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Detection and confirmation of α-cobratoxin in equine plasma by solid-phase extraction and liquid chromatography coupled to mass spectrometry

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ABSTRACT

α-Cobratoxin (CTX) is a large peptide (71 amino acids) with strong analgesic effect and may be misused in sports such as horse racing. To prevent such misuse, a sensitive method is required for detection and confirmation of the toxin in equine samples. CTX was extracted from equine plasma using an optimized mixed-mode solid-phase extraction (SPE) procedure. Extracted CTX was reduced with dithiothreitol and alkylated with iodoacetamide, and then was digested by trypsin at 56 °C for 30 min. The digest was analysed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), and tryptic peptides T2 (^{3}CFITPDITSK^{12}) and T4 (^{24}TWCDAFCSIR^{33}) were monitored for detection and confirmation of CTX. The limit of detection (LOD) was 0.05 ng/mL for CTX in plasma, and the limit of confirmation (LOC) 0.2 ng/mL. Unlike small peptides consisting of the 20 canonical amino acids, CTX was stable in equine plasma at ambient temperature for at least 24 h. The developed analytical method was successfully applied to analysis of incurred plasma samples; CTX was detected in plasma collected 15 min through 36 h post subcutaneous administration of CTX (2.0 mg dose) to a research horse, and confirmed 30 min through 24 h. Additionally, an approach named "reliable targeted SEQUEST search" has been proposed for assessing the specificity of T2 at product ion spectrum level for confirmation of CTX. T2 is uniquely specific for CTX, as evaluated with this approach and BLAST search. Furthermore, the effect of dimethyl sulfoxide (DMSO) as a mobile phase additive on electrospray (ESI) response of T2 and T4, background noise level and signal to noise ratio (S/N) was examined; DMSO increased signal intensity of T2 and T4 by a factor of less than 2. It is the first report that DMSO raised background noise level and did not improve S/N for the peptides, to the authors' knowledge. The developed analytical method may be applicable for analysis of CTX in plasma from other species such as greyhound dogs or even human beings.

1. Introduction

α-Cobratoxin (CTX) is a neurotoxin present in the venom of some snakes belonging to Naja genus. It consists of 71 amino acid residues, and its canonical sequence (Accession number: P01391) is documented in the UniProt protein database. Structurally, CTX forms three hairpin loops with its polypeptide chain [1]. It belongs to the snake three-finger toxin family [2]. Physiologically, the toxin can bind antagonistically to muscle- and neuronal-type nicotinic acetylcholine receptor (nAChR), with high affinity, leading to paralysis [2]. It is reported that the neurotoxin may have therapeutic potential as a treatment for lung cancer [3], multiple sclerosis [4] and arthritis [5]. Pharmacological studies have shown that CTX can induce potent analgesia in rodent pain models [6–8] and inhibit nociception in rat models of inflammatory [9] and neuropathic [10] pain. Due to its analgesic effect, the toxin was rumored to be illegally administered to race horses. In fact, CTX was found in the content of vials confiscated by horse racing jurisdictions in North America in 2008 [11]. In horse racing, no foreign substance is allowed to enhance or alter the performance of a racehorse. To maintain the integrity of fair competition in sports, to protect the well-being of racehorses and to deter misuse of CTX in horse rac-

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ing, an analytical method for the detection and confirmation of the toxin in equine samples collected post competition was needed.

There is no reported immunoassay such as enzyme-linked immunosorbent assay (ELISA) for detection of CTX in biological samples like plasma or urine, despite the need mentioned above. There has been only one reported analytical method for the detection and confirmation of the toxin in biological samples [12]. The reported method, which used liquid chromatography coupled to mass spectrometry (LC–MS), involved a two-day sample preparation procedure consisting of precipitating the target analyte at its isoelectric point from equine plasma with ammonium sulphate (a conventional technique in protein chemistry) and subsequent clean-up steps. This method is not amenable to batch processing of tens or hundreds of samples as is routinely performed in routine drug testing. In addition, the method used 3 mL of equine plasma for detection or confirmation of the toxin, and it took three days to complete the analysis including sample preparation.

In this paper, we report a novel LC–MS method for detection and confirmation of CTX in equine plasma. In the present method, the analyte was extracted from equine plasma by mixed-mode solid-phase extraction (SPE), and only 1 mL of plasma was used. With this method, sample preparation and analysis can be achieved in one day. Additionally, batch processing of large number of samples is feasible, and the current method is much more sensitive than the one published (limit of detection 0.05 ng/mL versus 1 ng/mL) [12].

It has been recently reported that dimethyl sulfoxide (DMSO) at a low percentage (5%) in mobile phases of liquid chromatography can enhance electrospray ionization (ESI) response and improve identification of low abundance proteins in proteomic experiments [13–15]. It was also shown in a recent publication that DMSO as a mobile phase additive (1%) increased electrospray ionization of small peptide hormones in diluted urine samples [16]. In the present study, effect of DMSO added at a low percentage to LC mobile phases on signal intensity of the analyte, background noise level and the matrix effect was examined.

2. Materials and methods

2.1. Chemicals and reagents

CTX (chromatographically purified) and iodoacetamide (IAA, Ultra grade) were purchased from Sigma (St. Louis, Missouri, USA). Dithiothreitol (DTT, Ultra grade) was obtained from Fluka (Buchs, Switzerland). Porcine trypsin (sequencing grade modified) was from Promega (Madison, Wisconsin, USA). Ammonium bicarbonate (Certified) and DMSO (purity ≥ 99.7%) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Phosphoric acid (Baker Analyzed Reagent) was from J.T. Baker Chemical Co. (Phillipsburg, New Jersey, USA). Acetonitrile (ACN, LC–MS grade), ammonium hydroxide (GR grade, 28%), formic acid (GR grade), methanol (HPLC grade) and water (LC–MS grade) were obtained from EMD Millipore Company (Billerica, Massachusetts, USA).

CTX stock solution (1.00 mg/mL) was prepared by dissolving accurately weighed powder of the analyte in ACN/water/formic acid (40/60/0.1, v/v/v), and was stored at 4 °C. Working solutions of CTX at concentrations of 100 to 0.02 μg/mL were prepared daily by diluting the stock solution in the same solvent mentioned above.

2.2. Sample pre-treatment

To each aliquot (1 mL) of blank equine plasma, 2.5 mL of 2% phosphoric acid was added for preventing possible degradation of CTX by enzymes present in plasma. Then a working solution of CTX at appropriate concentration was spiked to pre-treated blank plasma, resulting in the following concentrations of calibrators: 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ng/mL. For instance, 10 μL of 0.02 μg/mL CTX was added to 1.0 mL of blank plasma, leading to a calibrator of CTX at 0.2 ng/mL. The samples were briefly shaken by vortex. CTX-incurred plasma samples from administration of CTX to a research horse were similarly treated, without spiking of CTX. This sample pre-treatment was conducted immediately before SPE.

2.3. SPE of CTX from plasma samples

CTX was extracted from plasma samples using Oasis WCX cartridges (3 cc, 60 mg, 30 μm, 80 Å; Waters, Milford, Massachusetts, USA), on a Speedisk 48 Pressure Processor (J.T. Baker, Center Valley, Pennsylvania, USA). WCX cartridges were conditioned sequentially with 2 mL of methanol and 2 mL of water. The pre-treated plasma samples were then loaded onto the cartridges and allowed to pass through before rinsing. Each cartridge was rinsed with 2 mL of water, then with 3.4 mL of 0.5% concentrated ammonium hydroxide (28%, 14.8 mol/L) in water, and finally with 2 mL of ACN/water (80/20, v/v). The analyte in the rinsed cartridge was eluted with 1.0 mL of ACN/water/formic acid (40/60/2, v/v/v). The eluate was collected in a fresh glass culture tube (12 × 75 mm) and then transferred to a 2-mL plastic microcentrifuge tube (Fisher Scientific, Pittsburgh, Pennsylvania, USA). All the conditioning, loading, rinsing and eluting steps were carried out via gravity.

SPE of CTX from equine plasma was also attempted using Oasis MAX cartridges (3 cc, 60 mg, 60 μm; Waters, Milford, Massachusetts, USA). To 0.50 mL of plasma spiked with CTX, 0.50 mL of methanol/ACN (1:1, v/v) containing 1% acetic acid was added, and the mixture was centrifuged at 13,000*g* for 10 min (at 20 °C). The supernatant was transferred to a glass tube (13 × 100 mm) containing 2.0 mL of 5% concentrated ammonium hydroxide (28%) in water, and the mixture was briefly mixed with vortex. The pre-treated samples were loaded onto MAX cartridges that had been conditioned with 2.0 mL of methanol followed by 2.0 mL of water. After complete passage of the samples, the cartridges were rinsed with 2.0 mL of 5% concentrated ammonium hydroxide and then 2.0 mL of ACN/water (40:60, v/v). The target analyte was eluted from the cartridges using 1.5 mL of ACN/water/formic acid (70/30/2, v/v/v), and the eluates were collected.

The SPE eluates were dried using a Savant SC250 EXP SpeedVac Concentrator with a Savant RVT 5105 Refrigerated Vapor Trap (Thermo Fisher Scientific Inc., Asheville, North Carolina, USA). The settings for the vacuum concentrator were as follows: temperature, 50 °C; vacuum pressure, 0.4 Torr. The eluates were dried in the concentrator for 2.5–3 h, and the drying process was manually terminated when the vacuum pressure became lower than 0.4 Torr. It is worth to mention that complete drying of SPE eluates is critical for subsequent trypsin digestion. Otherwise, any acid or base residue in the dried samples will negatively affect succeeding step of trypsin digestion. The dried residue from each plasma sample was dissolved in 110 μL of 100 mM ammonium bicarbonate for the subsequent tryptic digestion to be described below.

2.4. Tryptic digestion of CTX

Disulfide bonds in the CTX molecule were reduced and alkylated before tryptic digestion of the analyte, and reduction and alkylation of the disulfide bonds were conducted using a reported procedure [17,18] with modifications. To the CTX extract in 110 μL of 100 mM ammonium bicarbonate prepared above, 4 μL of 500 mM DTT in water was added. The mixture in the vial was incubated in a water bath at 56 °C for 30 min. Then the vial was removed from the water bath and allowed to cool to ambient temperature, and 10 μL of 600 mM IAA in water was added. The mixture was incubated in the dark at ambient temperature for 30 min. To the sample, trypsin (2 μg in 10 μL of 100 mM ammonium bicarbonate) was transferred.

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