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

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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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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