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Development and validation of a method for the determination of a therapeutic peptide with affinity for the human B1 receptor in human plasma using HPLC-MS/MS

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ABSTRACT

The peptide described in this report (MW 1180 Da; 10-amino acid synthetic peptide) is a potent and selective antagonist of the human B1 receptor (B1) that has been investigated for the treatment of chronic pain. A method to quantitate this peptide in human plasma has been developed to support human clinical trials designed to evaluate the safety, pharmacokinetics, and efficacy of this compound. Plasma samples (0.2 mL) were extracted using a Waters Oasis MAX (10 mg) 96-well plate and the resulting samples were analyzed using an Applied Biosystems API-5000 HPLC-MS/MS with an electrospray ionization (ESI) source. The method was validated for the determination of the B1 peptide in human plasma over the concentration range of 1–50 ng/mL. Isotopically labeled B1 peptide ($^{13}C6^{15}N_2$-B1 peptide) was used as an internal standard. Interday precision and accuracy, determined from analysis of quality control (QC) samples, yielded coefficients of variation (CV) of less than 5.3% and accuracy within a 2.4%. Within batch precision and accuracy determinations provided CV values of less than 7.3% and accuracy within a 6.0% bias. Precautions had to be taken to prevent B1 peptide loss to container surfaces and contamination of the HPLC-MS/MS. The validated assay was used in support of human clinical trials.

1. Introduction

There is currently a renewed interest in the pharmaceutical industry for developing peptides as therapeutic compounds. This can be attributed to their having high potency, good selectivity and low toxicity profiles, however there are significant hurdles facing the development of peptides as drugs. One major issue has been that peptides are difficult to synthesize on a large scale. In addition to this, peptides can be difficult to deliver orally because of instability in the digestive tract and first pass metabolism [1,2]. Along with poor bioavailability, they often have poor pharmacokinetic characteristics and can be rapidly eliminated.

Many advances have been made recently in the areas of peptide synthesis [3], delivery [4] and pharmacokinetics [5,6] and these improvements have made it possible for peptides to become viable drug candidates. This has created a recent need for the development of bioanalytical methods. Historically, peptides have been quantitated using ligand binding techniques, although there have been past reports of HPLC-MS/MS methods which have been applied to the biological determination of peptides [7–9]. For example, enfuvirtide (Fuzeon®; a 36-amino acid synthetic peptide), an HIV-1 fusion inhibitor, was quantitated in human plasma using HPLC-MS/MS after solid phase extraction (SPE) and enzymatic cleavage with chymotrypsin [10]. In addition, Chang et al. [11] validated a method for this compound in human plasma using HPLC-MS/MS. Despite the fact that there are many reports of peptide analysis using HPLC-MS/MS in the literature, very few of these HPLC-MS/MS methods have been reported as validated according to current FDA guidelines on bioanalytical methods validation [12].

Peptide antagonists of Bradykinin B1 have previously been reported and have been investigated as drugs to treat various ailments including cancer, inflammation and pain [13,14]. The peptide (B1 peptide) in this report (D-Orn-Lys-Arg-Pro-Hyp-Gly-Cpg-Ser-D-Tic-Cpg, Fig. 1) is composed of ten alternating non-native and native amino acids (MW 1180 Da). It has been demonstrated to be a potent and selective antagonist of the human B1 receptor (B1) and was under evaluation for the treatment of chronic pain. A method to determine B1 peptide concentrations in human plasma was required to support human clinical trials designed to evaluate safety, pharmacokinetics, and efficacy of this compound. This report details the development and validation of an HPLC-MS/MS method for the determination of the B1 peptide in human plasma and the application of the method to support first in human (FIH) clinical studies.

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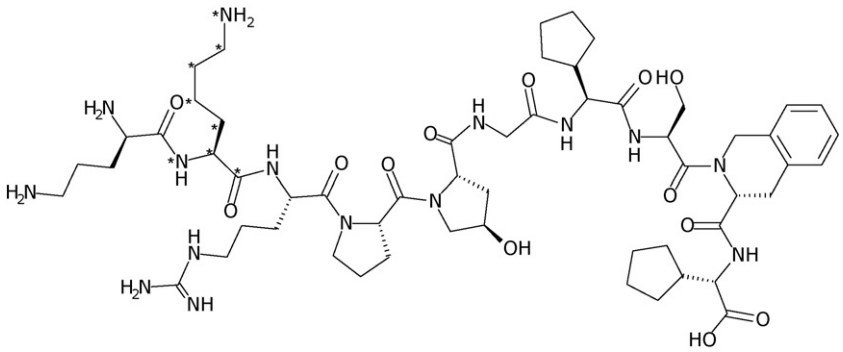

Fig. 1. Structure of the B1 peptide (D-Orn-Lys-Arg-Pro-Hyp-Gly-Cpg-Ser-D-Tic-Cpg) and stable-labeled internal standard. (*) Indicates site of stable isotopic label for the internal standard.

2. Experimental

2.1. Materials

The B1 peptide was purchased from PolyPeptide Laboratories (Torrance, CA). Stable label internal standard was synthesized in-house (Amgen Inc., Thousand Oaks, CA). HPLC grade acetonitrile, methanol, isopropanol, and water were purchased from Burdick and Jackson (Morristown, NJ). Glacial acetic acid and formic acid were purchased from J.T. Baker (Phillipsburg, NJ). Ammonium hydroxide was purchased from Alfa Aesar (Ward Hill, MA). Human sodium heparinized plasma was purchased from Bioreclamation (Hicksville, NY). Glass and polypropylene tubes were purchased from VWR (Brisbane, CA). Silanized glass tubes were purchased from Chase Scientific (Rockwood, TN).

2.2. Standard and sample preparation

Primary standard stock solutions (1 mg/mL) of the B1 peptide and internal standard were prepared in 25% methanol in polypropylene vials. Secondary standard spiking stock solutions were prepared in human plasma at concentrations of 1, 10, and 100 μg/mL in polypropylene tubes. The B1 peptide standards for the calibration curve were prepared in polypropylene tubes using the secondary standard spiking stock solutions by serial dilution to yield concentrations of 1, 2, 2.5, 5, 10, 20, 25, and 50 ng/mL. Working internal standard solution (15 μL, 1000 ng/mL) in 25% methanol was added to 200 μL of standards, quality controls, and all study samples. For internal standard free controls, 15 μL of 25% methanol was added. All these solutions were then diluted with 215 μL of 2% ammonium hydroxide before they were loaded onto the SPE cartridges.

2.3. Quality control preparation

A second weighing of the B1 peptide independent of the stock used for calibration standards was used for the preparation of the quality control samples (QCs). This primary QC stock solution (1 mg/mL, B1 peptide) was prepared in 25% methanol. Secondary quality control spiking solutions were prepared in human plasma in polypropylene tubes at concentrations of 1, 10, and 100 μg/mL. Quality control solutions (100 mL) were prepared in 100 mL polypropylene volumetric flasks at concentrations of 3, 25, 42.5, and 200 ng/mL human plasma. Smaller volumes (500 μL) were then aliquotted into separate polypropylene tubes and frozen at −70 °C for use during validation experiments and sample analysis.

2.4. Solid phase extraction

Oasis MAX solid phase extraction cartridges in 96-well format (10 mg, 30 μM, Waters, Milford, MA) were preconditioned with 1 mL of methanol followed by 1 mL of water. The prepared samples were loaded onto the cartridges and a low vacuum was applied. The cartridges were then washed sequentially with 1 mL of 2% ammonium hydroxide and then 1 mL of acetonitrile–methanol (50:50, v/v). After washing, the samples were allowed to elute under gravity for 30 min into a 96-well collection plate using 0.5 mL of 2% acetic acid in methanol–water (90:8, v/v). Gravity elution was necessary to obtain high and consistent recovery across extraction cartridges. Gravity elution was incorporated into the SPE procedure after it was observed that some wells in the 96-well SPE plate would begin eluting into the collection plate before vacuum was applied and that these wells would have higher recovery than slower flowing samples. A 30-min period was therefore allowed so that the majority of samples eluted under gravity. Vacuum was then applied to the plate to collect the remaining elution solvent. After vacuum elution, the samples were dried down with nitrogen at 30 °C using a 96-well MiniVap Evaporator (Porvair Science, Shepperton, UK). The samples were reconstituted by the addition of 200 μL of 0.1% formic acid in methanol–water (5:95, v/v), the collection plate was covered with a web seal mat, vortexed for 5 min, sonicated for 5 min and vortexed again for an additional 5 min.

2.5. Instrumentation

All analyses were performed using a Shimadzu LC-20 HPLC (Shimadzu Scientific Instruments Inc., Columbia Maryland) coupled to an Applied Biosystems API 5000 mass spectrometer (MDS Sciex, Concord, Ontario, Canada) with a Turboionspray® (TIS) interface. A Leap CTC PAL autosampler was used for sample injection (Leap Technologies, Carrboro, NC).

Table 1
Optimized MS/MS parameters for the B1 peptide and $^{13}C_6{}^{15}N_2$-B1 peptide.

Analyte	Precursor/product ion (*m*/*z*)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)	Dwell time (ms)	Retention time (min)
B1 peptide	591 → 132	131	55	20	300	2.1
$^{13}C_6{}^{15}N_2$-B1 peptide	595 → 132	36	61	20	300	2.1

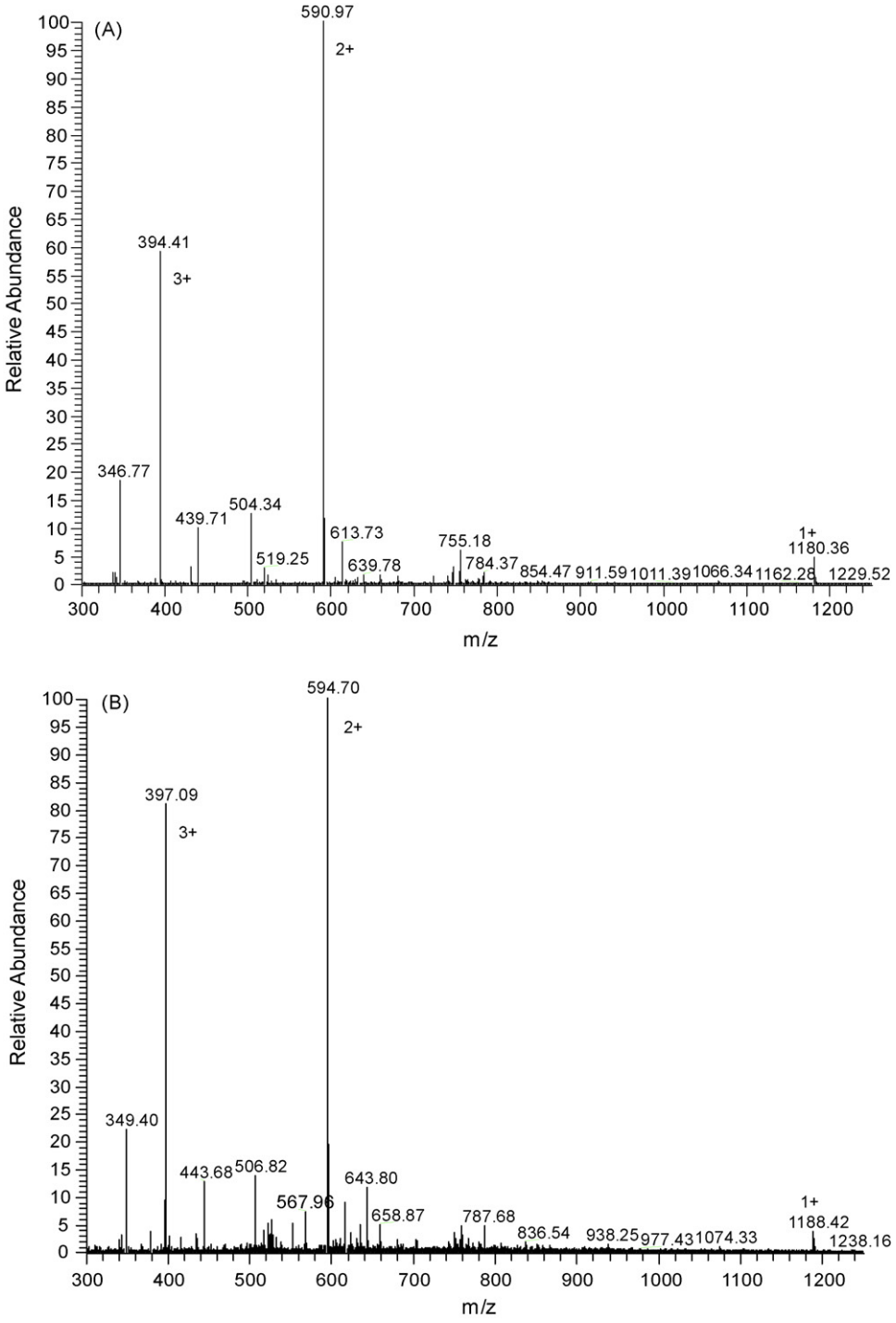

Fig. 2. Full scan mass spectra (Q1) of the B1 peptide (A) and $^{13}C_6{}^{15}N_2$-B1 peptide (B).

2.6. Chromatographic conditions

All analyses were performed on a 75 mm × 2.1 mm Polaris C18 analytical column with a particle size of 5 μm (Varian, Palo Alto, CA). Mobile phase A consisted of 0.1% formic acid in methanol–water (5:95, v/v). Mobile phase B consisted of 0.1% formic acid in methanol–water (95:5, v/v). The initial eluent composition was 10% B. The eluent was kept at 10% B for 0.8 min and increased to 95% B in 1.2 min, and held at 95% B for 2.5 min. It was then reduced to 10% B in 0.3 min and allowed to equilibrate at 10% B for 0.7 min. The total run time was 5.5 min. The eluent flow was 0.3 mL/min. The injection volume was 25 μL.

2.7. Mass spectrometric conditions

The B1 peptide and the internal standard $^{13}C_6{}^{15}N_2$-B1 peptide were dissolved and diluted in 50% methanol to give solutions with concentrations of 1 μg/mL. The neat solutions were infused separately into the API 5000 mass spectrometer using a syringe pump (Harvard Apparatus, Holliston, MA, USA) for parameter optimization. Precursor ions for the B1 peptide and $^{13}C_6{}^{15}N_2$-B1 peptide were determined from mass spectra using the TurboIonSpray® source operating in the positive ionization mode. The Quantitative Optimization function of the Analyst software (Applied Biosystems) was used to optimize the MS parameters followed by further man-

ual adjustment of source parameters to maximize the instrumental response. Analysis parameters for the B1 peptide and the internal standard ($^{13}C_6{}^{15}N_2$-B1 peptide) are listed in Table 1. The source temperature was kept at 500 °C. Curtain gas, Gas 1, and Gas 2 were all set at 50. The collision gas was set at 10. The ionspray voltage was +5000 V. Q1 resolution was set to unit and Q3 resolution was set to low.

3. Results and discussion

3.1. Mass spectrometry

Full scan mass spectra are shown in Fig. 2 for the B1 peptide and the internal standard ($^{13}C_6{}^{15}N_2$-B1 peptide). The +1, +2, and +3 charge states for the B1 peptide were *m/z* 1180.4, *m/z* 591.0, and *m/z* 394.4, respectively. The +1, +2, and +3 charge states for the internal standard ($^{13}C_6{}^{15}N_2$-B1 peptide) were *m/z* 1188.4, *m/z* 594.7, and *m/z* 397.1, respectively. The +1 charge state for both the B1 peptide and the internal standard demonstrated a lower intensity, therefore during method development only the +2 and +3 charge states were monitored. The +2 charged precursor ions were chosen for multiple reaction monitoring (MRM) of both the analyte and the internal standard based on experience gained during method development, which consisted of simultaneously monitoring MRM channels for the +2 and +3 precursor ions. The +2 precursor ion always provided lower limits of quantitation and improved precision when compared with the +3 precursor ion.

The Q3 product ion scans using the +2 precursor ion for both the B1 peptide and the internal standard, $^{13}C_6{}^{15}N_2$-B1 peptide are shown in Fig. 3. In addition to b1, y2 and y9 product ions, the most abundant product ions were immonium ions observed at *m/z* 132 and *m/z* 70, for both labeled and unlabeled compounds. The optimized MRM detection channel for the B1 peptide was *m/z*

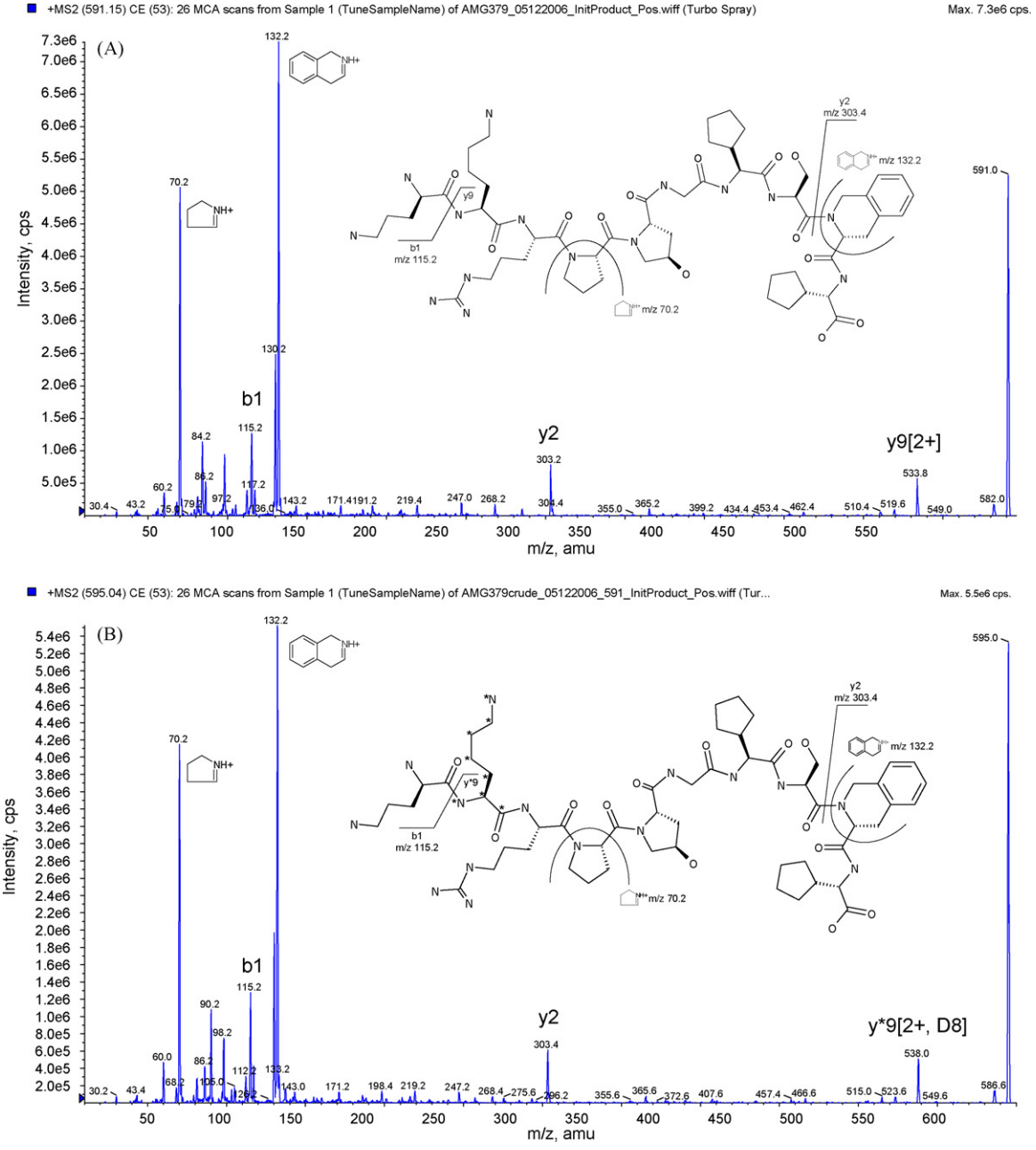

Fig. 3. Product ion mass spectra (Q3) of the B1 peptide (A) and $^{13}C_6{}^{15}N_2$-B1 peptide (B) for molecular ions of 591 and 595, respectively, and the proposed fragment structures.

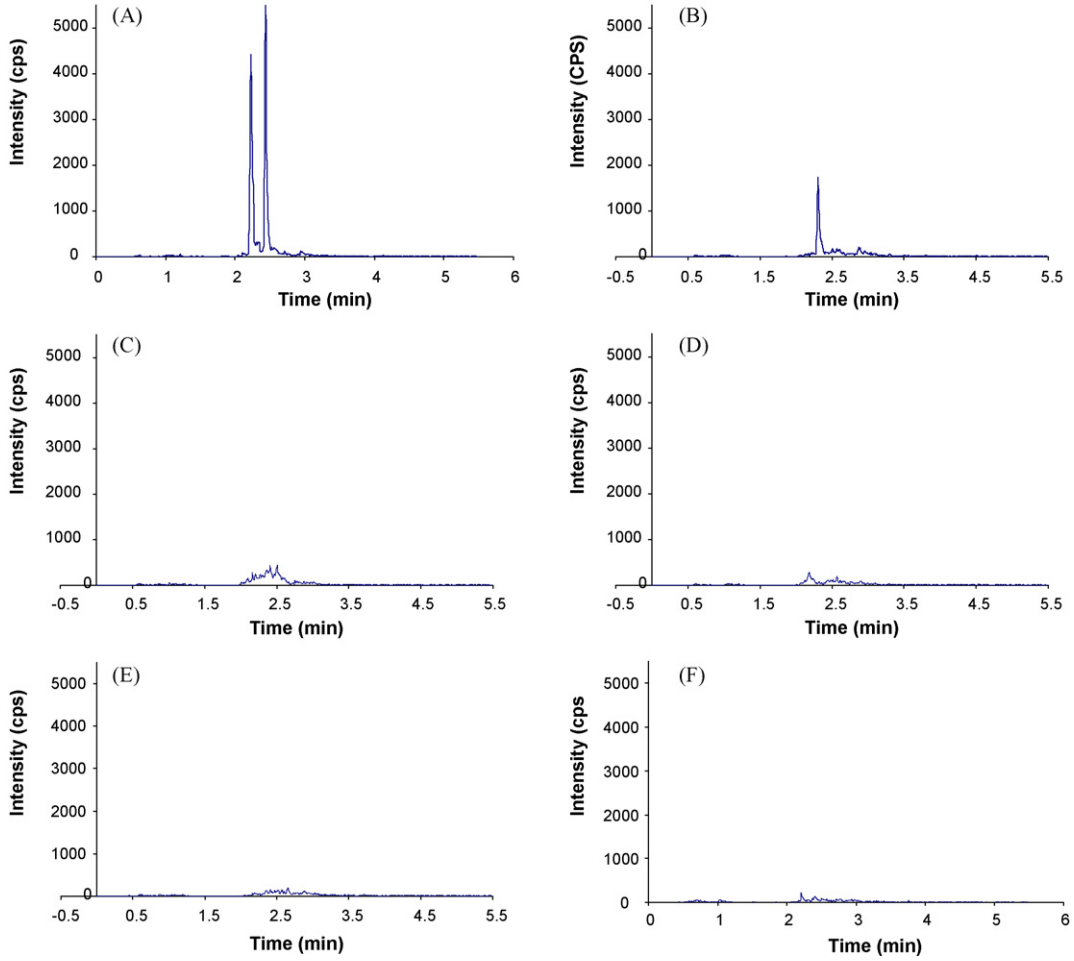

Fig. 4. Chromatograms of control plasma extracts of various matrices prepared using solid phase extraction and analyzed using HPLC-MS/MS. Chromatograms correspond to dog (A), mouse (B), rabbit (C), human (D), rat (E), cynomolgus monkey (F).

591 → 132 and the MRM detection channel for the internal standard was *m*/*z* 594 → 132.

3.2. Optimization of sample handling and preparation

Early experience with samples from preclinical species indicated that protein precipitation could be used to prepare samples for the determination of the B1 peptide in plasma. Using this technique, lower limits of quantitation (LLOQs) below 0.1 ng/mL were obtained from plasma samples collected from both monkey and rat. Unfortunately similar LLOQs for the B1 peptide could not be obtained when using protein precipitation to prepare mouse, dog or human plasma, due to interfering peaks and general baseline noise at the retention time of the B1 peptide.

Contamination of the HPLC-MS/MS system was also problematic when injecting human plasma samples after preparation using protein precipitation. Analysis of more than approximately one hundred injections on the API 5000 resulted in a significant decrease in instrument response and an inability to accurately measure the lowest points on the calibration curve. It has been previously published that the analysis of samples prepared using protein precipitation can cause contamination of the MS/MS source resulting in a general decrease in sensitivity [9]. For this compound, sensitivity was lost very rapidly on the API 5000 when using protein precipitated samples, making this sample technique impractical for this assay.

To provide additional sample clean-up, a solid phase extraction (SPE) method was developed. Chromatograms obtained from the preparation of control plasma from various species when using a generic SPE method are shown in Fig. 4. They indicated that baseline interference varied greatly between plasma from different species even when using the more rigorous SPE procedure for plasma preparation.

Based on these observations, sample preparation using an optimized solid phase extraction method was investigated both to minimize endogenous interference peaks as well as to reduce instrument contamination. A number of Waters SPE 96-well plates were evaluated including Oasis HLB, Oasis MCX, and Oasis MAX. In an attempt to further clean-up the sample, protein precipitation followed by SPE was also investigated; however, the additional step did not yield a cleaner sample than SPE alone. After evaluation, it was determined that the optimal signal to noise was obtained using an Oasis MAX 96-well extraction plate. Whereas all three of the SPE chemistries provided adequate recovery of the peptide, baseline noise when using Oasis HLB and MCX cartridges was higher when compared to the MAX chemistry. The fact that the MAX SPE cartridges provided a cleaner baseline suggests that a greater proportion of the interference peaks were either cationic or neutral. Samples prepared using the optimized MAX SPE method could be injected into the HPLC-MS/MS system with a minimum loss in sensitivity, provided that a high curtain gas flow was used, and the distance of the sprayer from the orifice was maximized. Both the

Table 2
B1 peptide standard samples prepared in glass, silanized glass, and polypropylene vials in either plasma or 25% methanol solutions.

Nominal (ng/mL)	Plasma[b] Glass	Plasma[b] Silanized glass	Plasma[b] Polypropylene	MeOH (25%)[c] Glass	MeOH (25%)[c] Silanized glass	MeOH (25%)[c] Polyporpylene
Accuracy (%)[a]						
1	87.8	93.6	101.0	11.1	9.1	40.3
2	85.2	97.3	98.4	12.3	15.0	48.8
2.5	90.4	96.7	92.9	16.0	13.1	34.7
5	91.4	94.1	114.0	18.1	21.3	58.1
10	87.3	93.7	98.9	19.4	19.4	60.5
20	85.8	93.4	97.2	19.7	20.8	65.0
25	91.6	95.7	98.8	24.2	22.3	68.7
50	90.2	92.1	98.9	20.0	22.0	74.4

[a] All values were compared to the best-fit regression line for the plasma/polypropylene calibrants.
[b] Serially diluted in plasma from an initial plasma stock.
[c] Serially diluted in methanol (25%) and spiked into plasma after dilution.

gas flow and the distance from the sprayer to the orifice were increased as much as possible while avoiding a significant reduction in instrument sensitivity.

Upon routine application of the newly developed preparation method, it was observed that frozen quality control samples containing the B1 peptide could not be measured accurately. Quality control samples prepared at 3, 25, and 42.5 ng/mL were analyzed and found to contain approximately 44%, 63%, and 76% of expected potency. Similar results were obtained after remaking all stock solutions, standards and quality controls, so an investigation was begun to identify the source of the low QC concentrations which were initially attributed to absorption onto the glass containers used to prepare solutions. Loss of peptide due to adsorption during sample handling has been previously reported [9], so based on this, and also on the past experience in this laboratory with the analysis of other miscellaneous peptides, experiments were initiated to measure loss in potency possibly caused by precipitation and/or container absorption.

Many types of approaches have been suggested to prevent absorption of peptides to surfaces. These have included the use of solvents, pH control, different container and tube material, or modifying or coating surfaces where absorption is problematic. Ponganis and Stanski [15] evaluated the use of pH and silanized versus unsilanized glassware to prevent peptide loss in serum and found that the benefits of silanizing the glassware were insignificant compared to benefits gained by modifying pH. Redeby and Emmer [16] also investigated the use of silanized, fluorinated, and polypropylene tubes to prevent adsorption of peptides. They found no effect on peptide absorption based on tube material. Due to concern about the peptide stability and absorption, approaches using different tube materials (glass, silanized glass, and polypropylene) and addition of plasma to stock and standard preparation were evaluated for the B1 peptide.

Standard curves were analyzed using each of the three vial materials as well as two standard curve preparations. One standard curve was prepared using serial dilution in 25% methanol, which was then spiked in equal volumes into plasma followed by SPE. The other standard curve was prepared using serial dilution in plasma followed by SPE. All values were compared to the best-fit regression line for the plasma/polypropylene calibrants. A summary of the results obtained from analyzing fresh standard curves is shown in Table 2. The highest relative recovery was found using polypropylene tubes when preparing the standard curve by serial dilution in plasma, while the worst recoveries were seen using aqueous solution (25% methanol) serial dilution in glass. The use of silanized glass offered only a modest improvement over unmodified glass containers.

These results were confirmed with a second experiment involving the preparation of plasma quality control samples (QCs) using secondary stocks made in either 25% methanol or plasma spiking solutions in both glass and polypropylene. Once made, the quality control samples were frozen and their potency was evaluated the following day using fresh standards prepared with calibration standards serially diluted in plasma using polypropylene tubes. The primary stock solution (1 mg/mL), made by weighing solid powder of the B1 peptide and diluting with 25% methanol in polypropylene vials, was used in the preparation of all secondary stocks for both quality controls and standards. It was observed that the 1 mg/mL solution could be stored for an extended period of time without the apparent loss in potency observed at lower concentrations, probably because the solution was concentrated enough to saturate non-specific binding sites on the polypropylene vial without a significant effect on its overall potency. Conditions to prepare secondary stock solutions were examined by diluting the primary stock in either human plasma or 25% methanol in polypropylene tubes at concentrations of 1, 10, and 100 μg/mL. Quality control samples (100 mL) were then prepared in 100 mL polypropylene or glass volumetric flasks at concentrations of 3, 25, and 42.5 ng/mL human plasma. The results are given in Table 3. The optimal recovery of the B1 peptide, based on these experiments, was obtained using plasma for all dilutions in polypropylene volumetric flasks after preparation of the primary (1 mg/mL) stock solutions.

In summary, based on this series of experiments, it was possible to make standards and QCs in a consistent fashion and measure

Table 3
Comparison of quality control samples in glass and polypropylene vials in plasma and aqueous solutions.

Nominal (ng/mL)	Aqueous Glass[a]	Plasma Glass[a]	Plasma Polypropylene[a]
Accuracy (%)			
3	43.6	92.1	100.8
25	62.8	88.6	102.6
42.5	75.9	93.3	95.2

[a] Average of three samples.

Table 4
Precision and accuracy data for the determination of the B1 peptide (1–50 ng/mL) in 6 lots of human plasma.

Nominal (ng/mL)	Determined mean (ng/mL, $n = 6$)	Accuracy[a] (%)	CV[b] (%)
1	0.99	99.00	6.40
2	2.02	100.95	8.20
2.5	2.54	101.76	5.70
5	4.96	99.10	5.00
10	9.86	98.58	6.60
20	20.42	102.09	4.70
25	25.29	101.14	2.70
50	48.62	97.23	7.90

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation.

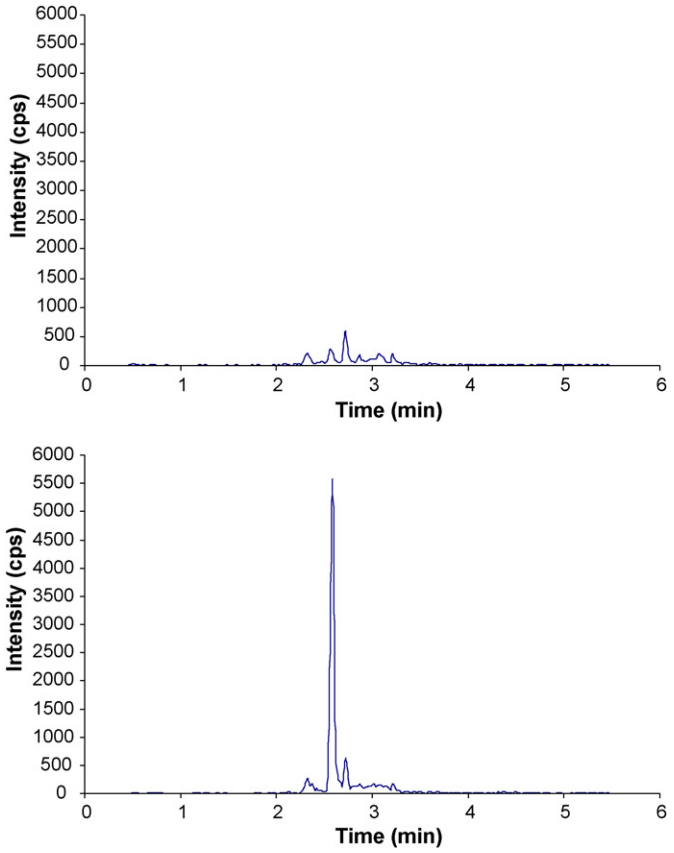

Fig. 5. Chromatogram of control plasma (A) and a standard at the LLOQ of 1 ng/mL (B), prepared using solid phase extraction and analyzed using HPLC-MS/MS.

QC concentrations within 90–106% of their expected concentration. This was accomplished by the consistent use of polypropylene tubes and plasma for all dilutions required for making standards or QCs. Only the high concentration (≥1 mg/mL) primary stocks could be diluted in 25% methanol without apparent loss of the B1 peptide due to binding to the container. Use of plasma for all further dilutions had the biggest effect on reducing absorption, although a small additional benefit was noted with use of polypropylene tubes.

3.3. HPLC-MS/MS method validation

This method was validated for clinical analysis using the current FDA criteria for bioanalytical methods validation [12]. The following validation parameters were evaluated for the B1 peptide: intraday standard curve accuracy and precision, quality control intraday and interday precision, recovery and matrix effects, bench-top stability, long-term stability and freeze–thaw stability.

3.3.1. Standard curve accuracy and precision

Replicate calibration standards using six different lots of sodium heparinized human plasma (male and female) were analyzed in one analytical run for accuracy and precision. Intraday accuracy is expressed by the deviation of the mean of standards in six different lots of plasma from the nominal concentration. Mean values were within ±3% of the nominal value and the coefficient of variation at each level was within ±9%. Results are given in Table 4. Sample chromatograms of a control human plasma extract and LLOQ (1 ng/mL) are shown in Fig. 5.

3.3.2. QC intraday and interday precision

For the individual experiments on each day, the mean of the replicate QCs were within 7% of the nominal value for LLOQ, low, middle and high QCs. The % CV of the replicates was within 8% (QC precision). Interday variation was determined by statistical comparison of QC results obtained on three separate days. The interday accuracy was expressed as the deviation of the mean of the experiments. Interday precision (% CV) was less than 6%. Results are given in Table 5.

3.3.3. Recovery and matrix effect evaluation

Experiments to assess recovery and matrix effect values for the B1 peptide were potentially unreliable due to the inability to maintain reasonable concentrations of the B1 peptide in solutions in the absence of plasma. The recovery experiments that were performed using samples for the B1 peptide prepared at concentrations of 3, 25, and 42.5 ng/mL in control human plasma resulted in recoveries of 79, 83, and 73%. Matrix effect experiments performed using the same concentrations resulted in enhancement of responses from 5 to 12%, although precision for the non-plasma comparator was poor, probably due to the absorption discussed previously. Relative matrix effect could be assessed using the standard curve experiment shown in Table 4. The good precision of the slopes (less than 5% CV) for the six standard curves indicated that there is minimal matrix variability in the assay caused by lot to lot differences in human plasma.

3.3.4. Stability

Freeze–thaw, bench-top, reinjection and long-term stability were evaluated and the results are summarized in Table 6. Quality control samples ($n = 4$ at each concentration) were left at room temperature for over 6 h prior to extraction. The B1 peptide appears

Table 5
Intraday and interday accuracy and precision of the B1 peptide quality control samples.

	LLOQ (1 ng/mL)	Low QC (3 ng/mL)	Mid QC (25 ng/mL)	High QC (42.5 ng/mL)	Dil. QC (200 ng/mL)
Day 1 mean ($n = 6$)	1.02	2.82	24.14	40.84	188.98
Accuracy[a] (%)	101.60	93.97	96.56	96.09	94.49
CV[b] (%)	3.59	7.30	4.50	5.80	3.10
Day 2 mean ($n = 6$)		2.96	25.12	41.94	196.38
Accuracy[a] (%)		98.80	100.47	98.67	98.19
CV[b] (%)		3.50	2.00	5.20	4.50
Day 3 mean ($n = 6$)		3.00	26.28	42.93	202.92
Accuracy[a] (%)		100.10	105.12	101.02	101.46
CV[b] (%)		4.30	3.80	4.70	3.80
Interday mean ($n = 18$)		2.93	25.18	41.90	196.09
Accuracy[a] (%)		97.63	100.72	98.59	98.05
CV[b] (%)		5.03	3.43	5.23	3.80

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation.

Table 6
Assessment of the stability of the B1 peptide.

Nominal conc. (ng/mL)	Bench-top stability at room temperature for 6 h 45 min			Storage at −60 to −80 °C for 63 (QC1) and 76 days (QC2 and QC3)		
	Determined mean (ng/mL, $n=4$)	Mean %accuracy[a] ($n=4$)	CV[b] (%) ($n=6$)	Determined mean (ng/mL, $n=4$)	Mean %accuracy[a] ($n=4$)	CV[b] (%) ($n=4$)
3	2.90	96.80	3.20	2.90	96.57	3.40
25	24.63	98.50	1.50	24.71	98.86	1.10
42.5	40.69	95.74	4.30	40.78	95.95	4.00

Nominal conc. (ng/mL)	Stability after 3 freeze–thaw cycles			Storage in the autosampler at 80–12 °C for 3 days			
	Determined mean (ng/mL, $n=6$)	Mean %accuracy[a] ($n=6$)	CV[b] (%) ($n=6$)	Determined mean (ng/mL, $n=6$)	Mean %accuracy[a] ($n=6$)	CV[b] (%) ($n=6$)	Deviation from first injection (%)
3	3.09	102.97	3.00	2.90	96.57	5.90	1.348
25	25.97	103.86	3.90	24.02	96.09	4.40	0.493
42.5	43.04	101.28	1.30	40.41	95.08	3.80	1.053

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation.

Table 7
Interday assay performance for B1 peptide determination from human plasma (12 consecutive assay runs).

Statistic	Standard concentration (ng/mL)								QC concentration (ng/mL)			
	1	2	2.5	5	10	20	25	50	3	25	42.5	200
Mean	0.98	2.06	2.55	4.93	10.17	20.06	24.92	48.37	2.75	24.01	41.22	196.11
Accuracy (%)	98.0	103.0	102.0	98.6	101.7	100.3	99.7	96.7	91.7	96.0	97.0	98.1
CV (%)	5.0	4.4	4.2	3.7	4.1	3.3	3.7	2.3	7.9	5.5	5.7	10.0
n	24	24	24	22	24	24	24	24	24	24	24	14

to be stable in plasma for at least 6 h on the bench-top at room temperature (CV ≤ 4.3% and accuracy within 95.74% of nominal). Quality control samples ($n=6$ at each concentration) were subjected to three freeze–thaw cycles consisting of storage at room temperature for approximately 4 h, vortexing, and then refreezing (−60 to −80 °C, for at least 12 h). After three freeze–thaw cycles the samples were analyzed using freshly prepared calibration standards. The B1 peptide appears to be stable in human plasma after three freeze–thaw cycles (% CV ≤ 3.9% and accuracy within 103.86% of nominal) QC samples were stored in the autosampler tray at 8–12 °C for 3 days to evaluate reinjection stability. The B1 peptide appears to be stable in the autosampler over a period of 3 days (% CV was ≤5.9% and accuracy was within 95.08% of nominal). Quality control samples ($n=4$ at each concentration) were stored frozen (temperature range −60 to −80 °C) for approximately two months (63 days for QC1 and 76 days for QC2 and QC3) prior to extraction to evaluate long-term stability. The quality control samples were evaluated against freshly prepared calibration curves. The B1 peptide appears to be stable in plasma for at least two months at −60 to −80 °C (% CV was ≤4.0% and accuracy was within 95.95% of nominal). All stability results met pre-specified acceptance criteria.

4. Clinical sample analysis

The validated bioanalytical method for the B1 peptide was used to analyze plasma samples from healthy subjects dosed with the B1 peptide in a single rising dose PK study. Twelve different analytical runs were performed over a period of 15 weeks to analyze 1026 plasma samples from 61 subjects receiving either intravenous or subcutaneous doses of the B1 peptide. Each set of clinical samples (unknowns) was bracketed by duplicate standard curves and sets of QC's. Dilution QC's (200 ng/mL) were included in 7 of 12 of the analytical runs to allow for a 1:10 dilution of samples expected to be above the standard curve range. Statistics for the 12 consecutive runs are shown in Table 7. The assay proved to be rugged and reliable as indicated by the consistent accuracy and precision values for both standards and QC's. The plasma concentration–time curves, following administration of the B1 peptide with a 6-h intravenous infusion, are shown in Fig. 6.

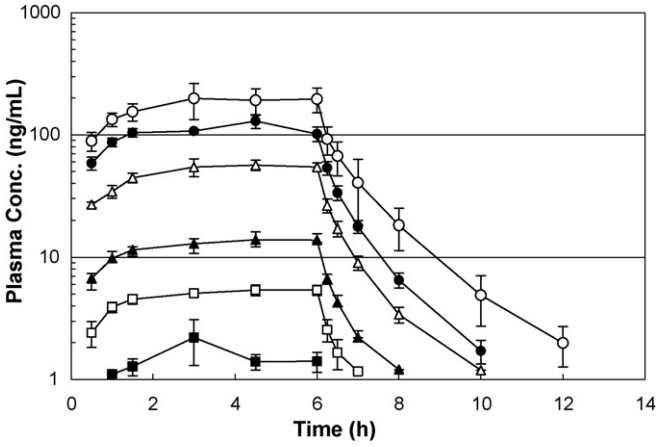

Fig. 6. Plasma–time concentration curves of patients dosed with an intravenous 6 h infusion of the B1 peptide (A). 0.2 mg/kg (■), 0.7 mg/kg (□), 2.0 mg/kg (▲), 7.0 mg/kg (△), 14 mg/kg (●), and 28 mg/kg (○). Error bars represent ± 1 standard deviation.

5. Conclusions

An HPLC-MS/MS method for the determination of the B1 peptide was developed and applied to the quantitation of the B1 peptide following parenteral dosing in human subjects. Pre-study validation of the method was performed and it was applied successfully to the analysis of samples generated during human clinical trials. The B1 peptide was shown to bind to container surfaces when dissolved in aqueous solutions and that this binding could be minimized through the use of polypropylene containers and the inclusion of plasma in dilution solutions.

Conflict of interest statement

All co-authors were in the employment of Amgen Inc. during the duration of the research detailed in this report. No additional financial or personal conflicts of interest have been identified that could have inappropriately influenced the work submitted.

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