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Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 695–702
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of potato carboxypeptidase inhibitor in African Green Monkey plasma using 96-well SPE and LC–MS/MS

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Accepted 24 September 2004
Available online 21 March 2005

Abstract

Potato carboxypeptidase inhibitor (CPI), a peptide with multiple isoforms (MW > 4000 Da) was determined from African Green Monkey plasma using a PE Sciex API-3000 LC–MS/MS in the positive ionization mode with the turbo ionspray interface (450 °C). Samples were prepared using an Oasis MCX 96-well solid phase extraction plate and chromatographed on an Allure C_{18} HPLC Column (50 mm × 1.0 mm, 5 μm) using gradient elution. Upon analysis of the extracts using LC–MS/MS, the concentration of CPI was calculated using a single MS/MS transition (*m/z* 830.5 → 221.0) that was reflective of the mass concentration (μg/mL) of main the CPI isoforms present in plasma from monkeys after they were given an intravenous dose of CPI. The assay was linear for CPI over concentrations of 0.05–10 μg/mL when extracting 200-μL aliquots of African Green Monkey plasma. The assay was applied to the determination of CPI in African Green Monkey plasma samples in two separate analytical runs (correlation of standard curves, $r_1 = 0.9991$ and $r_2 = 0.9953$). Quality control (QC) samples were run at 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μg/mL for each assay. Average ranges ($n = 12$) for accuracy and precision for all concentrations of QCs during the two runs were 92.0–102.0% of expected potency and 10.4–21.8% (coefficient of variations), respectively.

Keywords: Potato carboxypeptidase inhibitor; CPI; Peptide analysis; Protein analysis; LC–MS/MS

1. Introduction

Potato carboxypeptidase inhibitor (CPI, Fig. 1) is a known inhibitor of mammalian pancreatic carboxypeptidases [1] and is of recent interest in drug development due to its ability to act as an antithrombotic agent by inhibiting the thrombin activatable fibrinolysis inhibitor (TAFI) cascade [2]. In summary, TAFI acts on circulating fibrin protein and eventually brings about the formation of stabilized blood clots. TAFI inhibitors such as CPI prevent the formation of stabilized fibrin leading to an increase in fibrinolysis. The systematic effect of the in vivo administration of a TAFI inhibitor is therefore a decrease in thrombosis and a related decrease in blood viscosity.

CPI was used at Merck Research Laboratories as a positive comparator to small molecule TAFI inhibitors and it was necessary, as part of these studies, to develop a bioanalytical method to determine the concentration of CPI in African Green Monkey plasma. CPI can be present as one of three isoform groups (CPI-I, II and III, MW > 4000 Da), which occur due to the presence of three intramolecular disulfide bonds. Each isoform group is known to have at least two different peptide sequences contained within it. The isoforms have been found to possess nearly identical inhibitory activity [3]. The CPI dosed to the monkeys in this study was a commercial isolate from potatoes and consisted of a mixture of these isoforms of differing molecular weights. Since CPI was dosed as this naturally occurring mixture, multiple pharmacologically active compounds of different molecular weight had to

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doi:10.1016/j.jpba.2004.09.059

Pyr-Gln^{2}-His-Ala-Asp-Pro-Ile-Cys-Asn-Lys-Pro-Cys-Lys-Thr-His-Asp-Asp-Cys-Ser-Gly-Ala-Trp-Phe-Cys-Gln-Ala-Cys-Trp-Asn-Ser-Ala-Arg-Thr-Cys-Gly-Pro-Tyr-Val-Gly^{39}

Fig. 1. Amino acid sequence of potato carboxypeptidase inhibitor, CPI (39 amino acids, 4276.2 Da). Additional CPI sequences observed in this sample were probably due to the deletion of Gln^2 (38 amino acids, 4148.1 Da), the deletion of Gly^{39} (38 amino acids, 4218.6 Da), and the deletion of both Gln^2 and Gly^{39} (37 amino acids, 4091.0 Da). The various sequences of CPI are known to contain three disulfide bonds and a blocked N-terminus (Pyr).

be measured. Previous research has shown that this natural isolate is largely composed of a single isoform (CPI-II) that is divided into approximately equal amounts of two polypeptide chains containing 38 or 39 amino acid residues, respectively. These two chains differ in the sequence of amino acids that form their N-terminus and could be individually detected and measured in our experiments using LC–MS/MS.

There have been many experiments reported previously which use LC–MS/MS for the characterization of peptides or proteins, including proteomic analysis [4], identification of endogenous peptides [5], or general characterization of peptide and proteins obtained from biological isolates [6,7], and many of these applications have been recently reviewed [8,9]. Routine laboratory analysis of large peptides, and proteins by LC–MS/MS is made possible due to their often possessing a high net charge that forces their mass to charge ratio (*m/z*) into the relatively narrow mass analyzer range of bench top LC–MS/MS instruments ($m/z \leq 3000$ Da). Despite the significant number of publications detailing qualitative characterization of macromolecules using LC–MS/MS, there are a limited number of reports concerning the use of this technique for their quantitative bioanalysis. LC–MS/MS assays have been reported for endogenous endothelins [10] and bradykinin antagonist polypeptide B201 and substance P [11]. LC–MS assays have been reported for synthetic human calcitonin [12], the decapeptide cetrorelix [13] and the synthetic peptide, NR58-3.14.3 [14]. At present, it seems that quantitative analysis of macromolecules is still performed principally using immunoassay and bioassay techniques [15].

This report details the development of bioanalytical methodology for the quantitation of CPI in African Green Monkey plasma using LC–MS/MS to allow an accurate assessment of the concentration of inhibitor in circulation. Developing an analytical method that would provide relevant information on the sum of these active compounds in plasma represented a unique challenge. In addition, to having to measure a mixture of active compounds of unknown and uncharacterized composition, the targeted isoforms also possessed a large molecular weight (>4000 Da), intramolecular disulfide bonds contributing to tertiary structure, and multiple charge states. These combined factors added to the overall complexity of providing an accurate and relevant measure of inhibitor concentration. These issues and others encountered as part of method development are detailed in the following report.

2. Experimental

2.1. Materials

To appropriately evaluate potato carboxypeptidase inhibitor for potential in vivo antithrombotic effects through enhanced fibrinolysis, CPI isolate, obtained from Sigma–Aldrich Chemical Company (St. Louis, MO, USA) was subjected to additional purification before its use in analytical and pharmacological experiments. Antiplasmin activity was removed from the preparation to avoid its inhibitory effects on the primary profibrinolytic mechanism of the compound. In addition, endotoxin activity, which could complicate the interpretation of the study results, was also removed. The CPI was separated from endotoxin and antiplasmin activities in a 1.5 mL/min, 0–20% sodium chloride gradient over 140 min in 15 mM Tris, pH 9.0 buffer containing glycine (5 mM) using 15Q strong anion exchange chromatography on a 1.0 cm × 16 cm, XK16 column (Pharmacia, Uppsala, Sweden) using a Pharmacia LCC-501 fast performance liquid chromatograph. Peak fractions were detected at 254 nm and analyzed for their ability to inhibit TAFI using an in-house protocol. Pooled CPI fractions were dialyzed against ultra pure water (18 MΩ) using a SpectraPor MWCO 2000 CE membrane (Spectrum Medical Industries Inc., CA, USA) to remove salts before quantitation by amino acid analysis. Purified CPI was lyophilized using an SC21A Rotary SpeedVac Plus (Savant Industries Inc., NY, USA) and stored at −70° C. The CPI used for the preparation of analytical standards came from the same batch used for the intravenous dosing of monkeys. This was necessary due to the variable nature of each batch of CPI isolate, as well as the lack of meaningful purity information available for the individual isoforms. The lack of extensive purity information of the individual components of the CPI isolate limited the expression of CPI concentrations to mass per volume units (μg/mL).

Acetonitrile (Omnisolve, HPLC grade) was obtained from EM Science (Gibbstown, NJ, USA). Drug free African Green Monkey plasma was obtained in-house at Merck Research Laboratories (West Point, PA, USA). Oasis mixed phase cation exchange (MCX), 30 mg, 96-well extraction plates were obtained from Waters Inc. (Milford, MA, USA). All other reagents were ACS grade and were used as received.

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) LC200 HPLC pump and an HTS PAL Autosampler from CTC Analytics (Zwingen, Switzerland). The mass spectrometer was an AB/MDS Sciex (Toronto, Canada) API-3000 Triple Quadrupole LC–MS/MS with a turboionspray interface (450 °C). Data was collected and processed using AB/MDS Sciex Analyst data collection and integration software. Theoretical isotopic molecular weights of CPI were calculated with the aid of Molecular Weight Calculator

for Windows 9x/NT/2000/ME, Version 6.22 (freeware by Matthew E. Monroe, http://www.alchemistmatt.com/).

2.3. Chromatographic conditions

Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in methanol. The mobile phase was filtered through a 0.2 μm nylon filter prior to use. Mobile phase flow rate was 150 μL/min starting with 35% mobile phase B and ramping in a linear fashion to 95% mobile phase B over 4 min. After the gradient cycle was complete, the column was allowed to reequilibrate for 4 min at the starting mobile phase conditions (65:35 A:B) before the next injection. The HPLC column was a 50 mm × 1.0 mm, 5 μm Restek Corp. (Bellefonte, PA, USA) Allure C_{18} protected by an inline filter, which was changed after each batch run to maintain peak efficiency. The column was operated at ambient laboratory temperature.

2.4. MS/MS detection

Precursor ions for quantitating potato carboxypeptidase inhibitor were determined from mass spectra obtained during the infusion of a neat solution containing CPI (100 ng/mL, 50/50 MeOH/0.1% formic acid) into the mass spectrometer using the turbo ionspray source, operating in the positive ionization mode with the collision gas off. Upon manually identifying MS/MS transitions based on Q1 (Fig. 2) and product ion spectra (Fig. 3), auto optimization using the AB/MDS Sciex Analyst software was performed using the same infusion solution. This program was used to select declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE) and exit potential (CXP). Transition-specific API-3000 analyst parameters (DP/FP/EP/CE/CXP) for the transitions at *m/z* 1070.2 → 563.2, *m/z* 1038.2 → 249.0, *m/z* 856.2 → 563.4 and *m/z* 830.5 → 221.0 are listed in Table 2. All other parameters were optimized manually and were the same for the four monitored transitions. These settings were TEM 450 °C, NEB 8, CUR 8, IS 4700 V and CAD 12.

2.5. Preparation of standards

Standard and quality control (QC) stock solutions were separately prepared by weighing solid CPI (purified as described in Section 2.1) into 10-mL volumetric flasks and filling to the mark with 50/50 (v/v) methanol/water. The standard stock was then used to make working standard solutions with concentrations of 0.4, 0.8, 1.6, 4.0, 8.0, 16, 40 and 80 μg/mL. Twenty-five microliters of each standard stock solution was then spiked into 200 μL of drug-free African Green Monkey Plasma to form standards in plasma of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10 μg/mL. The QC stock solution was then diluted in the same fashion into QC working standard solutions of 0.4, 0.8, 1.6, 4.0, 16.0 and 40 μg/mL to form QC standards of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μg/mL in African Green Monkey plasma. Both standards and quality controls in African Green Monkey plasma were made on the day of sample preparation and stored at room temperature before extraction.

2.6. Plasma extraction procedure

A 0.2-mL aliquot of African Green Monkey plasma was pipetted into a Sarstedt (Newton, NC, USA) 5-mL polypropylene tube. Tubes containing unknown samples received an additional aliquot of 50/50 (v/v) methanol/water (25 μL) after which they were vortex mixed. Phosphate buffer (0.1 M, pH 2.0, 0.5 mL) was then added to each tube and they were vortex mixed again. All wells in a Waters Oasis™ 96-well mixed phase cation exchange extraction plate were conditioned by sequential washes of 1000 μL of

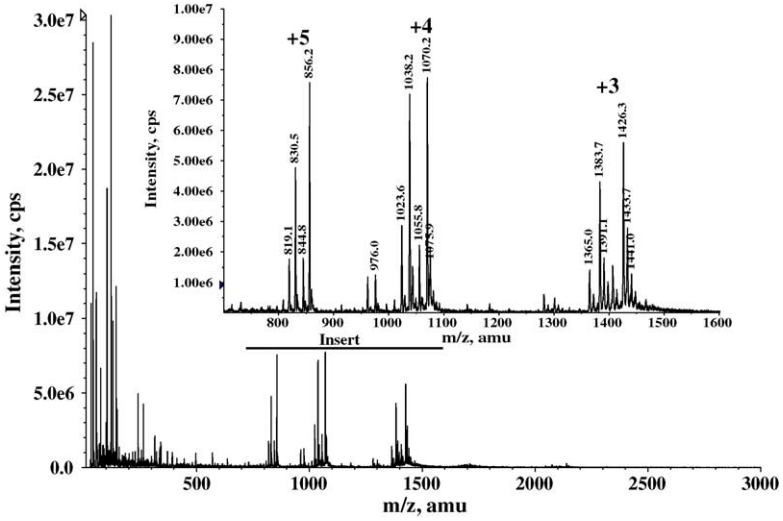

Fig. 2. Full scan mass spectrum of protonated potato carboxypeptidase inhibitor (CPI) over the range of *m/z* 10–3000 Da. (insert) Magnification of the major groups centered at *m/z* 840, 1050 and 1400 Da corresponding to the +5, +4 and +3 charge states of CPI.

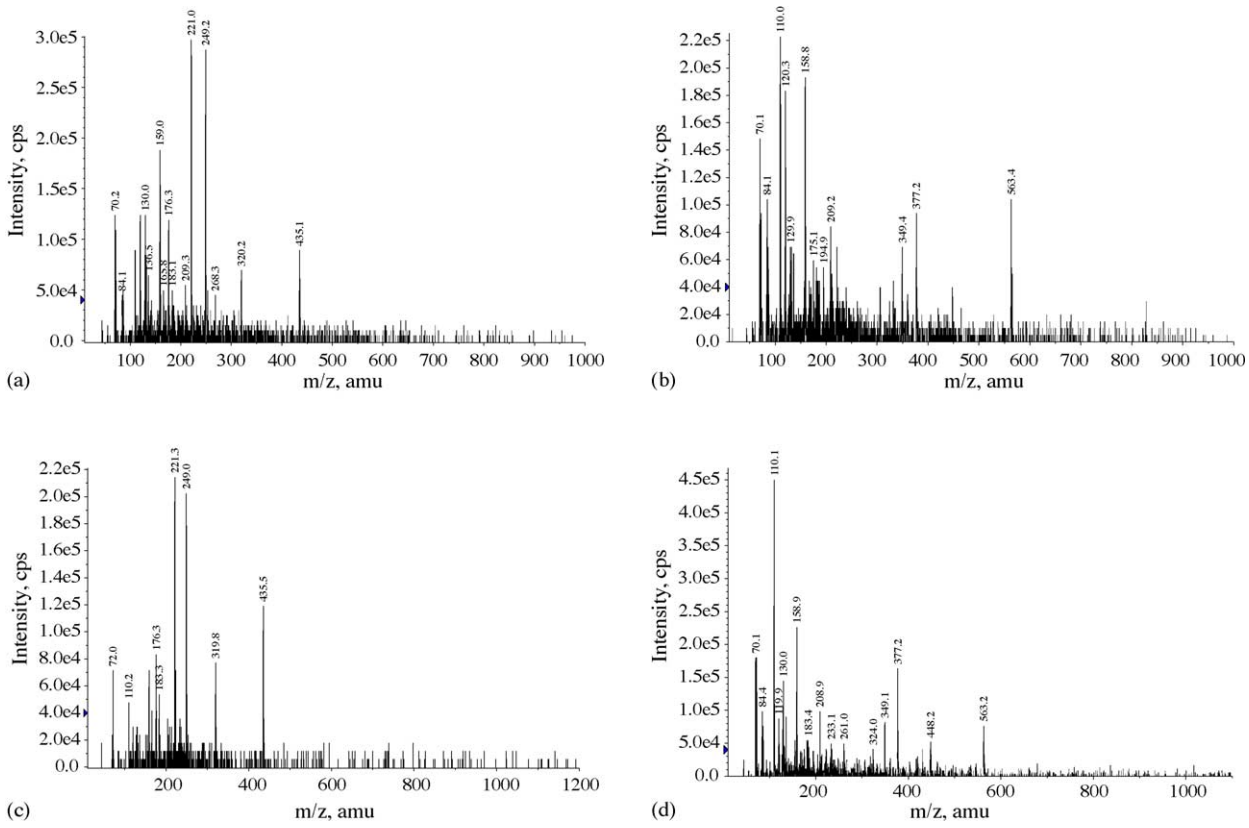

Fig. 3. (a–d) Product ion mass spectrum of potato carboxypeptidase inhibitor (CPI) for each of the precursor masses at: (a) *m/z* 830.5 Da; (b) *m/z* 856.2 Da; (c) *m/z* 1038.2 Da and (d) *m/z* 1070.2 Da.

methanol and 1000 μL of water. The entire volume of each sample (725 μL) was then transferred using an 8-channel pipetter (Matrix Technologies Corp., Hudson, NH, USA), applied to individual wells in the 96-well extraction plate and drawn through the plate using vacuum. The entire volume was transferred to obtain the best possible limit of quantitation (LOQ). The wells were then washed sequentially with 1000 μL of HCl (0.1 M) and 1000 μL of water. Washing with dilute HCl was recommended as a wash step for removing "unretained proteins" in the manufacturer literature. After rinsing the underside of the plate with distilled water, the plate was positioned on an ELISA plate (Corning Costar Corp., Cambridge, MA, USA), and then centrifuged ($360 \times g$, 5 min) to remove retained solvents. The extraction plate was returned to the extraction manifold containing a 96-position deep-well collection plate (MicroLiter Analytical Supplies Inc., Suwanee, GA, USA), and 900 μL aliquots of 5/95 (v/v) ammonium hydroxide/methanol were pipetted into each well of the extraction plate. Vacuum was used to elute retained analytes into the deep-well collection plate. The extracts were evaporated to dryness under nitrogen (50 °C) using an EvapArray 96-Well Sample Concentrator, and then reconstituted using 150 μL of 20/80 (v/v) methanol/water. Following reconstitution, the deep-well plate was sealed with Packard TopSeal™-S heat sealing film from Packard Instrument Company (Meridian, CT, USA) and transferred to a 96-well autosampler for injection (20 μL) onto the LC–MS/MS.

Recovery of this preparation method was determined by extracting samples of African Green Monkey plasma (200 μL) containing 5.0 and 0.5 μg/mL of CPI ($n = 5$), and comparing them to control samples of African Green Monkey plasma which were first extracted and then spiked to contain a concentration of CPI representative of 100% recovery. Recoveries of the two major isoforms were as follows: 5.0 μg/mL—4148.1 Da, 76.5 ± 7.5%, 4276.2 Da, 73.2 ± 5%; 0.5 μg/mL—4148.1 Da, 82.7 ± 7.6%, 4276.2 Da, 75.6 ± 5%. No assessment of the effect of sample matrix on MS/MS detection was performed.

Dilution integrity was tested by performing triplicate analysis of CPI in two different lots of African Green Monkey plasma (~5 μg/mL) and then diluting each 1:10 with African Green Monkey plasma and retesting. Multiplication of diluted sample concentrations ten-fold gave mean concentrations of CPI within 4.6% of those for the undiluted samples.

Stability of CPI in African Green Monkey plasma and was addressed by fortifying plasma from two different monkeys with 5.0 and 0.50 μg/mL of CPI. The sample concentrations were determined (triplicate) before and after one week of storage at −20 °C. The concentrations (using *m/z* 830.5 → 221.0) for high and low samples, were as follows:

Table 1
Molecular weights of CPI sequences calculated from the +5, +4, and +3 charge states

$[M+5H]^{+5}$	Found molecular weight	$[M+4H]^{+4}$	Found molecular weight	$[M+3H]^{+3}$	Found molecular weight	Average molecular weight	Theoretical molecular weight[a]
819.1	4090.5	1023.6	4090.4	1365.0	4092.0	4091.0	4094.68
830.5	4147.5	1038.2	4148.8	1383.7	4148.1	4148.1	4151.71
844.8	4219.0	1055.8	4219.2	1406.9	4217.7	4218.6	4222.74
856.2	4276.0	1070.2	4276.8	1426.3	4275.9	4276.2	4279.76

[a] Molecular weights before formation of up to three Cys–Cys intramolecular bonds.

plasma 1: initial 5.207 ± 0.179 and 0.533 ± 0.024 μg/mL, after storage, 4.810 ± 0.145 and 0.573 ± 0.019 μg/mL; plasma 2: initial 4910 ± 0.314 and 0.541 ± 0.035 μg/mL, after storage, 5.273 ± 0.124 and 0.553 ± 0.019 μg/mL. Mean CPI concentrations were within 15% of expected, before and after storage indicating that the analyte was stable in the matrix over the time of pharmacological experiments.

3. Results and discussion

3.1. MS/MS characterization of potato carboxypeptidase inhibitor (CPI)

Analysis of macromolecules using LC–MS/MS is often more difficult than that of small molecules (MW < 1000) due to their larger more complex structure and their often being observed in multiple charge states. CPI has been the subject of much past characterization due to its biological activity and these experiments have been summarized [16], but none of this information gave a strong indication of what could be expected while analyzing CPI in biological samples using LC–MS/MS. In this case, quantitation of circulating CPI presented some additional analytical challenges due to the presence of multiple active isoforms of this peptide, which possessed (Fig. 1) possibly 37, 38 or 39 amino acids [17]. Fig. 2 shows the full scan spectrum over the range of *m/z* 10–3000 Da for the CPI (purified natural isolate) that was used in this study. Notable characteristics of the spectrum included multiple peaks at less than *m/z* 400 Da, a series of groups centered at approximate *m/z* 840, 1050 and 1400 Da, with no significant peaks observed higher than *m/z* 1500 Da. It was immediately obvious from this information that the three groups centered at *m/z* 840, 1050 and 1400 Da corresponded to the +5, +4 and +3 charge states of CPI (MW approximately 4000 Da), respectively. A closer examination of the charge envelopes (Fig. 2, insert) indicated that they were each composed of at least four peaks (*m/z*), which originated from probable CPI isoforms. Table 1 shows the four most abundant peaks within each envelope, the molecular weight calculated from the individual peaks, and then the average molecular weight using all three charge states. An average of 4276.2 Da corresponds to the largest CPI peptide possessing the full 39 amino acid compliment as per Fig. 1, while the average of 4148.1 Da corresponds to the 38 amino acid peptide resulting from the deletion of Gln^2 from the N-terminus. The other two peptides observed in the mixture, with average molecular weights of 4218.6 and 4091.0 Da, corresponded respectively to the sequence of the these first two peptides, but each also without C-terminal glycine (Gly^{39}).

After examination of the full scan spectrum, collision induced dissociation (CID) was performed on the four most prominent molecular ion peaks within each of the three charge envelopes to determine if detectable product ions could be obtained. The best product ion spectra originated from the +5 and +4 charge groups (Fig. 3a–d), and many of these transitions were considered to be of sufficient magnitude for use in LC–MS/MS. CID performed on precursor ions chosen from the +3 charge group resulted in products ions that were too small as to allow MS/MS detection at the targeted LOQ of 0.05 μg/mL. This poor detectability excluded these transitions from further use during method development. The AB/MDS Sciex Analyst software was then used to optimize the MS/MS response of the two most prominent peaks in each of the product ion spectra shown in Fig. 3. Four of these eight transitions provided significantly larger signal than the others, so they were chosen to be monitored in the final assay. The four final detection transitions and their related detection parameters are listed in Table 2.

3.2. Chromatographic development

The choice of chromatographic method to be used for CPI was initially not an obvious one as it was assumed that

Table 2
Selected reaction monitoring transitions and instrument parameters for the API-3000

CPI (MW/charge)	Precursor ion (*m/z*)	Fragment ion (*m/z*)	DP/FP/EP/CE/CXP (eV)
4148.1/+5	830.5	221.0	41/200/–10/70/30
4148.1/+4	1038.2	249.0	46/280/–10/80/16
4276.2/+5	856.2	563.4	41/250/–10/57/36
4276.2/+4	1070.2	563.2	41/270/–10/73/34

size, secondary or tertiary structure, charge, or a combination of these would make it problematic to chromatograph this molecule using conventional, small pore, reverse phase HPLC packings. In the past, CPI has been separated from related compounds using large-pore (300 Å) packings tailored to macromolecules and these included a Zorbax 300SB C_{18} [18] and a Vydac C_{18} [19]. Sharp peaks were obtained for CPI when using a Nova-Pak C_{18} [20], although this column did not provide as much selectivity between related compounds and interferences as the wide pore columns. Based on these past reports, it was felt that gradient chromatography using a conventional C_{18} column should be attempted as there was not an immediate need to resolve the CPI from interfering matrix components due to the high specificity of MS/MS detection, which resulted in the chromatography shown in Fig. 4a.

3.3. Sample analysis

At the initiation of method development for CPI, there was not a firm expectation of what limit of quantitation would be required to provide relevant information during pharmacological studies. The assay development proceeded with the intent to obtain the best possible LOQ while using the API 3000 LC–MS/MS instrument to measure CPI in a 200-uL monkey plasma sample. The rationale behind this intended LOQ was to make the assay applicable to the full range of pharmacological experiments, which were initially planned using this peptide isolate. Ultimately, the best LOQ that could be obtained while consistently meeting assay acceptance criteria, was 0.05 μg/mL. The LOQ that was obtained for CPI is fully 1-2 orders of magnitude higher than that routinely obtained for small molecules using the same instrumentation. This was not surprising as a higher LOQ is commonly observed when using LC–MS/MS detection for the measurement of macromolecules due to both their larger molecular weight and their propensity to exist in multiple charge states during electrospray ionization. Molecular weight serves to reduce detectable molecules per mass of analyte as it increases, while multiple charges cause a dilution of the detection signal in proportion to the number of different ions (*m/z*) that are present. The LOQ of 0.05 μg/mL for CPI was obtained by fully optimizing the API-3000 on the most sensitive mass transitions that were identified per Section 3.1 and by using a small bore HPLC column which served to concentrate the sample, and also to enhance ionization by reducing mobile phase flow into the mass spectrometer. The response of the mass spectrometer to CPI was linear from the LOQ of 0.05 to 10 μg/mL.

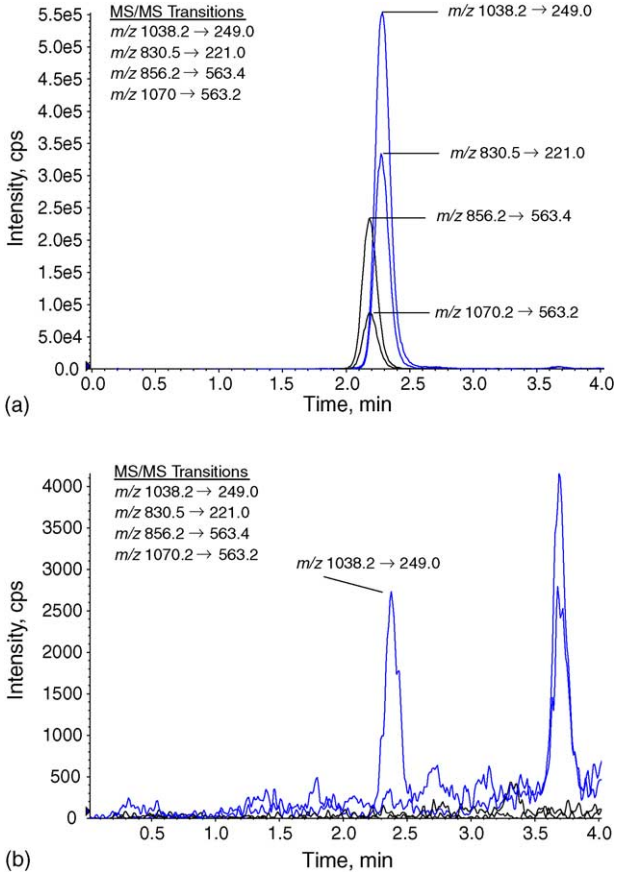

Fig. 4. Representative, overlaid selected reaction monitoring chromatograms for an African Green Monkey: (a) sample collected 4 h (C_{max}) after the administration of an intravenous dose of CPI (0.2 mg/kg bolus followed by an infusion of 0.05 mg/kg/h). The peak represents a concentration of 37.4 μg/mL CPI after a 1:10 dilution with control plasma and (b) predose.

While considerable effort was placed into optimizing the instrument to reach a LOQ of 0.05 μg/mL, this was eventually not important to the analysis of the plasma samples generated in the pharmacological studies. CPI was dosed intravenously to eight different African Green Monkeys using a 0.2 mg/kg bolus followed by an infusion of 0.05 mg/kg/h for 4 h. This dosing regimen resulted in high concentrations (μg/mL) of CPI in circulation relative to the LOQ of the method. Ultimately, all of the samples collected over the 4 h period had to be diluted 1–10 to bring them within the range of the calibration curve of the pre-developed method.

The assay for CPI was developed to meet pre-established criteria required to support discovery studies. These can be summarized as follows. The calibration curve used for the assay had to have a minimum of five points that were within ±20% of their calculated value. Assay acceptance was based on the quantitation of six sets of quality control (QC) samples at varying concentrations on the calibration curve, including the limit of quantitation. Two thirds of the QC concentrations had to be determined within 25% of their theoretical value at three concentrations along the standard line, and within 30% of their theoretical value at the limit of quantitation. The coefficient of variation for the QCs that were acceptable could not exceed 20%.

Circulating CPI concentration was monitored using the four most sensitive mass transitions that were identified during method development. Fig. 4a shows an overlaid chromatogram of the four mass transitions from an African Green Monkey after four hours of CPI infusion. Fig. 4b

shows chromatograms of the predose plasma sample from that same monkey. Upon performing the assay acceptance calculations using all four detection channels, accuracy and precision were best for the two most sensitive transitions (*m/z* 1038.2 → 249.0 and *m/z* 830.5 → 221.0), both of which originated from detection of the 4148.1 Da sequence. The *m/z* 830.5 → 221.0 transition was used to calculate the final concentrations of circulating CPI since an interference peak, shown in Fig. 4 (b), was observed in some of the predose samples in the *m/z* 1038.2 → 249.0 channel. CPI concentration was calculated from the equation $y = mx + b$, by weighted ($1/x^2$) linear least square regression of the calibration line constructed from peak areas versus nominal CPI concentration. The assay was applied to the determination of CPI in African Green Monkey plasma samples in two separate analytical runs (correlation of standard curves, $r_1 = 0.9991$ and $r_2 = 0.9953$). The performance of the assay during two analytical runs, when monitoring the transition *m/z* 830.5 → 221.0, is summarized in Table 3 (both runs combined). QC samples were run at 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μg/mL for each assay. Average ranges ($n = 12$) for accuracy and precision for all concentrations of QCs during the two runs were 92.0–102.0% of expected potency and 10.4–21.8% (coefficient of variations), respectively. Precision was within the acceptance criteria for all QC concentrations, although it was high when compared to the routine precision historically obtained on small molecules. Since the assay passed the pre-established acceptance criteria, no efforts were made during these experiments to identify a suitable internal standard. Identification and use of a suitable internal standard would be expected to offer some improvement in the precision of the assay for CPI.

The analytical methodology was developed with the assumption that any of the four transitions would be representative of the mass concentration of CPI in circulation and that the ratio of CPI peptides did not significantly change throughout their residence time in the body. This assumption was necessary due to the fact that there was not a characterized working standard of CPI available that contained a single isoform sequence. This lack of a working standard eliminated the ability to provide molar concentrations of

Table 3
QC precision and accuracy data for the determination of potato carboxypeptidase inhibitor (CPI) from African Green Monkey plasma using LC–MS/MS

Nominal concentration (μg/mL)	CPI determined concentration mean (μg/mL, $n = 12$)	Accuracy[a] (%)	Precision[b] (%CV)
0.050	0.046	92.0	21.8
0.100	0.102	102.0	16.3
0.200	0.194	97.0	16.2
0.500	0.494	98.8	11.8
2.000	2.012	100.6	12.3
5.000	4.924	98.5	10.4

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

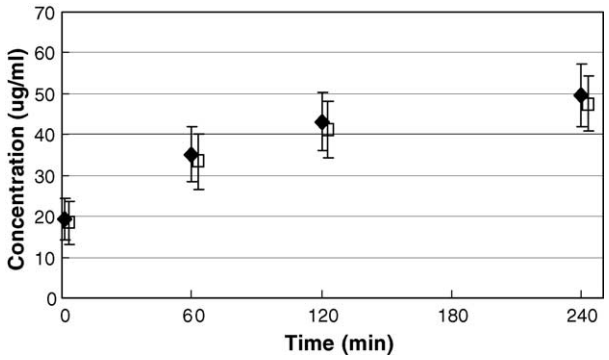

Fig. 5. Mean CPI plasma concentrations (μg/mL) in eight African Green Monkeys after the administration of an intravenous dose of CPI (0.2 mg/kg bolus followed by an infusion of 0.05 mg/kg/h) when calculated using the 4148.1 Da peptide sequence (♦, *m/z* 830.5 → 221.0). The CPI concentration when calculated using the 4276.2 Da peptide sequence (□, *m/z* 856.2 → 563.4) is shown (offset slightly) for comparison. Error bars represent ± standard deviation ($n = 8$).

circulating CPI. Ultimately, even with this limitation, the bioanalytical method provided the information that was required, which included a relative assessment of CPI concentrations in each monkey as well as the time to reach steady state concentration. The plasma-time concentrations (μg/mL), calculated using the *m/z* 830.5 → 221.0 transition (4148.1 Da), for the eight monkeys given an intravenous dose of CPI are shown in Fig. 5. Also included in this Figure are the mass concentrations (μg/mL) of CPI as determined based on the LC–MS/MS detection of the second CPI-II sequence, when using the *m/z* 856.2 → 563.4 transition (4276.2 Da). Particularly noteworthy are the approximately superimposable plasma concentration-time curves for the two sequences, indicating that the two forms of CPI-II are probably cleared at a similar rate. Despite this similarity there did seem to be a slight bias to higher concentrations when using the 4148.1 Da peptide sequence for calculating the total CPI in circulation. A comparison of chromatographic peak areas for the two isoforms in standards (5.0 μg/mL, $n = 8$) in control monkey plasma versus samples obtained from CPI dosed African Green Monkeys (240 min, $n = 8$) indicated that there was a statistically significant (unpaired *t*-test, $p = 0.01$) mean increase in the 4148.1/4276.2 Da peak area ratio in dosed samples (1.09 versus 1.16). These observations would account for the slight bias toward higher concentrations when using the 4148.1 Da isoform to calculate total CPI and indicated that the 4276.2 Da isomer may be cleared at a slightly higher rate than the 4148.1 Da isoform.

4. Conclusions

Potato carboxypeptidase inhibitor (CPI), dosed intravenously as its natural isolate was quantified in African Green Monkey plasma during pharmacological experiments. At least four different amino acid sequences of CPI were detected in the natural isolate when using LC–MS/MS. A single

isoform sequence was chosen for MS/MS detection and was shown to be representative of the total mass concentration of CPI in circulation. The LC–MS/MS assay developed was linear over the range of 0.05–10 μg/mL when extracting 200-μL samples of African Green Monkey plasma.

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