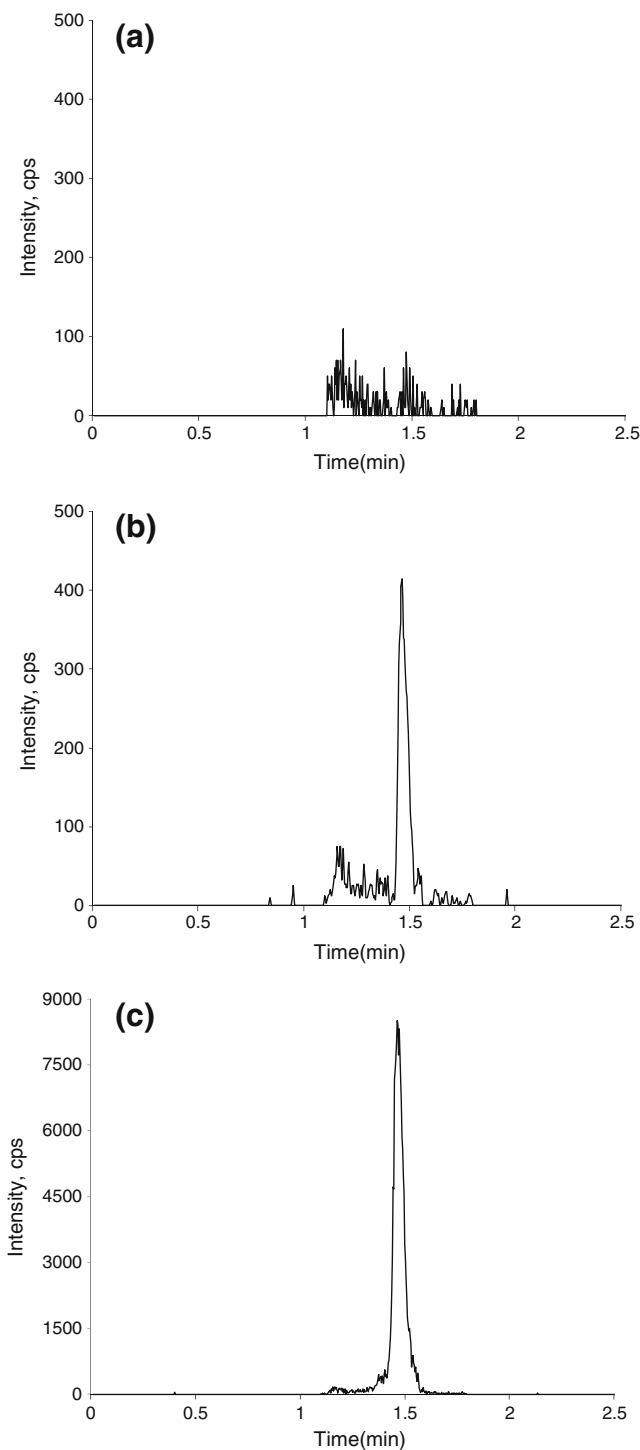

Figure 5. Ion chromatograms of **(a)** Control cynomolgus monkey serum blank; **(b)** 5.00 ng/mL LLOQ of 20 kDa PEGylated CGRP[Cit, Cit] standard; **(c)** 20 kDa PEGylated CGRP[Q, Q] internal standard

Table 1. Intraday and Interday Accuracy and Precision for the Determination of 20 kDa PEGylated CGRP[Cit, Cit] in Cynomolgus Monkey Serum

	QC	LLOQ	Low	Middle	High
Day	Concentration (ng/mL)	5.00	15.0	100	400
1	Intraday mean (ng/mL)	4.80	14.5	97.1	366
($n=6$)	% CV	10.6	6.8	1.7	2.0
	% RE	−3.9	−3.1	−2.9	−8.4
2	Intraday mean (ng/mL)	5.37	15.0	101	383
($n=6$)	% CV	1.6	8.2	2.9	2.6
	% RE	7.4	0.1	1.5	−4.3
3	Intraday mean (ng/mL)	4.82	14.4	98	387
($n=6$)	% CV	7.5	6.4	3.9	4.6
	% RE	−3.6	−3.9	−2.3	−3.2
1–3	Interday mean (ng/mL)	5.00	14.7	98.8	379
($n=18$)	% CV	8.7	7.0	3.5	4.0
	% RE	−0.1	−2.3	−1.2	−5.3

potential =180V). The same b_4, b_5, b_6, b_7, b_8, and b_9 and b_5-43Da ions were observed, confirming that the in-source CID generated the same peptide fragment ions from both non-PEGylated and PEGylated CGRP[Cit, Cit].

The internal standard, 20 kDa PEGylated CGRP[Q, Q] demonstrated similar in-source fragmentation to that observed for 20 kDa PEGylated CGRP[Cit, Cit] as shown in Figure 4b. The in-source CID spectrum contained singly-charged b_5, b_6, b_7, b_8, and b_9 fragment ions shifted to a lower mass by 29 Da due to the mass difference between citrulline (175.2 Da) and glutamine (146.1 Da), while the b_4 ions remained at the same m/z value due to the same amino acid sequence. The b_5-43Da ion unique to the Cit^5 residue was not observed for the internal standard due to the Glu^5 substitution.

In-source CID has the advantage of fragmenting all charged species simultaneously and rapidly. This parallel in-source fragmentation is especially useful for the oligomerically dispersed PEGylated peptides since all the oligomer ions retain the same peptide, but are different in PEG size. After in-source fragmentation, abundant peptide fragment ions can be produced instantaneously and be utilized as the surrogate peptide ions for LC-MS/MS quantitative analysis of PEGylated peptides without multiple-step trypsin digestion [27–29].

MS/MS Detection of Surrogate Peptide Ions for 20 kDa PEGylated Peptides

As shown in Figures 3b and 4b, the most abundant in-source CID fragmentation ions are the b_8 ions, these singly charged ions were selected as the surrogate peptide ions for both the 20 kDa PEGylated CGRP[Cit, Cit] and 20 kDa PEGylated CGRP[Q, Q] and were used as the precursors to generate MRM ion transitions for quantitative analysis. The ion transition ultimately chosen and optimized for 20 kDa PEGylated CGRP[Cit, Cit] was m/z 964.6 → 680.6 corresponding to further fragmentation of the b_8 ion to b_5-43Da ion. Similarly, the MRM ion transition for 20 kDa PEGylated CGRP [Q, Q] was m/z 935.6 → 694.6 corresponding to the fragmentation of the b_8 ion to b_5 ion.

Development of the UPLC-MS/MS Method for 20 kDa PEGylated CGRP[Cit, Cit]

Following development of the MS/MS conditions the method was further refined to allow for the best possible LLOQ, accuracy and precision by optimizing sample preparation and UPLC parameters as described in the Materials and Methods Section. Immunoaffinity precipitation (IAP) has been reported as a successful extraction method for PEGylated peptide in biological matrix [28], however, it requires anti-PEG antibody reagent preparation and incubation for immunocapture. Sample preparation using protein precipitation (PPT) was attempted, but only a 25 ng/mL LLOQ of 20 kDa PEGylated CGRP [Cit, Cit] could be achieved, likely due to the high background and ion suppression from the PPT extract. After evaluating the use of different types of SPE materials, solid phase extraction with an Oasis HLB µElution plate was found to be effective for the extraction of PEGylated CGRP[Cit, Cit] and the internal standard PEGylated CGRP[Q, Q] from cynomolgus monkey
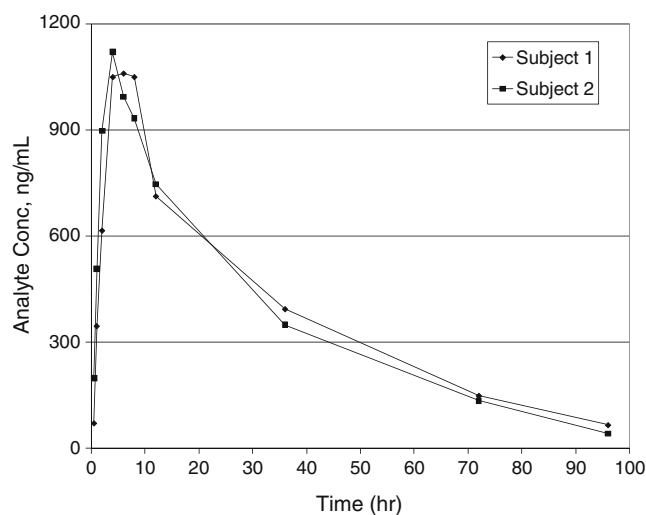

Figure 6. Concentration *versus* time plot of 20 kDa PEGylated CGRP[Cit, Cit] in cynomolgus monkey serum after subcutaneous administration of 20 kDa PEGylated CGRP [Cit, Cit] (2 mg/kg) to two animals

serum. As shown in Figure 5a, the SPE extract from blank serum demonstrated a clean background with no interference being observed from the control blank serum and no crosstalk from the internal standard. The LLOQ of 5.00 ng/mL 20 kDa PEGylated CGRP[Cit, Cit] exhibited good signal-to-noise ratio as shown in Figure 5b. The analyte was well-retained and eluted at 1.47 min as a sharp and symmetric peak despite the fact that this peak is composed of a mixture of PEG peptide oligomers. The internal standard co-eluted with the analyte at the same retention time (Figure 5c).

Assay Linearity and Performance

Assay linearity was evaluated using standard curves over the concentration range of 5.00–500 ng/mL. The 500 ng/mL upper limit of quantitation (ULOQ) was chosen to avoid saturation of the in-source CID. The calibration curve in cynomolgus monkey serum was linear with a correlation coefficient (r^2)> 0.99. No saturation of response was observed up to the ULOQ of 500 ng/mL. In addition, no carryover was observed when analyzing control serum blanks following the 500 ng/mL standard. Intraday and interday accuracy and precision results are shown in Table 1. The intraday % CV was between 1.6% and 10.6%, and the interday % CV was between 3.5% and 8.7%. The interday mean accuracy (% RE) was between −5.3 and −0.1% including the LLOQ (5.00 ng/mL).

Quantitation of 20 kDa PEGylated CGRP[Cit, Cit] in Serum Samples

The developed method was applied to the analysis of serum samples from two cynomolgus monkeys given a subcutaneous dose 20 kDa PEGylated CGRP[Cit, Cit]. Figure 6 shows the concentration-time profiles obtained. All concentrations from serum collected over the timeframe of the study were above the LLOQ of 5.00 ng/mL. Concentrations were relatively consistent between the two animals. The average maximum concentration (C_{max}) was 1090 ng/mL and was reached at 5 h (T_{max}). The apparent elimination half-life was 21.7 h, demonstrating that PEGylation significantly improved the pharmacokinetic properties of the CGRP peptide.

Conclusions

A novel UPLC-MS/MS method is presented for the direct quantitative measurement of 20 kDa PEGylated CGRP[Cit, Cit] in cynomolgus monkey serum. Gas-phase dePEGylation by in-source CID was utilized to generate peptide fragment ions as surrogate for quantitative analysis of 20 kDa PEGylated peptides. The described UPLC-MS/MS method was successfully applied to a cynomolgus monkey pharmacokinetic study. As an alternative to traditional enzyme-linked immunosorbent assay (ELISA), this method possesses unique advantages including not needing specific antibody-based reagents allowing for much faster and cost-effective method development; the method is also potentially applicable for testing multiple

different PEGylated peptide analogs. Future studies are planned to illustrate its applicability for other types of PEGylated peptides in different therapeutic areas.

Acknowledgments

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